

PROBLEMS OF THE BIOCHEMISTRY OF THE NERVOUS SYSTEM

Edited by

PROFESSOR A. V. PALLADIN

MEMBER OF THE ACADEMY OF SCIENCES OF THE U.S.S.R.
AND THE ACADEMY OF SCIENCES OF THE UKRAINIAN S.S.R.

Translated by

F. S. FREISINGER

Translation edited by

H. HILLMAN and R. WOODMAN

INSTITUTE OF PSYCHIATRY
LONDON

PERGAMON PRESS

OXFORD · LONDON · NEW YORK · PARIS

1964

PERGAMON PRESS LTD.
Headington Hill Hall, Oxford
4 and 5 Fitzroy Square, London, W.1

PERGAMON PRESS INC.
122 East 55th Street, New York 22, N. Y.

GAUTHIER-VILLARS ED.
55 Quai des Grands-Augustins Paris 6^e

PERGAMON PRESS G. m. b. H.
Kaiserstrasse 75, Frankfurt am Main

Distributed in the Western Hemisphere by
THE MACMILLAN COMPANY · NEW YORK
pursuant to a special arrangement with
Pergamon Press Limited
Translated by F. S. Freisinger, B.Sc., Ph.D

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Library of Congress Catalogue Card Number 63-10030

This book is a translation of the original Russian *Voprosy biokhimiyi nervnoi sistemy*, published in 1959 by the Academy of Sciences of the Ukrainian S.S.R., Kiev.

Printed in Poland
PWN-DRP

EDITORS' PREFACE

AT THE International Congress of Biochemistry in Moscow in 1961, many Western biochemists for the first time had an opportunity of seeing, meeting and hearing their Soviet opposite numbers. During the last few years, Soviet Biochemists have gradually expanded their interests beyond the Pavlovian formulations. This Symposium held at Kiev in 1957 under the chairmanship of Prof. A. V. Palladin collected some of the first fruits of the new policy.

Some of the contributors, like Palladin and Vladimirov are already well known in the West, but most have only published hitherto in Russian. It is hoped that these important papers will show the direction of Soviet biochemistry at the cross roads. As can be seen, particular stress is placed on "functional biochemistry"—the relationship between the biochemical, physiological and psychological properties of the nervous system. There are many descriptions of experiments relating animal conditioning to changes in brain chemistry. There are also others showing the effects of X-rays on different organs. Contributions on the effects of hypothermia and on nucleotide biochemistry are other studies in which Soviet biochemists have specialized. Palladin's contribution to the Biochemical Congress at Brussels in 1956 is included as a useful review of brain biochemistry with special reference to work done in laboratories in the U.S.S.R.

In view of recent reports that a much greater proportion of Soviet resources are being diverted to biological research, it is hoped that the translation of this Symposium will be a useful introduction to the problems, the approaches and the techniques of Soviet biochemistry.

*Institute of Psychiatry,
London*

HAROLD HILLMAN
RICHARD WOODMAN

PREFACE

THE biochemistry of the nervous system presents some of the greatest difficulties of both biochemistry and biology, conceptually as well as methodologically. At present, the amount of work devoted to these studies in the Soviet Union is expanding every year, and this is an interim survey of work done recently in this field. The first fruits of the studies at the Pavlov Session of the Academy of Sciences and the Soviet Academy of Medical Sciences were presented at the Kiev Conference on "Biochemistry of the Nervous System" in December 1953, and were subsequently published in 1954. Since then, further important advances have made it necessary to review the subject again. This symposium contains papers given at the Second Conference on the "Biochemistry of the Nervous System" organized by the Institute of Biochemistry of the Ukrainian Academy of Sciences on 12 to 16 February 1957, and it is hoped that these articles give some indication of trends of work at present done in the Soviet Union.

The relation of function to biochemistry remains, as hitherto, the main interest of our experimental studies. These aim at characterizing biochemically the main functional states of the nervous system. Attention has also been paid to dynamic aspects of the biochemistry of the central nervous system.

Soviet biochemists are now using a wide range of methods, such as radioactive tracers, paper electrophoresis, paper chromatography and other physico-chemical methods.

The symposium begins with papers on proteins and their metabolism in the brain and peripheral nerves, and the effect of functional states such as excitation and inhibition upon them, as well as changes occurring in their metabolism during growth. Then follow articles about phosphorus-containing substances and methods of their investigation. Findings about the chemical nature of a brain glycogen and its different fractions, about carbohydrate metabolism during excitation and inhibition, and about adrenaline metabolism are presented. In other papers ammonia metabolism is discussed. Several papers discuss developmental biochemistry of the brain.

The histochemical approach is illustrated by the article on the nucleoproteins of the neurone. Several papers are devoted to hypothermia in view of its growing clinical importance. In recent years considerable attention

has been paid in this country to the biological effects of radiation and this is reflected in the articles in this symposium. Finally, the symposium deals with cerebral metabolism in some pathological conditions.

This symposium was printed at a time when the Soviet Union was preparing to celebrate the Fortieth Anniversary of the October Revolution, which created exceptional opportunities for the development of science and its application for the benefit of the people. The advances of biochemistry in the past forty years are only one aspect of the scientific successes achieved in the Soviet Union.

We hope that this symposium will give Soviet scientists some idea of trends in neurochemistry, and encourage further research to the benefit of the Soviet people, successfully building Communism.

A. V. PALLADIN

Member of the Soviet Academy of Sciences

PROTEINS OF THE NERVOUS SYSTEM

A. V. PALLADIN, YA. V. BELIK, N. M. POLYAKOVA
AND T. P. SILICH

Institute of Biochemistry,
Ukrainian Academy of Sciences, Kiev

STUDIES have been going on for many years on the proteins of the nervous system; for example, D. Petrovskii (1) in 1873 published the first work on the isolation of cerebral albumens and globulins. In 1891, A. Y. Danilevski (2) described a method for the fractionation of cerebral proteins and he isolated neuroglobulin and neurostromin. Subsequent work has been inhibited by the technical difficulties involved in the isolation and the purification of proteins in the presence of lipids. To overcome these difficulties, we have used zonal electrophoresis on paper.

This method was chosen for its suitability although it had previously only been applied to serum proteins. It was important not to denature the proteins during preparation. The method had previously been applied in 1954 to rat brain, by Demling *et al.* (3) who spun down the extracts at 2200 r.p.m. Kaps (4) has also used paper electrophoresis to fractionate the proteins from patients with cerebral oedema. The method used by Palladin and Polyakova (5) was first applied to rabbit brain and then to various parts of the central and peripheral nervous system of the cat.

METHOD

The tissue was freed from membranes and blood vessels, the blood washed off and then homogenized in an equal volume of saline. The suspension was then kept at 2–4°C for 1 hr, frozen solid by liquid air for 15 min and stored overnight in the cold. After thawing, it was centrifuged for 20 min at 8000 r.p.m. and then the saline-soluble proteins were decanted off, put on to paper and electrophoresced in a barbiturate buffer at pH 8.6 for 6–7 hr at 240–280 V. The paper was dried, stained with amido Schwarz 108 and the protein bands scanned by a densitometer. Blood serum from the same animal was run simultaneously.

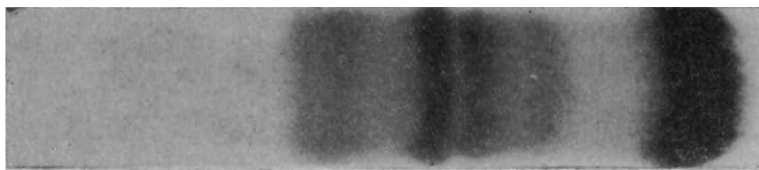


FIG. 1. Electrophorogram of the serum proteins of a cat.

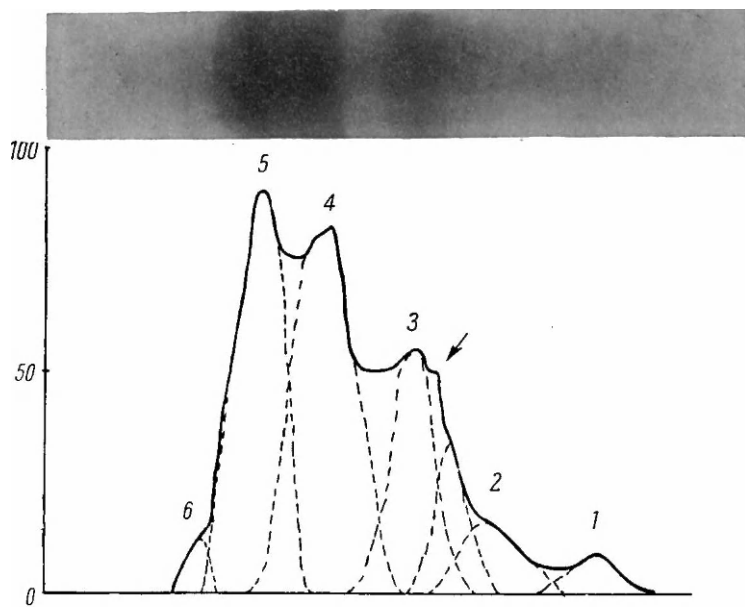


FIG. 2. Electrophorogram and the corresponding protein curves of cortical grey matter in cat brain.

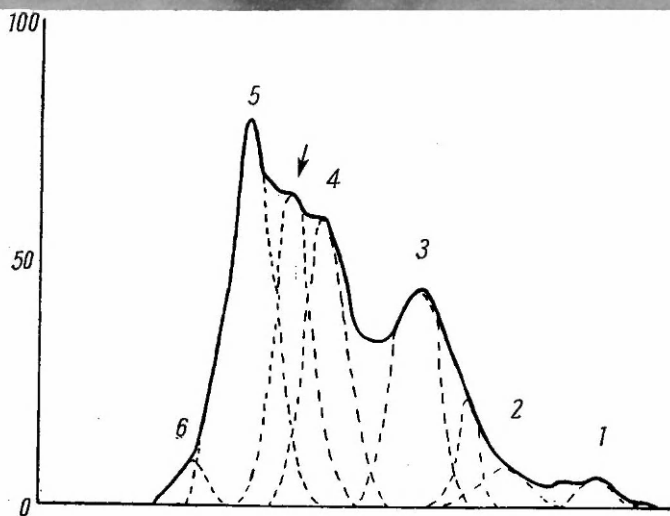


FIG. 3. Electrophorogram of the proteins in cortical white matter of cat brain.

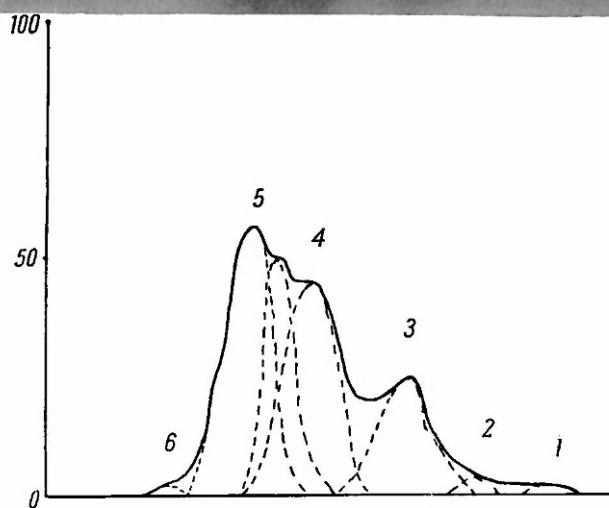


FIG. 4. Electrophorogram of the spinal cord proteins of cat brain.

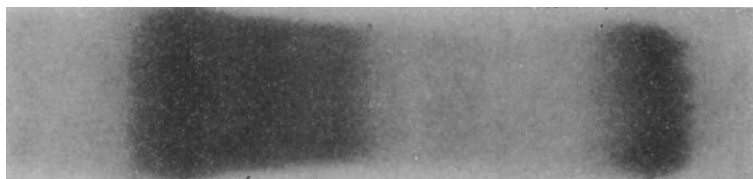


FIG. 5. Electrophorogram of the proteins in cat sciatic nerve.

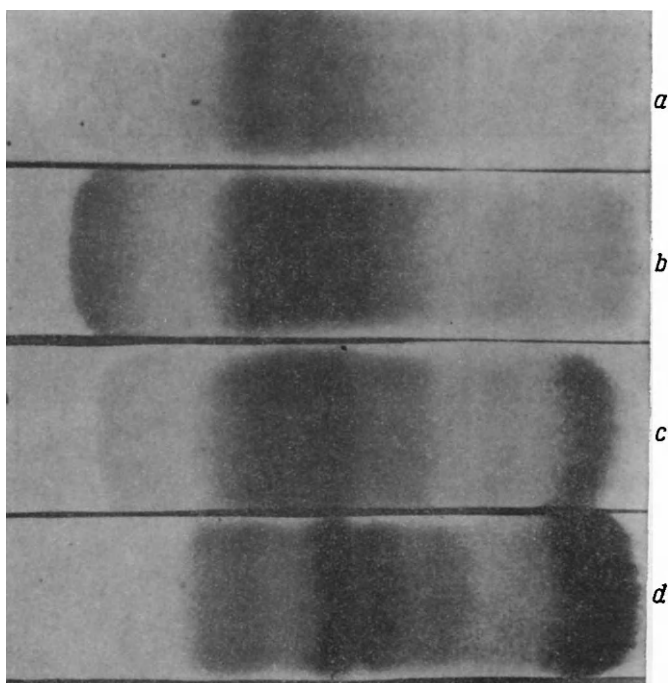


FIG. 6. Electrophorograms of proteins from cow nervous tissue; a—in white matter of the spinal cord; b—in the spinal roots; c—in the sciatic nerve; d—in the blood serum.

RESULTS

Electrophoresis of Cat Cerebral, Cerebellar and Spinal Cord Proteins

In each of these tissues 6 main bands could be identified. Comparison with the serum bands showed that most of these proteins from the central nervous system have an electrophoretic mobility similar to serum globulins and a few of them were similar to serum albumins. Similar results have been obtained by Hofmann (6), Kemp (7), Demling *et al.* (3) and Kaps (4), all confirming the low proportion of albumins, a phenomenon observed by Demling (4) in non-neural tissues using this method.

Electrophoresis of Proteins from Cat Sciatic Nerve (Fig. 5)

These results in the electrophorograms show that peripheral nerve contains a much larger proportion of albuminoid proteins as characterized electrophoretically. A similar finding was made by Keil (9) using ox palpebral nerve.

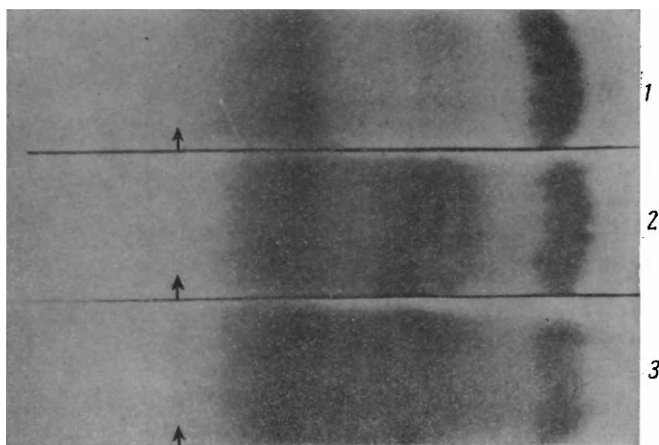


FIG. 7. Electrophorograms of proteins from cow nervous tissue: a—in the sciatic nerve of an adult; b—in sciatic nerve of a seven-month-old embryo; c—in sciatic nerve of a two-month-old embryo.

Electrophoresis of Proteins from Ox Nervous Tissue

Grey and white cerebral matter, cerebellum, medulla, spinal cord grey and white matter and spinal roots were studied by Polyakova (10). Nearly all of the 8 fractions found had the mobilities of globulins and contained even

less albuminoid proteins than cat nervous tissue, especially in the white matter of the spinal cord. Study of the ox spinal cord roots showed an interesting relationship between the histological nature of the tissue and its protein composition. Spinal cord roots may be regarded as intermediate between central and peripheral nervous tissue. Electrophoresis also demonstrated the intermediate character of spinal cord roots on the basis of their protein constituents since the albuminoid proteins were more abundant than in the central nervous system, but less abundant than in sciatic nerve. Furthermore, cationic proteins could be extracted from ox peripheral nerve, spinal cord roots and cord white matter but not elsewhere in the nervous system.

Several authors (11–14) have found proteins to be widely distributed in the nervous system, reaching their highest concentrations in those parts which are functionally most complex and phylogenetically youngest. White matter contains a lower proportion of soluble proteins.

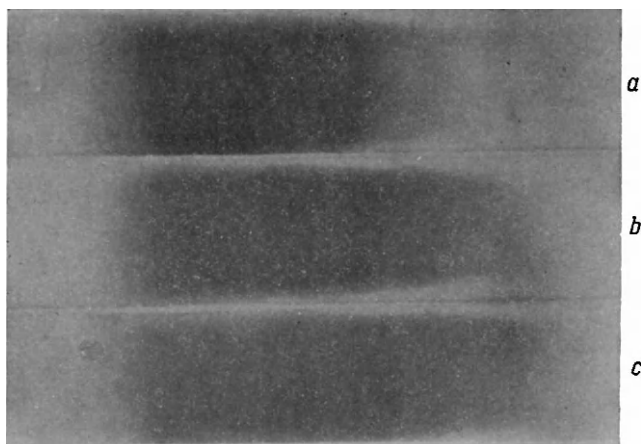


FIG. 8. Electrophorograms of proteins from cow nervous tissue: a—in the brain of an adult; b—in the brain of a seven-month-old cow embryo; c—in the brain of a two-month-old embryo.

Electrophoresis of Ox Embryo Nervous Tissue

Electrophoresis of the peripheral nerve proteins of 2- and 7-month-old ox embryos shows that they contain nearly as much albuminoid proteins as adult tissue (14) but separation of the globulins is less distinct, suggesting that they differentiate gradually during embryonic growth. Cerebral proteins from 2- and 7-month-old embryos, like adult tissue, show mainly globulin

composition. The number of fractions is much less than the adult (especially the 2-month-old sample) and is similar in this respect to embryonic peripheral nerve, thus suggesting once more that proteins become more numerous and well differentiated as the embryo develops.

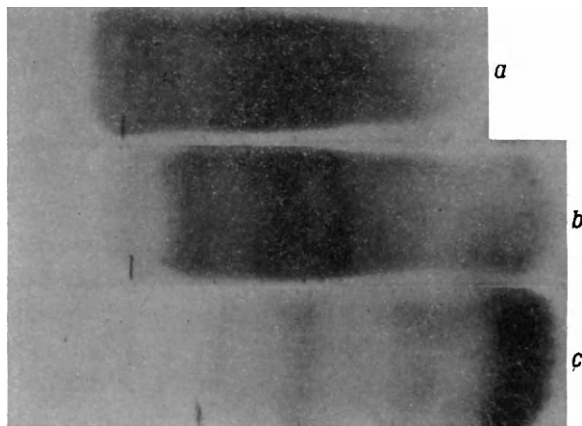


FIG. 9. Electrophorograms of precipitated proteins: a—by semi-saturation of a cerebral protein solution with ammonium sulphate; b—after full saturation of the filtrate remaining after semi-saturation of a cerebral protein solution with ammonium sulphate; c—after full saturation of a filtrate remaining after precipitation of the dissolved proteins from nerve tissue by semi-saturation with ammonium sulphate.

Ammonium Sulphate Precipitation and Paper Electrophoresis

Paper electrophoretic analysis depends upon the simultaneous processing of identifiable serum proteins in order to classify the unknown fractions. One cannot, therefore, necessarily presume that an unknown protein with the same electrophoretic mobility as a known serum protein is identical to it (14).

Fifty per cent saturated ammonium sulphate precipitates out globulins and saturated ammonium sulphate precipitates out albumins. Accordingly brain extracts prepared as described previously were saturated to 50 per cent with ammonium sulphate, centrifuged and the supernatant brought to 100 per cent saturation. The two precipitates were redissolved, dialysed and electrophoresed. Electrophoretograms of brain and peripheral nerve extracts treated in this way showed that globulins had the same mobility and precipitation characteristics as serum globulins. The ammonium sulphate fraction supposedly comprised of albumins contained a major portion

of material with the mobility of serum globulins. The 100 per cent saturated ammonium sulphate fraction of peripheral nerve contained mostly proteins with electrophoretic mobilities of albumins but again some corresponded to globulins.

RATES OF PROTEIN TURNOVER IN NERVOUS TISSUE

This has recently been studied by following the rates of incorporation of radioactive glycine and methionine into neural proteins by Palladin and Vertaimer (15). Peripheral nerve had not previously been studied and in view of the interesting differences found in the protein composition of central and peripheral nerve, this problem was investigated by Palladin *et al.* (14) by following the rate of ^{35}S -labelled methionine into the proteins of cat sciatic nerve. The isotope was injected at a dose of 0.023 mc/kg body weight 24 hr before the animals were decapitated. The sciatic nerve was removed from both hind limbs, washed in chilled saline, dried between filter paper and homogenized in 10 per cent TCA; the residue was well washed with 3 per cent TCA, dissolved in 0.2 N NaOH and reprecipitated. This residue was then extracted for 2 hr with methanol-chloroform at 68°C to remove lipids, dried by acetone, alcohol, ether and kept at 37°C until constant weight was reached (about 15 hr). It was then powdered and suspended for radioactivity measurements. This preparation also included nucleic acid nitrogen so a total nitrogen estimation was performed on one portion, nucleic acid phosphate content on another, and the protein nitrogen content obtained by subtraction. Serum protein, radioactivity and nitrogen content were measured on the same animal. Relative specific activity (RSA) is expressed as follows:

$$\text{RSA} = \frac{\text{counts (min)/mg tissue protein nitrogen}}{\text{counts (min)/mg serum protein nitrogen}} \times 100$$

Results from cat sciatic nerve gave values ranging from 3.7 to 9.3 (average 6.5). These values are compared with those from cerebral tissues in Fig. 10. The data of Kravchinsky and Silich (18) give RSA values of 22.6 for cerebral white and 34.5 for grey matter. These data are consistent with those of Palladin and Vertaimer (15) and Gaitonde and Richter (16). The rate of turnover thus increases from peripheral nerve to cerebral grey matter.

It is possible that certain protein fractions may have a much higher rate of turnover than others in various parts of the nervous system so Kravchinsky and Silich (18) have investigated the rate of incorporation of ^{35}S -labelled methionine into solvent fractionated protein extracts.

Protein fractions were prepared according to the method of Mirsky and Pollister (19), as follows:

- (a) fraction I extracted with 0.14 M NaCl containing mostly ribonucleoproteins,
- (b) fraction II extracted with 1 M NaCl containing desoxyribonucleoproteins,

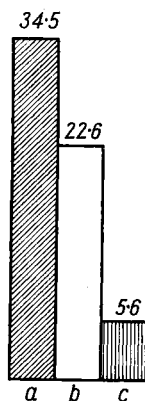


FIG. 10. The relative specific activity of proteins from cat nervous tissue: a—grey matter of the brain; b—white matter of the brain; c—peripheral nerve.

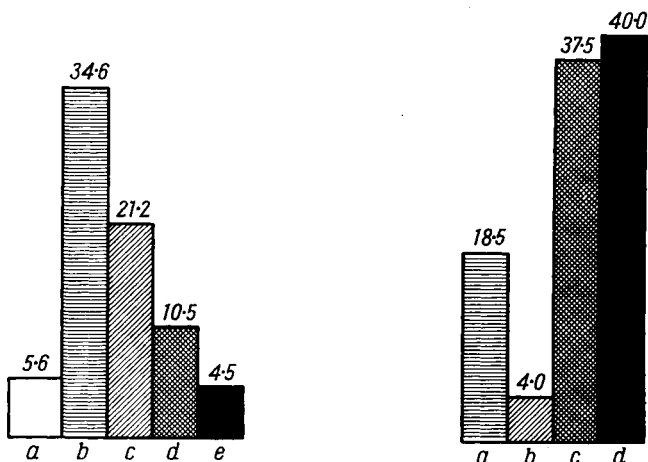


FIG. 11. Relative specific activity of proteins in fractions obtained from cat peripheral nerve: a—proteins from the whole tissue; b—extracted by 0.14 M NaCl; c—extracted by 1 M NaCl; d—extracted by 1 N NaOH; e—residue.

FIG. 12. Content of various protein fractions in cat peripheral nerve: a—extracted by 0.14 M NaCl; b—extracted by 1 M NaCl; c—extracted 1 N NaOH; d—residue.

- (c) fraction III extracted with 1 N NaOH,
- (d) fraction IV insoluble in the above solvents consisting of scleroproteins, neurokeratin and other material.

These fractions were handled as described by Kravchinsky and Silich (18). Figure 11 shows the rates of ^{35}S incorporation into various protein fractions, Fig. 12 gives the relative proportions of these fractions in peripheral nerve. The most rapid rate of turnover was found in fraction I (which contains 18.5 per cent of the protein) and the slowest in fraction IV (which contains 40 per cent protein).

Figure 13 shows the results of similar studies on cerebral tissue. The relative rates of ^{35}S incorporation into the 4 fractions follow the same order as in peripheral nerve; but the values are much higher.

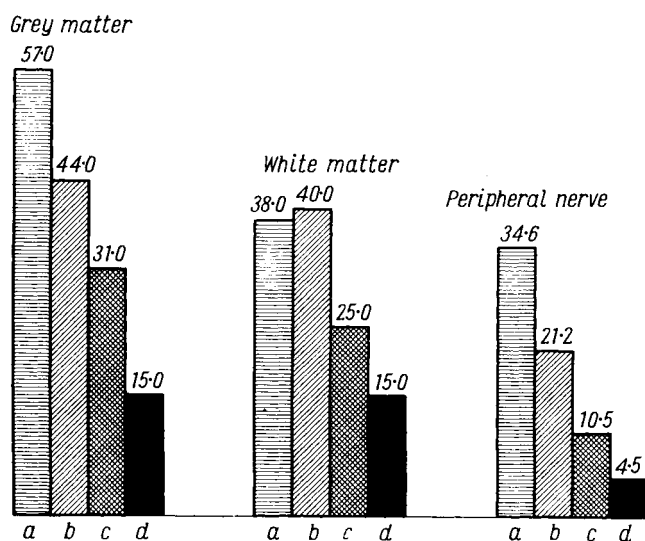


FIG. 13. The relative specific activity in various protein fractions from cerebral grey and white matter and peripheral nerve of cat: a—extracted by 0.14 M NaCl; b—extracted by 1 M NaCl; c—extracted by 1 N NaOH; d—residue.

RATE OF PROTEIN TURNOVER IN CEREBRAL TISSUES DURING EXCITATION

Several papers have been published on the effect of excitation upon the rate of protein turnover, using radioactive isotopes. Gaitonde and Richter (16) found a slight decrease in the rate of ^{35}S incorporation into the proteins of electrically stimulated rats compared with unstimulated controls.

Nechayeva (17) in similar experiments, found an initially higher rate in the stimulated rats which later fell to the value of the controls. Rozengardt and Maslova (21) found that some convulsants produced an initially increased rate of ^{35}S incorporation but others did not.

It was felt necessary to reinvestigate this problem, using varying periods of exposure to the isotope. Palladin *et al.* (22) used white rats of similar sex and weight (150–200 g), divided them into 2 groups, one of which received subcutaneous injections of amphetamine. A constant state of excitation was maintained for 24 hr by dividing the total dose of convulsant (0.6 mg/100 g body weight) into 3 parts injected at 8 hr intervals. These contained successively 40, 35 and 25 per cent of the total dose (10,000 counts/g body weight).

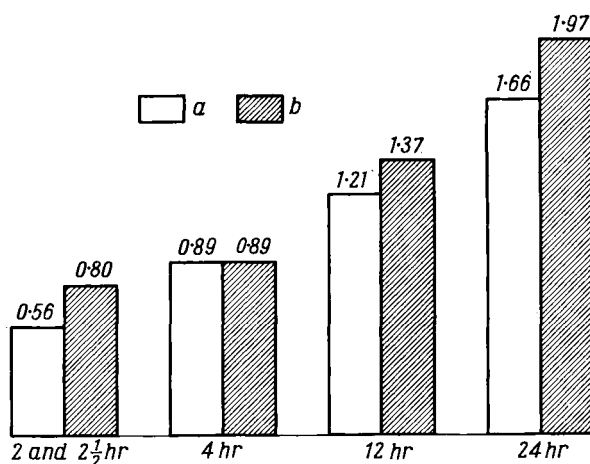


FIG. 14. The relative specific activity of cerebral proteins of cat under normal conditions and after stimulation measured at various intervals after the administration of radioactive methionine: a—normal state; b—stimulated.

The animals were decapitated 2½, 4, 12 and 24 hr after methionine injection. The brain was treated as described by Palladin *et al.* (22) and radioactivity measured in the acid-soluble fraction and residue separately, from which the RSA was calculated. Treated animals exposed 4 hr to the isotope incorporated it at the same rate as controls, while those exposed 12 and 24 hr had a slightly higher rate of incorporation than the controls which was not statistically significant. Thus increased protein turnover due to excitation would be expected to occur within 4 hr (see Fig. 14).

RATE OF PROTEIN TURNOVER DURING NARCOSIS

Animals were injected with 4 mg barbiturate and 50 mg urethane per 100 g body weight every 8 hr until sacrificed 24 hr after the 1st injection. ^{35}S -labelled methionine was administered simultaneously with the narcotic.

Protein turnover during narcosis had been previously studied by Gaitonde and Richter (16) who anaesthetized rats by means of ether or Nembutal. They observed a decrease in the rate of ^{35}S -labelled methionine incorporation into proteins of the nervous system and this decrease was accentuated by hypothermia. Nechayeva (17) found similar effects but Fridman-Pogosova (23), studying rabbits anaesthetized with urethane and sodium diethyl barbiturate, found no effect on the rate of incorporation into cerebral proteins, but an increased rate into spinal cord proteins. This work was reinvestigated by Palladin *et al.* (22) by the following method.

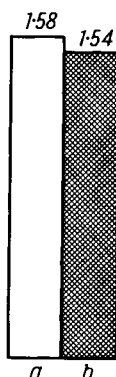


FIG. 15. The relative specific activity of the cerebral proteins of rat under normal conditions and during inhibition (narcotic sleep): a—normal conditions; b—narcosis.

The results of these experiments (see Fig. 15), showed no significant effect of narcosis on the rate of radioactive methionine incorporation into neural proteins. The different results of Nechayeva and Gaitonde and Richter may be due to variation in the type and depth of narcosis.

RATES OF PROTEIN TURNOVER RELATED TO AGE

Cerebral protein metabolism may differ not only in respect to the physiological state of the brain, but also the age of the animal. Protein turnover in the brains of rats has been studied in rats of varying ages (16) and found to be much higher in 20 g animals than 200 g ones. Similar findings were

obtained by Greenberg (24) using embryonic, young and adult brain homogenates. Palladin *et al.* (22) examined 4 rabbit age groups for rates of ^{35}S -labelled methionine incorporation into cerebral proteins.

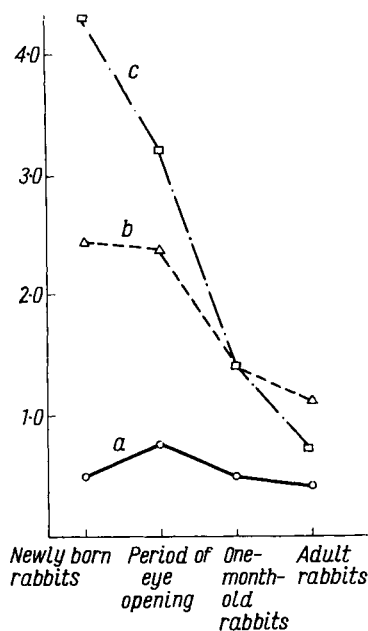


FIG. 16. Relative specific activity of the cerebral proteins in rabbits of different age: a—2½ hr; b—14 hr; c—24 hr, after administration of radioactive methionine.

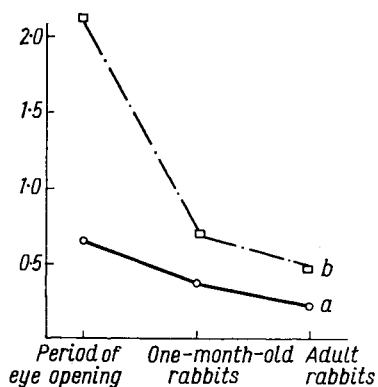


FIG. 17. The relative specific activity of sulphur-containing lipids in rabbit brain of different age: a—2½ hr; b—24 hr after the administration of radioactive methionine.

They were (i) newly born; (ii) 11–12 days old; (iii) 1 month old; (iv) adult. Radioactivity was measured $2\frac{1}{2}$, 14 and 24 hr after administering the isotope. Results are given in Figs. 16 and 17.

Where exposure to ^{35}S was for 24 or 14 hr, the rate of incorporation decreased with age. In animals exposed for $2\frac{1}{2}$ hr, however, the maximum rate of incorporation was observed in 11–12-day-old animals. This latter result may be due to the appearance of the visual function, since proteins associated with this process have a high turnover rate during this $2\frac{1}{2}$ hr exposure period. Although protein turnover decreases with age, it is interesting to note that this experimental result is not affected by varying the exposure period to radioactive methionine. The rates of ^{35}S -labelled methionine incorporation into cerebral sulpholipids was also measured in the last 3 age groups and found to decrease with age.

SUMMARY AND CONCLUSIONS

Paper electrophoresis has enabled further information to be obtained concerning the distribution of saline-soluble proteins in the nervous system. Although they constitute but 20 per cent of the total proteins present, they have been shown to have the highest rate of turnover. The two main functional components of the nervous system, the central and peripheral, could be differentiated on the basis of their protein composition, both by direct paper electrophoresis and when electrophoresis was preceded by ammonium sulphate fractionation. A small fraction of these proteins had a much higher turnover rate than the rest and the total turnover rate was higher in the central than in the peripheral nervous system. Turnover rates were studied under excitation and narcosis and values were shown to vary according to the exposure period to radioactive methionine, thus preventing any significant conclusions being drawn. Ageing was shown to decrease protein turnover rates, although the isotope exposure period did not affect the experimental results.

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THE RATE OF INCORPORATION OF AMINO ACIDS INTO CEREBRAL PROTEINS DURING VARIOUS FUNCTIONAL STATES

S. A. NECHAYEVA, N. V. SADIKOVA AND V. A. SKVORTSEVICH

Neurochemistry Laboratory, Pavlov Institute of Physiology, Soviet Academy of Sciences, Leningrad

THE incorporation of ^{35}S -labelled methionine (and $1\text{-}^{14}\text{C}$ -labelled glycine) into cerebral proteins has been studied by several authors (1-5). In this present work the rate of incorporation of $1\text{-}^{14}\text{C}$ -labelled glycine and ^{35}S -labelled methionine into the cerebral proteins of rats was studied during normal consciousness, electrical stimulation and anaesthesia.

METHODS

Electrical stimulation was applied to the paws in a chamber designed by Vladimirova (6). Anaesthesia was produced by a subcutaneous injection of 7-10 mg/100 g body weight of sodium amylobarbitone, and radioactive methionine or glycine were injected subcutaneously at a dose of 50,000 counts/min and 100,000 counts/min/g body weight respectively. After exposure to the isotope the animals were decapitated, the brains removed and blotted free of excess blood and frozen in liquid oxygen. The tissue was then homogenized in 10 per cent TCA and the insoluble residue washed with 5 per cent TCA until no further radioactivity appeared in the washings. One washing was conducted at 90°C (to remove nucleic acids which might have combined non-enzymically with amino acids, 7, 8) glutathione was removed from the acid-soluble fraction with cadmium, and it was washed with water, dissolved, and reprecipitated to free it from radioactive methionine. The number and specific activities of ^{14}C -labelled amino acids were determined by paper, chromatography the RSA of the individual amino acid being expressed as the proportion of the total radioactivity in the acid soluble fraction from 1g of tissue. For ^{14}C -labelled proteins radioactivity was expressed as counts per minute per 100 mg protein. In experiments using ^{35}S -labelled methionine, sulphur was extracted from the proteins and glutathione, and radioactivity expressed per unit of sulphur. To extract the sulphur, the protein of glutathione was digested

in a mixture of nitric, perchloric and hydrochloric acids containing copper nitrate. The resultant sulphuric acid was precipitated as benzidine sulphate, part being estimated spectrophotometrically and part used for radioactivity measurement.

It has previously been found that maximal incorporation of simultaneously injected radioactive methionine and glycine into cerebral proteins occurs within 2 hr. After 4 hr, protein radioactivity began to decrease. In view of this animals were sacrificed after not more than 2 hr exposure to the radioactive amino acids. Two hours exposure to ^{35}S gave highest SA values in the acid-soluble fraction followed by glutathione, proteins and sulphatides (see Fig. 1). The presence of ^{35}S -labelled glutathione indicates conversion of methionine into cysteine and cystine. This conversion appears to occur independently in the brain since it continued at an even higher rate in the brains of eviscerated than in whole animals (see Fig. 2).

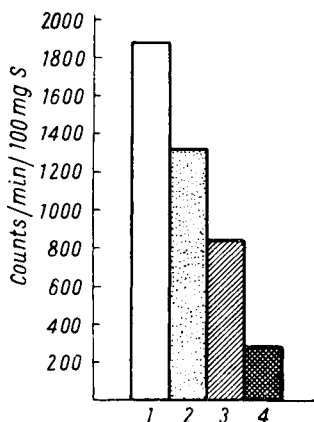


FIG. 1. Average values of the specific activity in the sulphur of various acid-soluble fractions in the brain of rat two hours after administration of ^{35}S -labelled methionine (50,000 counts/min/g body weight): 1—acid-soluble fraction; 2—glutathione; 3—proteins; 4—sulphatides.

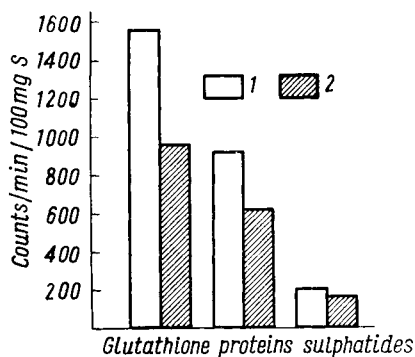


FIG. 2. Average values of the specific activity of protein sulphur, glutathione and sulphatides in normal animals and animals after hepatectomy and removal of abdominal organs 2 hr after administration of ^{35}S -labelled methionine (50,000 counts/min/g body weight): 1—normal animals; 2—operated animals.

Electrical Stimulation

After injection of ^{35}S -methionine a period of 3–5 min stimulation does not increase the proteins' specific activity but 20 min or 1 hr of stimulation does produce a slight increase compared to resting controls. Maximal

effects are observed after 90 min stimulation or after 1 hr if ^{14}C glycine is used. For prolonged periods of stimulation 15–30 V pulses were applied every 3 min. Studies on stimulated rates compared to resting ones and others under anaesthesia were conducted in parallel using animals of near identical weight. Results are given in Fig. 3. These show that 90 min of stimulation produce a protein SA 40 per cent higher, and a glutathione SA 35 per cent higher than in resting animals. These differences were only observed where animals responded to stimulation. Some animals ceased to respond and became unconscious in which case SA values were not affected either for proteins or glutathione.

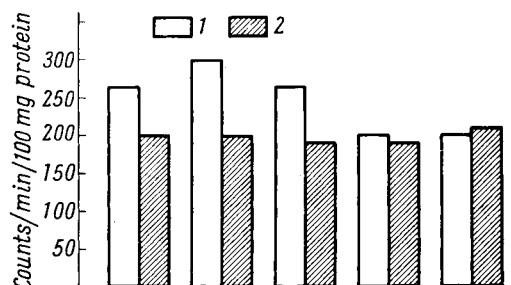


FIG. 3. Radioactivity of rat cerebral proteins in a state of relative rest and excitation 1½ hr after the administration of ^{35}S -labelled methionine: 1—excitation; 2—state of relative rest.

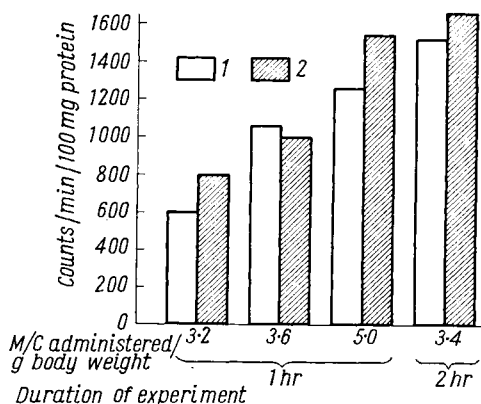


FIG. 4. Radioactivity in rat cerebral proteins in a state of relative rest and excitation after administration of ^{14}C -labelled glycine: 1—state of relative rest; 2—excitation.

After ^{14}C -glycine injection, stimulation for 1 hr produced a slight increase in the protein SA value compared to resting controls (Fig. 4). This effect was diminished if stimulation was continued for 2 hr. The SA of the acid-

soluble fraction was somewhat less in stimulated animals compared to resting controls. Chromatographic fractionation of the TCA extract revealed incorporation of ^{14}C glycine into serine, aspartic acid, glutamic acid, and glutamine thus indicating that protein radioactivity is not due to incorporated glycine alone (see Fig. 5).

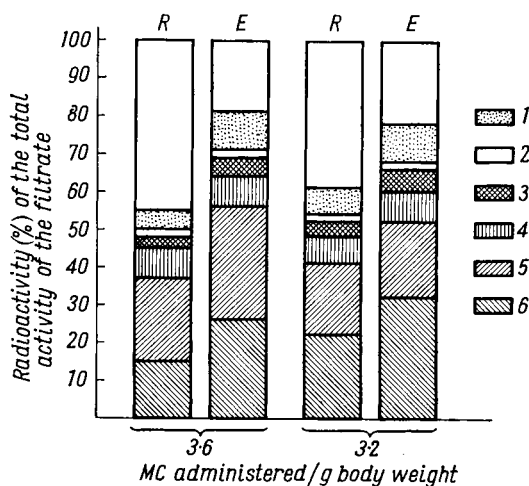


FIG. 5. Distribution of the radioactive carbon after the administration of ^{14}C -labelled glycine between non-protein aminoacids of rat cerebral tissue, R—state of rest, E—state of excitement: 1—glutamine; 2— γ -aminobutyric acid and valine; 3—aspartic acid; 4—glutamic acid; 5—serine; 6—glycine.

The ratio of radioactivity in glycine, serine, aspartic acid, glutamic acid and glutamine to that in the total acid soluble extract from stimulated animals was much higher than the equivalent ratio in resting animals. This suggests that stimulation prevents the conversion of ^{14}C from these amino acids into certain unknown compounds. Stimulation also causes increased conversion of ^{14}C glycine into glutamine without affecting the SA of glutamic acid suggesting enhanced glutamine synthesis. This is consistent with the data of Vladimirova (10) who studied the effects of stimulation on cerebral ammonia production.

Anaesthesia

The SA of the cerebral proteins of anaesthetized rats are much lower than that of stimulated ones (Fig. 6). This difference ranges from 0 to 99 per cent averaging 53 per cent and is obtained by reference to the values for resting animals. A similar, more marked difference was found in the ^{35}S -

labelled glutathione. Since these two abnormal physiological states had no effect on the total levels of cerebral protein or glutathione sulphur, the differences observed seem to be due to the altered turnover rates of these two substances.

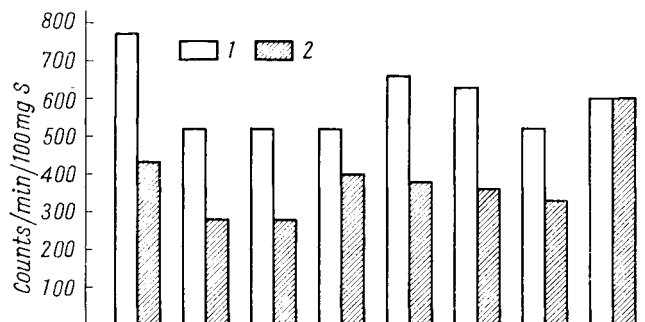


FIG. 6. Values of the specific activity in the sulphur of proteins in the brain of rats in a state of excitation and drug-induced sleep after administration of ^{35}S -labelled methionine (50,000 counts/min/g body weight): 1—excitation; 2—drug-induced sleep.

To validate these conclusions it is clear that the turnover of amino acids and proteins is established only when peptide linkages are formed.

Recently Melchior and Tarver (11) Winnik *et al.* (12) and Borsook *et al.* (14) have shown that when liver homogenates are incubated with radioactive amino acids the SA of the TCA-insoluble fraction depends not only on genuine polypeptides but also on adsorbed and disulphide-linked amino acids. In view of this, experiments were conducted in which the TCA-insoluble fraction from rat brain tissues were dissolved in alkali, reprecipitated, treated with ninhydrin and finally with formic acid and hydrogen peroxide. This treatment effectively releases all adsorbed and disulphide-linked amino acids and was used in 7 experiments where the effects of stimulation and anaesthesia on protein turnover after ^{35}S -methionine injection were studied. The SA value of the proteins was slightly reduced in both cases, by 5.5 per cent following reprecipitation from alkaline solution and by 7.7 per cent after formic acid/hydrogen peroxide treatment (Fig. 7). In experiments employing carboxyl ^{14}C -labelled glycine the protein was further treated with ninhydrin resulting in lowering of the SA value by 10 per cent (Fig. 8). Decreases due to the treatment are slightly higher in stimulated animals compared to resting controls but the total SA value is still significantly higher in stimulated animals. Since the proportion of amino acids not joined by peptide linkages is less than 10 per cent the use of tracer techniques as

described for studying protein turnover seems valid provided this correction is made. Pasynskii *et al.* (15) and Kuzin *et al.* (16) found that non-enzymic incorporation of radioactive amino acids by homogenates into proteins occurred only when the proteins had been denatured by non-physiological conditions. One can therefore assume that *in vivo* incorporation is by way of peptide bonding.

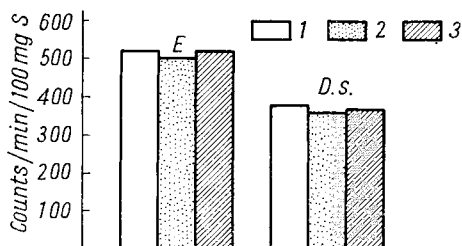


FIG. 7. Relative activity of the sulphur in the cerebral proteins of rats before and after treatment with alkali and formic acid and hydrogen peroxide: E—excitation, D.S.—drug-induced sleep: 1—original level; 2—after the action of alkali; 3—after the action of formic acid with hydrogen peroxide.

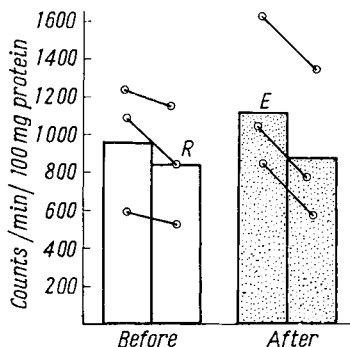


FIG. 8. Radioactivity of carbon in the cerebral proteins of rats before and after treatment with ninhydrin; R—state of rest; E—state of excitation.

QUANTITATIVE STUDIES OF THE RATE OF CEREBRAL PROTEIN TURNOVER

After injection of radioactive amino acids the SA values of cerebral proteins are much lower than those of non-cerebral tissues (17–19). Gaitonde and Richter (3) using ³⁵S-methionine found that this was due to the slower transport of amino acids into the brain than other organs, but incorporation of the methionine from the acid-soluble to the acid-insoluble fraction

of brain tissue was much more rapid than in liver tissue. In our own experiments using $1\text{-}^{14}\text{C}$ -labelled glycine, 1 hr after injection the SA value of the acid-insoluble fraction was 20–25 per cent of the acid-soluble fraction and after 2 hr nearly 50 per cent. Since there are 0.4 mg of free glycine and 10 mg of protein glycine per gram of brain tissue and half the free glycine is exchanged with the protein every 2 hr then the protein is exchanged once every 100 hr. Since 70–50 per cent of the glycine ^{14}C is incorporated into other free amino acids the validity of these calculations depends on the certainty of all the amino acids being incorporated into proteins at the same rate, making the above value for the protein exchange period only approximate. In experiments with ^{35}S -methionine the SA value of cerebral protein sulphur reached 45 per cent of the acid-soluble fraction value in 2 hr. Were all the acid-soluble sulphur in free methionine, cysteine and cystine, then according to the amounts of these amino acids present per gram of brain tissue the protein would have a life of only 4–5 hr. Since a large proportion of the acid-soluble ^{35}S becomes part of glutathione, taurine and inorganic sulphate (which were not measured) no reliable calculation can be made. For more accurate results it would be necessary to compare the SA values of the individual acid-soluble amino acids with the acid-insoluble ones.

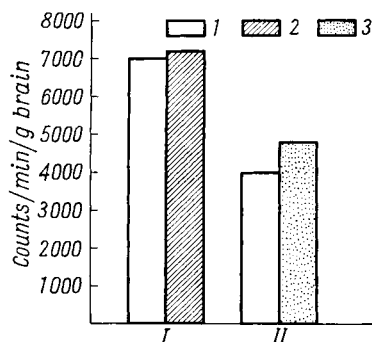


FIG. 9. Activity of trichloroacetic acid extract from the brain of rats in a state of relative rest, excitation and drug-induced sleep in experiments with ^{35}S -labelled methionine (I) and with ^{14}C -labelled glycine (II): 1—excitation; 2—drug-induced sleep; 3—state of relative rest.

Such methods as have been described are adequate for the comparison of protein turnover rates during different physiological states. Since electrical stimulation and anaesthesia do not appear to affect the rate of entry of injected amino acids into the brain (Fig. 9) the increased SA values of proteins following stimulation and decreased values during anaesthesia must be due to altered protein turnover rates.

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PROTEIN TURNOVER AND TISSUE RESPIRATION IN DIFFERENT PARTS OF THE BRAIN DURING AND AFTER DRUG-INDUCED SLEEP

K. I. POGODAYEV AND A. YA. MEKHEDOVA

Institute of Higher Nervous Activity, Soviet Academy of Sciences, Moscow

PAVLOV's theory of the protective and curative role of inhibition provides a theoretical basis for the use of sedation in various diseases. The mode of action of hypnotic drugs used clinically, particularly the barbiturates, has not received much attention, and studies of brain metabolism should help to elucidate their mode of action. We have attempted to study those metabolic processes which are most important for nervous activity, tissue respiration and protein metabolism, following the administration of therapeutic doses of hypnotic drugs, in view of previous demonstrations (Kotlyarevskii (1), Gorsheleva (2), Khozak (3)) that drug-induced sleep is very similar to physiological sleep.

In our experiments we used the same doses and methods of administration of amylobarbitone as the authors cited.

Studies of the brains of animals killed during drug-induced sleep can be used for the biochemical characterization of the state of protective inhibition. In one of his Wednesday seminars, Pavlov stated that, "After the administration of a hypnotic drug, higher inhibition disappears and the drug acts by directly decreasing the working capacity of a cell, and this is the phase during which transliminal inhibition takes place. We see here a result of transliminal inhibition by hypnotic drugs" (4). This view was confirmed by many other Soviet (5-10) and foreign (11-14) authors, who showed that inhibition slows down the processes of decomposition, and creates better conditions for those synthetic processes which restore the working capacity of the brain. However, all narcotic drugs have some toxic effect and cause metabolic disorders, particularly in oxidative-reductive processes (15-21).

We were able to observe a decrease in oxidative processes in patients during amylobarbitone sleep lasting 4 days with interruption; the level of incompletely oxidized substances—particularly organic acids—increased

considerably in the blood and urine. Administration of vitamin B-complex improved the oxidative processes during sleep and so increased its therapeutic value (22). In view of this, we studied the effect of ascorbic acid, riboflavine and nicotinic acid on respiration of brain tissue 1 and 2 days after amylobarbitone-induced sleep. The rate of protein resynthesis in the brain during drug-induced sleep has been studied, but the results are somewhat equivocal (7-10). This led us to carry out experiments with radioactive tracers on the rate of protein turnover in the brain during and after such sleep.

METHODS

We used 250 male laboratory white rats and guinea pigs, weighing 100-200 g and about 500 g, respectively. For 3 days the animals received sodium amylobarbitone orally—1 mg per g for rats and 0.1 mg per g for guinea pigs. The animals were kept asleep 12 hr a day for 3 days as well as having their natural sleep; in this state the animals reacted to sensory stimuli, such as noise or touch. Another group of guinea pigs, in addition to sodium amylobarbitone, were given the following daily doses of vitamins: ascorbic acid 5 mg, riboflavine 0.25 mg, and nicotinic acid 1 mg, for 3 days. Some animals were decapitated on the 3rd day of drug-induced sleep when they awakened, and others 1 or 2 days later. Controls were kept under normal conditions.

On the day of the experiments the rats were given subcutaneous injections in the back, of radioactive methionine in a dose of 30,000-50,000 counts per min per g. The animals were killed 2 hr later, their brains quickly removed and freed from membranes and blood vessels, and homogenates were made of the different parts of the brain with equal parts of 10 per cent trichloroacetic acid. The precipitate was washed twice with 5 per cent trichloroacetic acid. The lipids were extracted by routine methods. The protein so obtained was dried to constant weight, and its sulphur content estimated by the method of Pogodayev and Krasnova (23). The specific activity of the radioactive sulphur was found from the number of counts per minute in a barium sulphate precipitate of the protein, and the sulphur content in milligrams found by chemical analysis of 100 mg of the protein suspension.

The oxygen uptake of the homogenates was estimated in a Warburg apparatus in a Krebs-Ringer phosphate solution (24). The method of Thunberg (24) and a redox indicator were used to study the degree of oxidation and reduction under anaerobic conditions. The reducing capacity of the tissue was estimated by the rate at which the homogenates decolorized methylene blue in phosphate buffer at pH 7.6 under anaerobic conditions.

The ratio of 100–200 mg of tissue suspension and methylene blue was calculated approximately to decolorize the stain completely within 5–30 min. The reducing capacity was expressed in micrograms of methylene blue decolorized by 1 g moist tissue in 1 min. The oxidation-reduction ratio of the tissue depends upon the relative activities of the enzymes which act on it.

RESULTS

Figure 1 shows the specific activity of ^{35}S in radioactive methionine of proteins extracted from the cortex, subcortex, brain stem and cerebellum, of rats killed on the 3rd day of drug-induced sleep, and 1–2 days after the end of sleep, respectively. Individual experiments, as well as the average of the 10–12, show that the specific activity of all parts of the brain of rats during sleep was lower than the activity of the controls: in the brain stem it was lower by 5.3 per cent, in the cerebellum by 48.6 per cent and in the cortex and subcortex by 34 per cent.

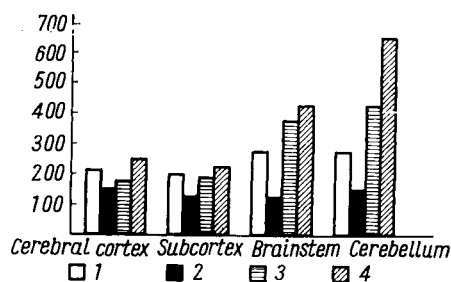


FIG. 1. The specific activity of ^{35}S -labelled methionine in rat brain proteins under various conditions as follows: 1—normal state; 2—during sleep; 3—one day after sleep; 4—two days after sleep.

After the drug-induced sleep the specific activity of the brain proteins increased: for example, 1 day after awakening the activity was 48 per cent above the controls in the brain stem, 52 per cent in the cerebellum, while in the subcortex it was approximately normal and in the cortex it was 21 per cent lower than the controls. Two days after awakening the activity of the proteins in the experimental animals was considerably higher than in the controls. In the brain stem it was up by 60.9 per cent in the cortex by 23.1 per cent, in the subcortex by 7.8 per cent, while the cerebellum was as high as 117 per cent above normal. These values are significant to $p = 0.05$.

The increased protein turnover in the parts of the brain examined by us—particularly in the brain stem and cerebellum—indicates intensification of protein resynthesis after drug-induced sleep. During sleep itself, however, the methionine utilization decreases so that we may presume that decreased protein resynthesis occurs.

Figure 2 shows the oxygen absorption of homogenates of the different parts. As in previous experiments, the animals were killed on the 3rd day of sleep or 1–2 days after awakening. The oxygen consumption of homogenates was decreased from all parts of the brain, except the cerebellum, which was increased by 28 per cent. One day after sleep the oxygen absorption of homogenates of cortex decreased by 20 per cent, and the brain stem by 10 per cent. The cerebellar homogenates had decreased their rate of oxygen uptake which was then only 5 per cent higher than normal. In the subcortex absorption was 4 per cent above normal. Two days after awakening the cortex homogenates were 15 per cent below normal and the subcortical ones 2 per cent. The cerebellum was 40 per cent higher and the brain stem 28 per cent.

It seems that during drug-induced sleep the oxygen absorption of homogenates of all parts of the cerebrum is depressed and remains low for 1–2 days after the end of sleep. Cerebellar and brain stem homogenates increase their oxygen absorption 2 days after awakening.

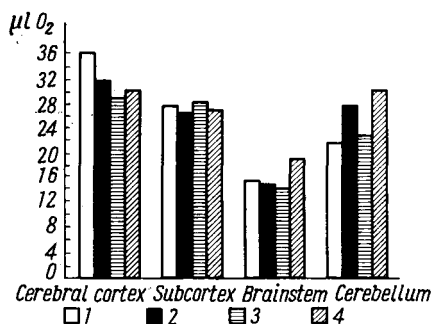


FIG. 2. Consumption of oxygen by homogenates of guinea pig brain under various conditions (values given as $\mu\text{l. O}_2/100 \text{ mg wet tissue/hr}$): 1—normal conditions; 2—during sleep; 3—one day after sleep; 4—two days after sleep.

Figure 3 shows the effect of some vitamins which play a part in the oxidation reduction reactions which are the basis of respiration, namely, ascorbic acid, riboflavine and nicotinic acid. After the end of sleep vitamins enhanced the oxygen absorption of homogenates of all parts of the brain, compared

with those experiments in which no vitamins had been given. Their administration for only 3 days was insufficient to prevent the marked decrease in the oxygen absorption after the end of the drug-induced sleep.

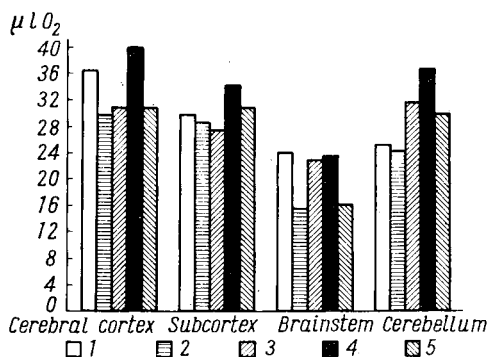


FIG. 3. Consumption of oxygen by guinea pig brain under various conditions as follows: (values given as $\mu l O_2$ 100 mg wet tissue/hr): 1—normal conditions; 2—one day after sleep; 3—three days after sleep; 4—one day after sleep and after administration of vitamins; 5—three days after sleep with administration of vitamins.

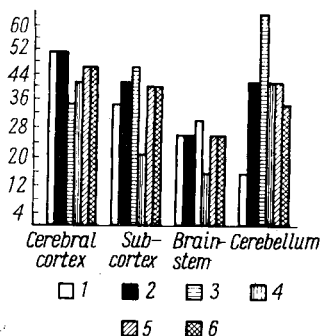


FIG. 4. The reducing capacity of guinea pig brain (expressed in μl of methylene blue decolorized by 1 g wet tissue in one minute): 1—normal conditions; 2—sleep; 3—three days after sleep; 4—one day after sleep with vitamin administration; 5—two days after sleep; 6—two days after sleep with vitamin administration.

Figure 4 shows the reducing properties under anaerobic conditions of the homogenates to methylene blue. In normal animals the cortex possesses the highest reducing activity, followed by the subcortex, while the brain stem is 48 per cent lower than the cortex and the cerebellum 70 per cent lower. Sleep had no significant effect on the reducing capacities except

in the cerebellar homogenates, where it produced a slight increase. The reducing activity of the cerebellar, brain stem and subcortical homogenates increased slightly a day after awakening, but the cortex decreased markedly. In animals which had received vitamins within 1 day of awakening, the subcortex, brain stem and cerebellum had much lower reducing activity, but no significant change could be seen in the cortex.

After 2 days awakening the reducing activity of homogenates from all parts of the brain, whether or not the animals had had vitamins, returned to normal. The only exception was the cerebellum which remained high.

It thus appears that drug-induced sleep as well as vitamins administered during sleep, have a greater effect on the rate of oxygen absorption than on the reducing capacity of homogenates of parts of guinea pig brain. Furthermore, changes in rate of oxygen absorption mirror changes in reducing activity. When the oxygen uptake increases the anaerobic reducing processes become less intense, and vice versa.

CONCLUSIONS

1. Radioactive isotopes studies show that on the 3rd day of amylobarbitone-induced sleep the rate of protein resynthesis in the brain decreases, but increases above normal 1 or 2 days after awakening, particularly in the brain stem and cerebellum.

2. Oxygen uptake of homogenates of all parts of the brain, except the cerebellum, is lowered by sleep and remains low 2 days after. Uptake in the cerebellum and brain stem is somewhat higher than normal 2 days after the end of sleep.

3. Oxygen uptake of all parts of the brain of animals which have been given vitamins during the 3 days of sleep markedly increases a day after awakening, but this increase reverts the next day.

4. Under anaerobic conditions, the normal cortex possesses the highest reducing activity to methylene blue followed by the subcortex, the brain stem and the cerebellum. Sleep increases reducing activity only in the cerebellar homogenates. One day after awakening the reducing capacity of the different homogenates increases (except for the cortex, which decreases) and remains high for 2 days.

5. Vitamins decrease the reducing activity 1 day after the end of sleep, but it returns to normal the next day.

6. Under the experimental conditions described above, aerobic respiration is more labile than anaerobic, and changes observed are, as a rule, opposite.

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COMPARATIVE STUDIES ON THE NUCLEIC ACIDS AND PHOSPHOLIPIDS OF THE NERVOUS SYSTEM

E. B. SKVIRSKAYA AND T. P. SILICH

Institute of Biochemistry, Ukrainian Academy of Sciences, Kiev

THE importance of phosphoproteins and phospholipids in the biochemistry of the nervous system and their connections with nucleotide phosphates in the whole nervous system and in individual nerve cells led us to interest ourselves in them. We started by using Schmidt and Thannhausers' method (1) but since other workers have found that the phosphoproteins separated by this method are contaminated with unidentified phosphorus compounds, we changed to the method of Vladimirov, Ivanova, and Pravdina (4) based on that of Davidson *et al.* (3) and Hammersten (2).

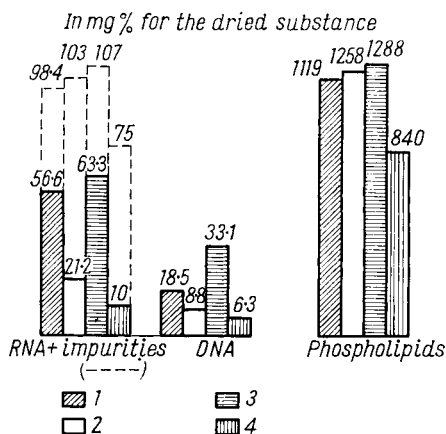


FIG. 1. The content of some phosphorus compounds in cat nervous tissue: 1—grey matter; 2—white matter; 3—cerebellum; 4—peripheral nerve.

Cats were given ^{32}P at a dose of $0.23 \mu\text{c/g}$ body weight 24 hr before sacrifice. Figure 1 shows that the cerebellum contains most RNA phosphorus $63.3 \text{ mg \%}$ then comes grey matter with 56.6 , then white matter 21.2 , finally peripheral nerve with 10.0 . DNA phosphorus is most abundant in the cerebellum ($33.1 \text{ mg \%}$) then grey matter with 18.5 , then white matter with 8.8 then peripheral nerve with 6.3 . The high values in cerebellum are due to

the abundance of nuclei in this tissue (5,6). The phosphorus-containing substance referred to as RNA occurs in about the same amounts throughout the nervous system. The phospholipids are distributed in similar proportions and concentrations as the "RNA".

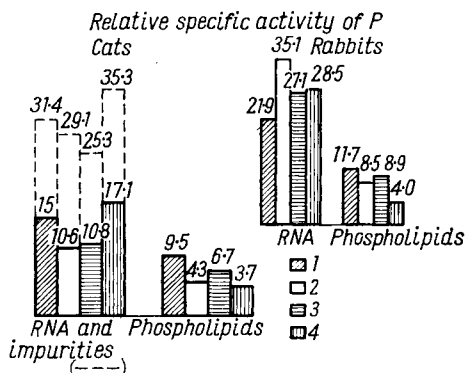


FIG. 2. The rate of incorporation of ^{32}P into phosphate compounds of nervous tissue: 1—grey matter; 2—white matter; 3—cerebellum; 4—nerve.

RATE OF INCORPORATION OF ^{32}P INTO PHOSPHATE COMPOUNDS OF THE NERVOUS SYSTEM

I. Nucleic Acid

Vladimirov's procedure, unlike that of Schmidt and Thannhauser differentiates between pure RNA and the phosphorus compound referred to as "RNA". Although the nucleic acids are not completely extracted by this method by careful standardization one can study the relative distribution and turnover rate of the nucleic acids and other high molecular weight phosphorus compounds fairly accurately.

The rate of ^{32}P incorporation into phosphorus compounds is expressed

$$\text{RSA} = \frac{\text{SA of phosphorus of the compound}}{\text{SA of tissue inorganic phosphorus}} \times 100$$

where RSA is the relative specific activity and SA the specific activity.

The RSA of pure RNA in the grey matter was much higher than in the white matter or cerebellum and the value for "RNA" was similar in all these tissues although it should be noted that ^{32}P is incorporated into "RNA" at $2\frac{1}{2}$ times the rate for pure RNA.

The RSA of pure RNA and "RNA" in peripheral nerve is higher than either cerebellum, grey or white matter. This is due to the fact that 40 per cent of the ^{32}P enters peripheral nerve in 24 hr compared to 10 per cent for the central nervous system. This is probably due to a blood/brain barrier.

These turnover rates for pure RNA in the grey and white matter of cats appear at first to be at variance with earlier experiments on rabbits. In the experiments on rabbits, when pure and contaminated RNA were not differentiated a higher RSA was found in the white matter after 24 hr exposure to ^{32}P than in grey matter. This could be explained in either of two ways. The preponderance of radioactivity in the "RNA" fraction which is equally distributed between grey and white matter, when added to the radioactivity of the pure RNA in the cortex would tend to level out any differences in the RSA values from grey and white matter. Alternatively, since cats are more developed in evolution than rabbits one would expect their turnover rates of grey matter substances to be more rapid. The data of Kreps *et al.* (8) who compared dogs and rabbits are consistent with this theory.

II. Phospholipids

The ratio of the turnover rates of phospholipids in the grey and white matter of cat cerebral cortex is 2.25 which is much higher than in rabbits (1.25). Such a species difference is even higher than that for the RNA values.

The cerebellum which consists of grey and white matter (not separated in these experiments) gives values intermediate between those for grey and white matter of both cats and rabbits. The peripheral nerve showed the lowest turnover rates which is not surprising as this tissue contains large proportions of inert phospholipids such as sphingomyelin.

THE NITROGENOUS BASES OF THE NUCLEIC ACIDS

If one takes adenine content as unity the relative distribution of the other nitrogenous bases are as follows:

	Ad.	Guan.	Cytosine.	Thym.
Brain	1.0	1.0	0.6	0.8
Nerve	1.0	1.0	0.5	0.5
Liver	1.0	0.4	0.5	0.8

The method used was developed by Rukina (9). Tissues were prepared by Schmidt and Thannhauser's method, followed by the separation of the pure nucleic acids. Table 1 shows the distribution of the nitrogenous bases in DNA of the brain, peripheral nerve and liver. The one notable difference is the low guanine level in the liver compared to nervous tissue.

TABLE 1. THE QUANTITY OF DNA NITROGEN BASES IN DIFFERENT TISSUES

$$\left(\frac{\text{Bases}}{\text{P in nucleic acid}} \right)$$

Tissue	Guanine	Cytosine	Adenine	Thymine
Brain	1.19	0.65	1.14	0.87
Nerve	1.17	0.62	1.19	0.63
Liver	0.48	0.67	1.28	1.00

The distribution of the nitrogenous bases from the RNA of brain, peripheral nerve and liver are given in Table 2. The exceptionally high guanine content of peripheral nerve is suspect, since the UV absorption of the eluate of the chromatographic spot corresponding to guanine shows two maxima. Only small quantities of uracil could be detected in peripheral nerve.

TABLE 2. THE QUANTITY OF RNA NITROGEN BASES IN DIFFERENT TISSUES

$$\left(\frac{\text{Bases}}{\text{P in nucleic acid}} \right)$$

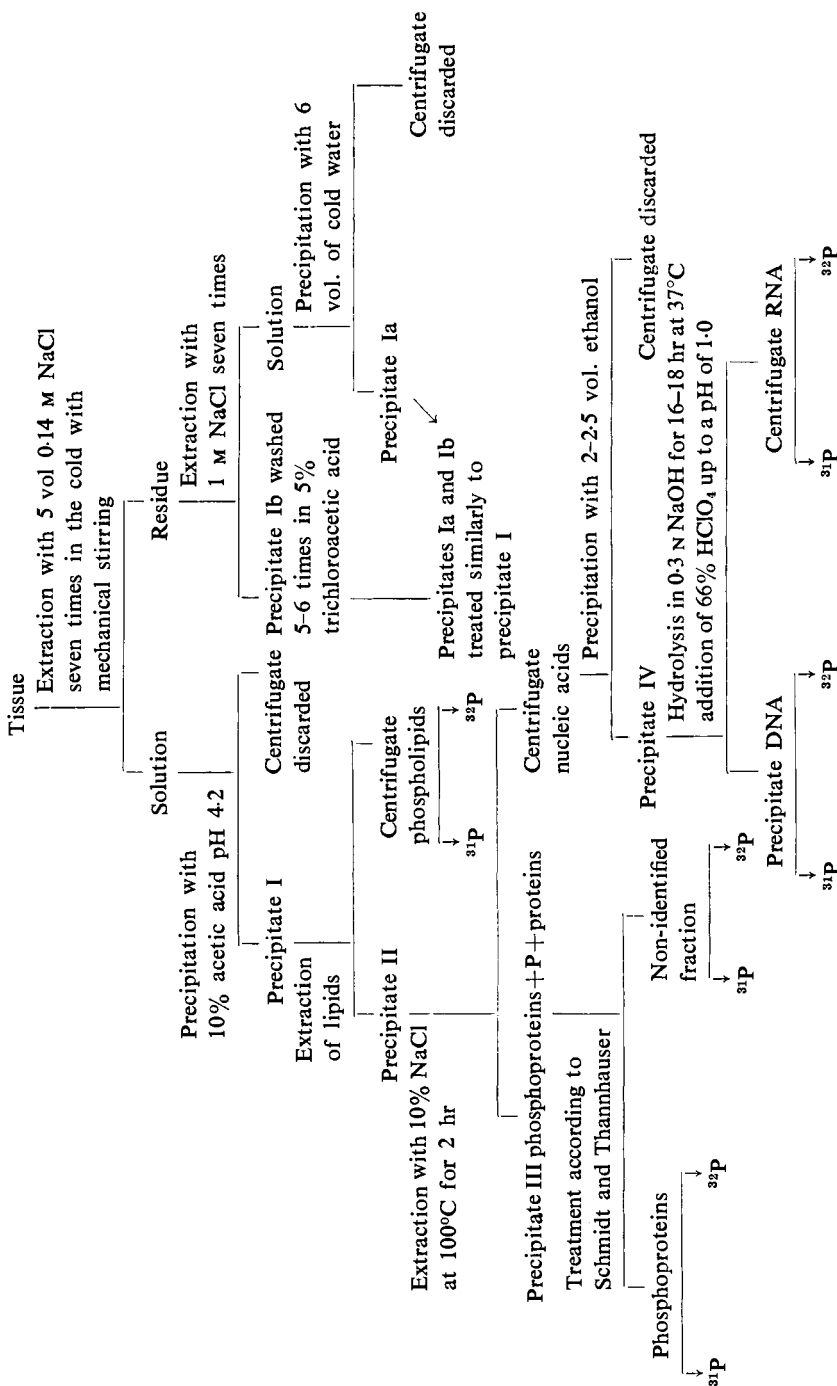
Tissue	Guanine	Cytosine	Uracil	Adenine
Brain	1.02	0.58	0.6	0.94
Nerve	6.83	0.69	0.13	0.78
Liver	1.61	0.30	0.44	1.34

The isolation of pure nucleic acids has enabled us to make a far more accurate comparative study of these compounds in various parts of the nervous system and also to assess the relative quantities of the nitrogenous bases composing the nucleic acids. We concluded from these results that the distribution of phosphonucleotides and phosphoproteins was related to differences in structure and function of different parts of the nervous system. The functionally complex and phylogenetically younger cerebral cortex is characterized by a greater abundance and turnover rate of phosphorus compounds than elsewhere in the nervous system.

NUCLEOPROTEINS AND OTHER PHOSPHORUS COMPOUNDS EXTRACTED BY NaCl

A great variety of nucleoproteins is known to exist (10-14) not only in the same individual but within the same tissue or even cell. The purpose of these studies was to determine how varied the distribution and turnover of nucleoproteins could be in the brain. Cortical grey and white matter

SCHEME OF ISOLATION OF NUCLEOPROTEIN FRACTIONS FROM THE BRAIN



and cerebellar tissue from cats was studied using ^{32}P injected at a dose of $0.23 \mu\text{c/g}$ body weight 24 hr before sacrifice. To separate cytoplasmic nucleoproteins from those in the nuclei, a method for the preliminary fractionation of the tissues by differential solvent extraction was used. This method, based on that of Mirsky and Pollister (15) consisted first of extraction by 0.14 M NaCl , then 1 M NaCl . We believe that 0.14 M NaCl extracts only ribonucleoproteins, leaving the desoxyribonucleoproteins in the nuclei; these are then extracted with 1 M NaCl .

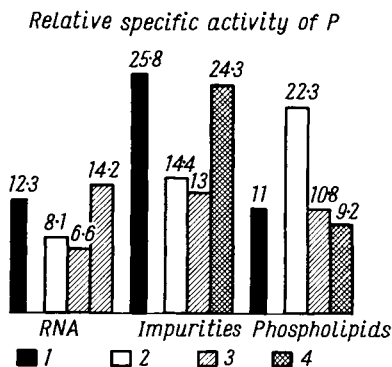


FIG. 3. Rate of incorporation of ^{32}P into phosphorus-containing compounds of various fractions of the grey matter of the brain of cats: 1—tissue; 2—fraction in 0.14 M NaCl ; 3—fraction in 1 M NaCl ; 4—residue.

Figure 3 shows how the ^{32}P differs markedly in its distribution between the NaCl fractionated compounds. Relative specific activity is expressed thus.

$$\text{RSA} = \frac{\text{SA of phosphorus of sample compound}}{\text{SA of tissue inorganic phosphorus}} \times 100$$

The RSA of the saline-soluble RNA was much lower than the RSA of the saline-insoluble residue. A similar difference was observed with the "RNA" substances. The phospholipids present quite a different picture. The RSA was much higher in the 0.14 M NaCl -soluble fraction than in the 1 M NaCl one. It seems that 0.14 M NaCl extracts only a few phospholipids but these have a very high turnover rate. The work of Khorkova (16) in which protein fractions from rabbit brain were studied is of great interest in connection with our results. Although the experimental conditions were different, the pattern of ^{32}P turnover rates in the various nucleic acids and phospholipids of the different solvent fractions were similar to those found by us (Fig. 4). The pattern was also similar from all parts of the brain although

the ^{32}P turnover rate of the combined tissue fractions could vary according to the tissue's source; for example the lower values for cerebellar tissue and white matter.

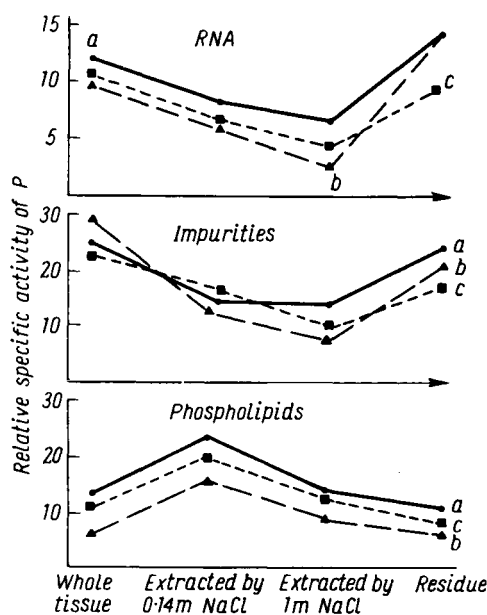


FIG. 4. Rate of incorporation of ^{32}P into phosphorus containing compounds of tissues and various fractions obtained from different parts of the brain of cats: a—grey matter; b—white matter; c—cerebellum.

These results led us to investigate the ^{35}S turnover rates of similarly fractionated tissues, using ^{35}S -methionine, and we found the highest rate of incorporation in the saline-soluble fraction. This is of interest regarding the theories of protein synthesis, in which RNA participates, since RNA protein complexes are thought to have higher turnover rates than other proteins (14, 18). As our data show greater ^{32}P and ^{35}S incorporation into the RNA fraction we conclude that protein synthesis is more active in the cytoplasm than the nucleus. This conclusion is further supported by preliminary experiments in which the rate of ^{35}S incorporation into cytoplasmic proteins of cat cerebral tissue has been found to be greater than that of the nuclear proteins. A similar possibility for non-cerebral tissues has also been put forward (15).

Since it has been found that in many types of tissue, nuclear RNA turnover is faster than in the cytoplasm, these variations between cellular components are hardly surprising. Our experiments in which the rate of ^{32}P

incorporation was found to be higher in the NaCl-insoluble RNA than NaCl-soluble RNA confirms this conclusion. On separating the nuclei by the method of Palladin *et al.* (19) we found that the rate of ^{32}P incorporation into the pure RNA extractable from the white cell was 35–40 per cent less than the corresponding value for nuclei only. Nuclear phospholipids are also characterized by a ^{32}P incorporation rate 3 times as great as for whole cells (Fig. 5).

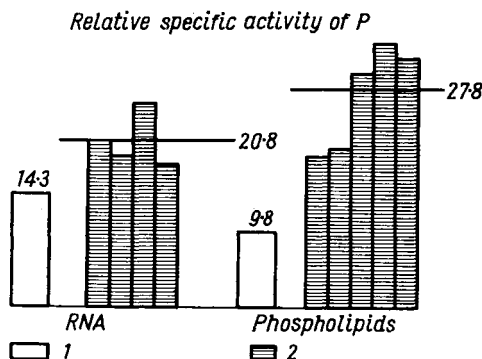


FIG. 5. Rate of incorporation of ^{32}P into RNA and phospholipids of whole tissue and nuclei of cat cerebral cortex: 1—whole tissue; 2—nuclei.

These experiments demonstrate the marked differences in the metabolic activity of nucleoproteins in various chemical fractions of the same tissue and also differences related to the source of tissue. For the phosphorus compounds described in general we can say that their metabolic activity varies at 3 levels; first, depending on the source or type of tissue, secondly, on the chemical means of fractionation and thirdly on the subcellular source of the material.

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ASPECTS OF LIPOPROTEIN TURNOVER IN THE BRAIN

G. YE. VLADIMIROV, T. N. IVANOVA AND N. I. PRAVDINA

Pavlov Institute of Physiology, Soviet Academy of Sciences, Leningrad

WHEN ribonucleic acid is extracted by the method of Schmidt and Thannhauser, it is contaminated by a considerable amount of organic phosphorus which we called "fraction X" (1). It has also been described as "inositide phosphorus" (2) or "residual organic phosphorus" (3). This fraction is of great interest, as it has a high rate of turnover, which is about 10 times greater than that of phospholipid and about 8 times that of ribonucleic acid in brain tissue. The phosphorus content of the fraction extracted from cerebral tissue is much higher than when it is extracted from other tissues.

The importance of the fraction in the total phosphorus metabolism of the brain is shown by the following calculation. The quantity of phosphorus exchanged in a phosphorus compound is proportional first to the relative specific activity (RSA) of the compound's phosphorus and secondly to its phosphorus content. To estimate the total quantity of phosphorus which is turned over $\text{RSA}\%$ has to be related to the total quantity of phosphorus in the compound in question. For an experiment lasting 3 hr one obtains the values shown in Table 1.

TABLE 1. COMPARISON OF TURNOVER RATES IN VARIOUS PHOSPHORUS-CONTAINING COMPOUNDS IN THE BRAIN TISSUE

Phosphorus fraction	P mg %	$\frac{\text{RSA}}{100}$	Replaced P mg
Phospholipids	160	0.021	3.36
Fraction "X"	19	0.24	4.56
Ribonucleic acid	10	0.029	0.29
Phosphoproteins	3.5	0.48	1.68

We have attempted here to characterize fraction X, its total amount and distribution in cerebral tissues, and its rate of phosphorus turnover.

METHODS

Rabbits and white rats received 0.3–0.5 $\mu\text{c/g}$ body weight of ^{32}P -labelled sodium diphosphate subcutaneously. The animals were decapitated and the brain homogenized in ice-cold 10 per cent TCA. The insoluble residue was washed repeatedly with 5 per cent TCA, water, acetone, ether, hot ether-alcohol and hot chloroform-methanol to remove acid-soluble and lipid phosphorus.

The phosphorus compounds in "fraction X" were separated from nucleotides and phosphoproteins by repeated extraction with acidified chloroform-methanol similar to Folch's method (4). The dried protein residue, freed from acid-soluble and lipid phosphorus was twice extracted at room temperature for 20 min with a 1:1 mixture of chloroform and methanol acidified with ion HCl to a concentration of 0.03 M. Control experiments in which the protein residue was hydrolysed by alkali, showed that such treatment led to almost complete extraction of the phosphorus present in "fraction X" by acid chloroform-methanol. Only about 20 $\mu\text{g/g}$ tissue remained unextracted.

If the residue is extracted with non-acidified chloroform-methanol only minute amounts of phosphorus with a low radioactivity are removed. The effect of acidifying the solvent can be explained in two ways. Either the HCl accelerates the breakage of the link between the protein and the lipid component or the lowered pH modifies the phosphorus compounds so that they become soluble in non-polar solvents. The former was found to be the correct explanation, since after extraction with the acidified solvent, if alkali was added to neutralize, as much phosphorus was extracted as without the neutralization step.

Alkaline hydrolysis of the dried chloroform-methanol extract (N NaOH) for 18 hr at 37°C separates the lipid from the phosphorus-containing portion. If the samples are neutralized, then acidified, an oily layer separates which is completely soluble in chloroform and contains no phosphorus. All the phosphorus remains, organically bound, in the aqueous phase. This suggests that the compound extracted is a lipoprotein.

The substance isolated by Schmidt and Thannhauser's method consists of 5 or more different compounds (5, 6) possessing different electrophoretic mobilities on paper. Of these, only inositol-diphosphate and phosphoserine have been identified.

We fractionated lipoproteins by differential solubility in chloroform into two types which had different rates of phosphorus turnover. For this procedure 9 ml water were added to 10 ml of the acid chloroform-methanol extract and after 2 min shaking they were centrifuged and the upper layer

of the aqueous methanol fraction was removed, leaving the chloroform-soluble fraction. Figure 1 shows that the variation in phosphorus content is about 18–19 mg%. About two-thirds of this is extracted by the chloroform and one-third by the aqueous methanol. The phosphorus extracted by the latter solvent has an extremely high turnover rate. Its RSA rises within 3 hr to about 41 per cent approaching the phosphoproteins' value which is the highest. The RSA of the chloroform-extracted phosphorus rises to 14.6 per cent 3 in₁ hr which is still much higher than the phospholipids (2 per cent).

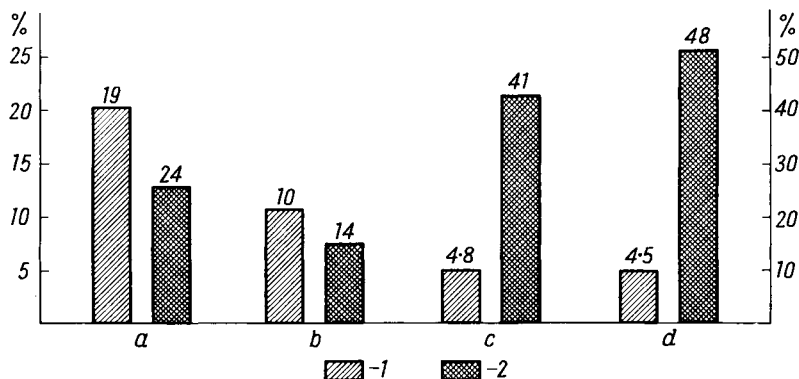


Fig. 1. Content (mg %) and relative specific activity (%) of lipoprotein phosphorus and phosphoprotein phosphorus in the cerebral cortex of rats: a—total lipoproteins; b—chloroform fraction; c—methanol fraction; d—phosphoproteins; 1—content of lipoprotein phosphorus and phosphoprotein phosphorus; 2—RSA.

The inositol-containing phosphorus compounds associated with RNA extracted by Schmidt and Thanhauser's method are known to have a very high rate of turnover (2). In view of this, we tried to identify inositol in the methanol-chloroform fraction; we did this by hydrolysing the extract with 6N HCl at 100°C for 25 hr conditions that Folch has shown to liberate inositol (7). The concentrated hydrolysate was put on to paper with saturated phenol solution and isopropanol : acetic acid : water (3 : 1 : 1) as solvents, and developed by ammoniacal silver nitrate. A standard 1 per cent solution of inositol, a hydrolysate sample from the chloroform extract, and a hydrolysate sample from the methanol extract (the latter two samples each being mixed with 1 per cent inositol), were placed on the chromatogram. The results showed that only the chloroform extracts contained material with an *R_f* value corresponding to inositol. Since the phosphorus compounds with a very high rate of turnover were extracted by methanol, we concluded that inositol-containing phosphorus compounds are not responsible for this high rate.

Dawson (8) has suggested that a possible cerebral component with an even higher turnover rate than diphosphoinositol is glycerophosphate, a product of phosphatidic acid. In view of this we endeavoured to detect phosphatidic acid by the action of phosphatases from young rats' blood on our extracts. Table 2 gives the results of the action of acid and alkaline phosphatases upon dried samples of the chloroform and methanol extracts. These show that both types of extract contained small quantities of glycerophosphate or similarly phosphorus ether-linked compounds (about 7 per cent of their total phosphorus). The inorganic phosphorus released had a much higher RSA than the total phosphorus in the extract.

TABLE 2. CONTENT AND RELATIVE SPECIFIC ACTIVITY (RSA) OF INORGANIC PHOSPHORUS CLEAVED FROM TOTAL LIPOPROTEINS BY PHOSPHATASES

Used for the sample mg	Phosphorus mg		RSA of phosphorus	
	Split off by the enzyme		Of the total extract	Split off at pH 10
	At pH 10	At pH 5		
188	12	3	14	20
188	14	2	14	20
188	10	3	14	19
188	15	—	14	22
100	10	1.5	26	45

We next considered the following phylogenetically and functionally differentiated regions of the brain: cortical grey and white matter, mesencephalon, corpora quadrigemina, cerebral peduncles, medulla oblongata, cerebellum and spinal cord.

The distribution of lipoprotein phosphorus is given in Fig. 2. The smallest amount (14 mg %) occurs in the grey matter and the greatest (37 mg %) in the spinal cord. Similar relations could be observed in the methanol- and chloroform-fractionated lipoproteins. The RSA values were lowest in the spinal cord (about 2.5 times less than in the grey matter) and highest in the white matter. The RSA values for the mesencephalon, medulla oblongata and cerebellum were similar.

The considerable variation in distribution and turnover of lipoprotein phosphorus in the brain is well illustrated by comparing the myelin-rich tissues of the white matter and spinal cord. Here the lipoprotein contents are similar (308 and 370 $\mu\text{g}\%$), but the RSA values for the methanol and chloroform extracts were 25 and 14 per cent for cerebral white matter and 8 and 4 per cent for spinal cord.

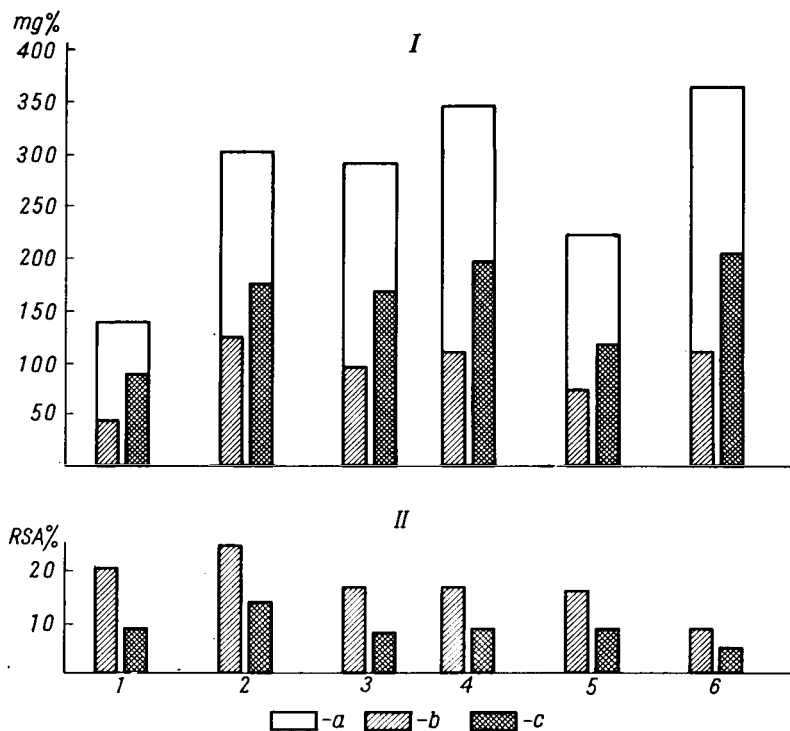


FIG. 2. Content (I) and relative specific activity (II) of lipoprotein phosphorus in various parts of the rabbit brain: a—total lipoproteins; b—methanol fraction; c—chloroform fraction; 1—grey matter; 2—white matter; 3—mesencephalon; 4—medulla oblongata; 5—cerebellum; 6—spinal cord.

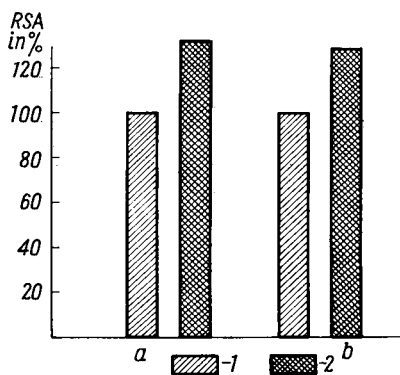


FIG. 3. Influence of stimulation of the central nervous system upon the rate of turnover of lipoprotein phosphorus: 1—narcotic sleep (100%); 2—stimulation; a—chloroform fraction; b—methanol fraction.

From previous studies (9) we found that the turnover rate of "fraction X" varied with the functional state of the central nervous system. It became slower during narcosis and more rapid during excitation. Similar results were obtained in the present experiments where lipoproteins were fractionated by chloroform and methanol. White rats were anaesthetized with sodium amylobarbitone (7 mg/100 g body weight), another group were stimulated by 20–40 V electrical stimulation of the paw, and a third group was anaesthetized under hypothermia. In this last group, as soon as the rectal temperature had dropped to the desired value, ^{32}P was administered and the animals killed 2 hr later. Results are given in Figs. 3 and 4. The values of the animals anaesthetized at normal body temperature are taken as 100 per cent and other results referred to it. These results confirm views expressed earlier, and the effects of hypothermia should be particularly noted. Figure 4 shows that a drop in body temperature from 37°C to 27°C causes a decrease in the methanol fraction phosphorus RSA value by 58 per cent and in the chloroform fraction by 70 per cent. Even under deep hypothermia, however, the lipoprotein RSA value reaches 5–7 per cent in 2 hr which indicates an important role in the function of the brain of this substance.

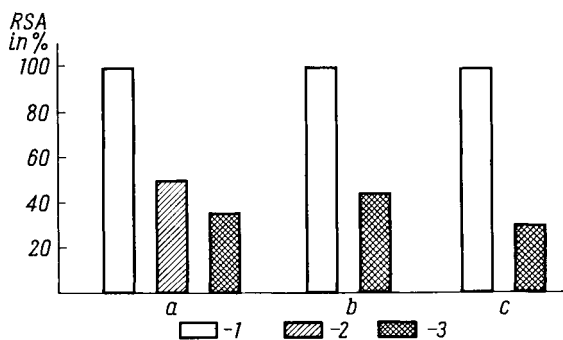


FIG. 4. The influence of hypothermia upon the rate of turnover of lipoprotein phosphorus: 1—temperature $37\text{--}35^\circ\text{C}$ (100%) 2— $33\text{--}26^\circ\text{C}$; 3— $20\text{--}13^\circ\text{C}$; a—total lipoproteins; b—methanol fraction; c—chloroform fraction.

CONCLUSIONS

From brain tissue, a lipoprotein type phosphorus compound was isolated which contained 16–19 mg % phosphorus and showed an RSA value of 24 per cent in 3 hr.

This substance could be fractionated into two parts on the basis of differential solubility in chloroform and methanol. The chloroform fraction

contained the greater amount of phosphorus with an RSA of 14 per cent in 3 hr and contained inositol. The methanol fraction contained no inositol and a lower amount of phosphorus reaching an RSA of 41 per cent in 3 hr.

This substance was unevenly distributed throughout the brain and varied in its turnover rate. Lower levels (15 mg %) were found in the grey matter and the greatest amount (up to 38 mg %) in the spinal cord.

Changes in the functional state of the nervous system altered the turnover rate of the lipoprotein phosphorus, stimulation leading to an increase in RSA and hypothermia producing a marked fall.

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METABOLISM OF PHOSPHORYLCHOLINE ESTERS AND ETHANOLAMINE ESTERS IN THE BRAIN

P. A. KOMETIANI, L. K. TKESHELASHVILI AND T. A. OVSYANKO

Department of Biochemistry, Beritashvili Institute of Physiology,
Georgian Academy of Sciences, Tiflis

IN 1953, at the first conference on the biochemistry of the nervous system organized in Kiev, we reported preliminary results of studies of the distribution and the metabolism of cerebral phosphorylcholine and ethanolamine compounds (1). The present paper summarizes further studies on the subject.

Phosphorylcholine and ethanolamine esters (phosphorylcholine (PCH) and phosphorylethanolamine (PE)) occur in the body both in a free state and linked to phospholipids. Phosphorylserine (PS) is closely related biochemically to these substances. The latter substance is incorporated into the structure not only of phospholipids, but also of phosphoproteins. PCH, PE and PS are, furthermore, found as double esters which form with glycerol, glycerylphosphorylcholine, glycerylphosphorylethanolamine and glycerylphosphorylserine respectively. These compounds are abundant in those tissues which have a high turnover of phospholipids and phosphoproteins (2).

The PCH and PE are of especial interest in view of their possible role in the synthesis of phospholipids as well as their participation in the synthesis of acetylcholine (8, 26). Until recently, their tissue concentrations had not been measured. In 1950, Awapura, Landua and Fuerst (3) published data on the tissue content of PE measured by chromatography. They found that the PE in rat tissues varied between 6–116 mg per 100 g fresh tissue, the greatest quantities being present in the spleen and in the pancreas.

In our laboratory, Tkeshelashvili (4) studied the distribution of PE and PCH in various organs and found that the PCH content varied between 2–20 mg % of fresh tissue. The greatest quantity of PCH was found in the liver, and the least in muscle. The brains of rats, rabbits and dogs contained respectively 300, 900 and 700 μ g of PCH per gram fresh weight. According to Tkeshelashvili (4) PE, at 140–400 μ g per gram, is always present in higher quantities than PCH. Unlike PCH which was most abundant in the liver, the greatest quantities of PE were found in the spleen and in the medulla

oblongata. According to earlier data of Ansell and Dawson (5), the content of PE in the brain varied between 4 and 13 mg % which seemed rather low. The more recent values of PE and PCH obtained by Ansell and Richter (6) and by Dawson (7) are in closer agreement with those of Tkeshelashvili.

The phosphorylation of choline by cerebral tissue observed in our laboratory (8) has been confirmed by other authors (9). Labelled phosphorus is incorporated into PCH and PE more rapidly (5, 10, 11) than into phospholipids. Phospholipids, as is well known, are not characterized by a high rate of turnover (12, 13) which suggests that PCH and PE are synthesized in the brain and liver by direct phosphorylation of choline and ethanolamine rather than from the hydrolysis of phospholipids.

Ansell and Dawson (5) and Norman and Dawson (14) have shown that after the injection of ^{32}P the radioactivity of PCH and PE in the liver is always higher than the radioactivity of the phospholipids.

Tkeshelashvili (4) studied the turnover of PCH and PE in the brain by injecting ^{32}P -labelled sodium phosphate suboccipitally into dogs and subcutaneously into dogs, rabbits and rats. When the sodium phosphate was injected subcutaneously into rabbits and rats, three hours later the maximal activity of PCH and PE was seen in the liver; in the brain, the relative activity of these compounds did not reach very high levels during this period. If one calculates the relative specific activity, however, the brain shows the greatest rate of PCH and PE turnover followed by the liver, spleen, kidney and muscle.

We studied the different rates at which labelled phosphorus was incorporated into PCH and PE in different parts of the brain of dogs. Labelled inorganic phosphate was injected subcutaneously in doses of $0.5\ \mu\text{c}$ per kg weight. Twenty-four hours later the animals were killed under anaesthesia, the brain quickly taken out, freed from the membranes and blood vessels, and analysed. PCH and PE showed the highest relative activity in the white matter and medulla oblongata, i.e. in those parts which contain the greatest quantity of phospholipids. The fact that the phosphorus in PCH and PE of the brain had a greater turnover rate is in agreement with the findings of Dawson (11).

The question of whether PCH and PE are used directly for the synthesis of phospholipids is still unanswered, however. If the phosphorus in PCH and PE has a higher turnover rate than the phosphorus in the phospholipids, and PCH is formed as a result of phosphorylation of choline and not as a result of decomposition of phospholipids, it would seem that the above compounds play a direct part in the synthesis of phospholipids. This conclusion, however, does not agree with earlier findings of Chaikoff (15),

Chargaff and Keston (16) or Kornberg and Pricer (17). These latter workers studied the synthesis of phosphatidylcholine in liver mitochondria and were led to conclude that choline was incorporated into di-acyl phosphatidic acid, without prior phosphorylation. They also found that coenzyme A participates in the esterification of glycerophosphate by fatty acids in the presence of ATP. On the other hand, Kornberg and Pricer (18) showed that ^{14}C - and ^{32}P -labelled PCH is incorporated by liver mitochondria into phosphatidylcholine without changes in the relative activity of the radioactive carbon and phosphorus. Furthermore, Rodbell and Hanahan (19) found that PCH could be incorporated into phosphatidic acid as a whole with simultaneous release of inorganic phosphate.

The apparent discrepancy is resolved by the demonstration of Kennedy and Weiss (20) that cytidine-5-triphosphate (CTP) participates in the synthesis of phosphatides. It appears that PCH in the presence of CTP can couple with the acceptor, forming phosphatidylcholine. To decide whether PCH and PE play a direct part in the synthesis of cerebral phospholipids, we carried out experiments to study the rate of uptake of labelled inorganic phosphate into the phospholipids as compared with the uptake of labelled PCH and PE. Dogs received suboccipital injections of IP in doses of 2–3 μC per kg body weight. Particular care was taken to standardize the experimental conditions. Relative specific activity (RSA) was expressed as a percentage of the radioactivity of inorganic phosphate. Phosphorus compounds were fractionated by the method of Schmidt and Thannhauser (21) as modified by Ivanova and Pravdina (22). PCH and PE labelled with ^{32}P were prepared by heating with labelled orthophosphoric acid in the presence of P_2O_5 at 165°C for 12 hr (23).

Twelve hours after injecting the radioactive phosphorus compounds at doses of 0.2–10 μC per kg body weight, the animals were killed by injecting ether into the heart, the brain quickly removed and homogenized in 10 per cent TCA at 0°C . Table 1 gives comparative data from experiments using labelled phosphoryl choline and labelled inorganic phosphate.

We found no difference in the radioactivity of the lipid fraction nor of the acid-soluble fraction, whether PCH or inorganic phosphate was used. Higher radioactivity was found in phosphoproteins and nucleic acids when inorganic phosphate was used. Had the lipid fraction been more radioactive when labelled PCH was used, this would have meant that PCH participated without preliminary hydrolysis in the synthesis of phospholipids. This was obviously not the case.

In the experiments described above, the individual radioactivities of the phospholipids were not differentiated so that the fates of injected PE

TABLE 1. THE RSA OF PHOSPHORUS COMPOUNDS IN BRAIN AS PER CENT OF THE RADIOACTIVITY OF INORGANIC PHOSPHORUS AFTER SUBOCCIPITAL INJECTION OF LABELLED INORGANIC PHOSPHATE AND PHOSPHORYLCHOLINE
(Results of one of four similar experiments)

Part of brain	Phosphorus fraction			
	Acid-soluble fraction	Lipid fraction	Ribonuclein fraction	Phosphoprotein fraction
After injection of labelled inorganic				
Grey matter of the cerebral hemispheres	56.8	10.7	13.7	100.2
Cerebellum	59.6	8.5	5.7	121.7
After injection of labelled phosphorylcholine				
Grey matter of the cerebral hemispheres	55.2	10.9	8.5	39.4
Cerebellum	57.4	7.7	4.5	40.0

and PCH were not fully known. Experiments were therefore carried out on rabbits, killing them 5 hr after suboccipital injection of the labelled compound ($2.5 \mu\text{c}$ per kg body weight). The brain was analysed for the substances quoted above, as well as for phosphatidyl choline, total cephalin fraction and phosphatidylethanolamine (see Table 2). Control animals received inorganic phosphate. The individual phospholipids were isolated and estimated by the method of Dawson (24).

TABLE 2. SPECIFIC ACTIVITY OF PHOSPHORUS COMPOUNDS ($\times 10^3 \text{ mg P}$) AFTER SUBOCCIPITAL INJECTION OF LABELLED INORGANIC PHOSPHATE, PHOSPHORYLCHOLINE AND PHOSPHORYLETHANOLAMINE
(Analysis carried out 5 hr after the injection)

Phosphorus fraction	Injected		
	Inorganic phosphate	Phosphorylcholine	Phosphorylethanolamine
Total lipid fraction	8.1	2.5	—
Phosphatidylcholine	9.8	8.8	—
Ratio:			
Phosphatidylcholine			
Total lipid fraction	1.1	3.5	—
Total cephalin fraction	25.6	—	10.5
Phosphatidylethanolamine	9.3	—	8.8
Ratio:			
Phosphatidylethanolamine			
Total cephalin fraction	0.36	—	0.84

Table 2 shows the relative activity of individual phospholipids compared with the total lipid content. Where inorganic phosphate was administered, the ratio of the specific activity (SA) of phosphatidylcholine to the RSA of total lipids was 1:1, and where PCH was administered 3:5. In experiments where inorganic phosphate was administered, the ratio of the RSA of the phosphatidylethanolamine to the total cephalin fraction was 0.36 and where PE was administered, the value was 0.84. The higher values obtained with PCH and PE indicate that these compounds may play a direct role in the synthesis of phosphatidylcholine and phosphatidylethanolamine.

It must be remembered that these differences tend to be minimized *in vivo*. Also the permeability of the cell membrane to phosphorylated esters is low. In view of this, we decided to study the possible role of PCH and PE in the synthesis of phospholipids in a simpler experimental system, namely cerebral homogenates.

For each experiment 300 mg of tissue homogenized in 1.2 ml of isotonic KCl was used. Final concentrations of other components were: barbiturate buffer, pH 7.2—0.1 M; phosphate buffer pH 7.2—0.001 M; ATP—0.001 M; $MgCl_2$ —0.017 M; NaCl—0.019 M; glutamate—0.02 M; radioactive phosphorus compound—25–30 μ c. Aerobic incubation was for 1 hr at 37°C.

TABLE 3. THE INFLUENCE OF ACETYLCHOLINE, PHOSPHORYLCHOLINE AND CHOLINE UPON THE RATE OF INCORPORATION OF LABELLED INORGANIC PHOSPHORUS INTO THE PHOSPHOLIPIDS OF THE BRAIN

(Relative activity $\times 10^3$ mg P)

Reaction medium	Phosphorus fraction		
	Total lipid fraction	Total cephalin fraction	Phosphatidylcholine fraction
^{32}P	16	12	1
^{32}P + acetylcholine	32	31	2
^{32}P + phosphorylcholine	8	7	0.7
^{32}P + choline	25	18	1.4

When radioactive inorganic phosphate was used, considerable incorporation into phospholipids occurred. The SA of phosphatidylcholine reached 50 per cent of total phospholipid activity and phosphatidylethanolamine reached 20 per cent of the cephalin fraction. On the other hand, with labelled PCH and PE, no incorporation into phospholipids occurred, presumably because of deficiencies in the composition of the reaction mixture, e.g. absence of CTP.

PCH and PE are polarized compounds and such properties affect the physico-chemical state of lipoproteins in the protoplasm. Hokin and Hokin (25) found that acetylcholine increases the rate of incorporation of ^{32}P labelled inorganic phosphate into phospholipids of cerebral homogenates. Accordingly we studied the effects of adding choline, acetylcholine or PCH to the reaction system at 0.5×10^{-4} M on the rates of ^{32}P -labelled inorganic phosphate incorporation into phospholipids. 20 μc of ^{32}P were added and aerobic incubation lasted 2 hr. Table 3 shows that both choline and acetylcholine increased the rate of incorporation into total phospholipids as a whole, as well as in concentrations of the cephalin and lecithin fractions. PCH however, had an inhibitory effect on the incorporation into phospholipids as a whole and into the lecithin and cephalin fractions separately.

SUMMARY AND CONCLUSIONS

1. PCH is primarily formed in the brain as a result of the phosphorylation of choline.

2. PCH and PE can be utilized directly for the synthesis of the corresponding phospholipids, without prior hydrolysis.

3. PCH inhibits the incorporation of inorganic phosphate into phospholipids of the brain.

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GLYCOLYSIS AND PHOSPHOPROTEIN METABOLISM IN BRAIN SLICES

N. P. LISOVSKAYA

Bakh Institute of Biochemistry, Soviet Academy of Sciences, Moscow

It is well known that the main source of energy for the brain comes from blood glucose during glycolysis and aerobic oxidation. Both processes yield energy-rich phosphates ($\sim P$) but with very different efficiencies. Glycolysis yields 2 molecules of $\sim P$ per molecule of glucose, whereas aerobic oxidation yields 24–38 $\sim P$ per molecule (1). In spite of the much greater efficiency of aerobic oxidation to synthesize $\sim P$, glycolysis of sufficient degree might be able to satisfy the energy requirements of the brain under conditions of low demand. It seems, however, that differences between glycolysis and aerobic oxidation of nervous tissue are not only quantitative but qualitative. Certain processes cannot occur in the brain in the absence of aerobic oxidation in spite of complete facilities for glycolysis.

One of the processes is the incorporation of ^{32}P into cerebral phosphoprotein (PP). In a previous paper (2) it was shown that a distinct relationship existed between PP metabolism and the preservation of aerobic oxidation in the brain, since on inhibiting such oxidation, PP turnover was stopped. The question arose as to what role was played by glycolysis which remained uninhibited, so the effects of the process on PP metabolism were accordingly studied, using rat cortical grey matter.

Slices were cut and incubated in a McIlwain saline to which ^{32}P had been added. Respiration, glycolysis (via lactate accumulation in the medium using Kuttner's method (3)), tissue inorganic phosphate (Pi), ATP, PP (including incorporated ^{32}P), were measured. Phosphoprotein was isolated by Schmidt and Thannhauser's method. In order to measure respiration and glycolysis separately, the slices were incubated under anaerobic conditions to inhibit respiration, or with $1-2 \times 10^{-5}M$ bromacetic acid to inhibit glycolysis. Bromacetic acid at this concentration does not affect respiration appreciably (Fig. 1).

Figure 2 gives values for lactic acid from slices incubated under an oxygen or nitrogen atmosphere, with and without bromacetic acid. They show that

glycolysis is lower under aerobic than under anaerobic conditions. Estimation of the final lactic acid content is, however, not necessarily proof of its speed of formation since some of it might be oxidized further aerobically but preserved anaerobically. Figure 2 is meant only to show the inhibitory effect of bromacetic acid on aerobic and anaerobic lactic acid formation. Maximal ATP levels are attained under aerobic conditions dropping to about a half in the presence of bromacetic acid. This latter set of conditions gives the same ATP levels as under anaerobic conditions without bromacetic acid: that is, when aerobic oxidation occurs in the absence of glycolysis, one gets the same levels of ATP as when anaerobic glycolysis alone occurs. With anaerobiosis and bromacetic acid (both respiratory processes inhibited), minimal amounts of ATP are found.

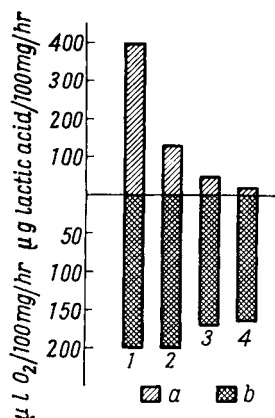


FIG. 1. The influence of various concentrations of bromacetate upon glycolysis and respiration of tissue slices from rat cerebral cortex: a—glycolysis; b—respiration; 1—without bromacetate; 2— 10^{-5}M bromacetate; 3— $2 \times 10^{-5}\text{M}$ bromacetate; 4— 10^{-4}M bromacetate.

With regard to PP metabolism, the maximum rate of ^{32}P incorporation is observed under aerobic conditions being greatly reduced in the absence of aerobic oxidation (Fig. 2 b, column 3). Hence glycolysis alone cannot maintain the normal PP turnover rate, but this does not mean to say that it plays no part in the PP metabolism. This is because inhibition of glycolysis under aerobic conditions causes a fall in the PP turnover rate, showing the need for simultaneous glycolysis and aerobic oxidation for maximal PP turnover. Can these relationships be explained purely on an energetic basis? Apparently not, since on comparing columns 2 and 3 of Fig. 2 b, one sees that although the ATP levels are the same under the two types of condi-

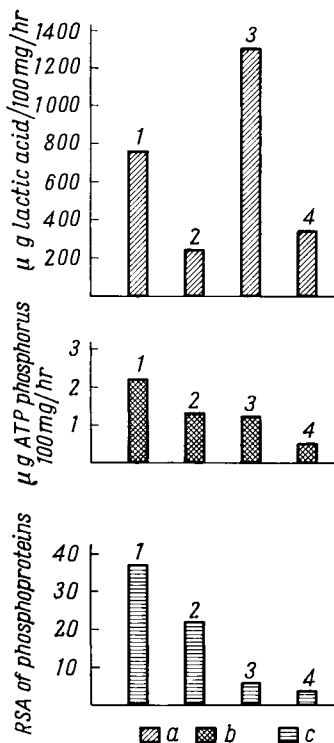


FIG. 2. Metabolism of phosphoproteins, resynthesis of ATP and formation of lactic acid during inhibited respiration and glycolysis in rat brain slices; a—lactic acid; b—ATP; c—phosphoproteins; 1—without inhibitor, O_2 ; 2— 10^{-5}M bromacetate; 3—without inhibitor, N_2 ; 4— 10^{-5}M bromacetate, N_2 ;

$$\text{RSA}-(\text{relative specific activity}) = \frac{\text{relative radioactivity of phosphoprotein phosphorus}}{\text{relative radioactivity of inorganic phosphorus}}.$$

tions, prevention of aerobic oxidation (column 3) produces a sharp fall in PP turnover. It is, therefore, apparent that optimal PP turnover is dependent upon some factor other than an adequate supply of energy and to investigate this problem further, a variety of oxidizable substrates have been studied with regard to their ability to sustain ATP synthesis and PP turnover. The results of these studies are given in Table 1.

These substrates can be divided into 4 groups. The first, containing glucose and mannose, are actively oxidized by cerebral slices and there is maximal ATP synthesis with high PP turnover. The second, containing fructose and pyruvic acid gives maximal respiration and ATP synthesis but PP turnover is almost nil. In the third group the substrates are oxidized but there is no ATP

TABLE 1. EFFECT OF VARIOUS SUBSTRATES ON RESPIRATION, RESYNTHESIS OF ATP AND PHOSPHOPROTEIN METABOLISM IN BRAIN SLICES

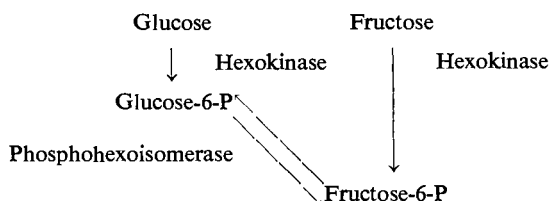
Substrate class	Preparatory substrate	Respiration	Resynthesis of ATP	Phosphoprotein metabolism
1	Glucose	++	++	++
	Mannose	++	++	++
2	Fructose	++	++	—
	Pyruvic acid	++	+	—
3	Glutamic acid	++	—	—
	Succinic acid	+	—	—
4	Fumaric acid	—	—	—
	Malonic acid	—	—	—
	Galactose	—	—	—

synthesis or turnover. In the fourth group neither oxidation, ATP synthesis nor PP turnover occurs.

The absence of PP turnover without ATP synthesis is understandable, but the substrates of the second group are remarkable in that ATP is synthesized but PP turnover is absent. Findlay *et al.* (4) have also found a very slow PP turnover in cat cerebral slices if fructose is used as substrate.

It appears then that glycolysis may well be essential for PP turnover since on using fructose as substrate no glycolysis occurred, in spite of its being oxidized at a similar rate to glucose (see also (5)). The results of an experiment employing fructose as a substrate are given in Fig. 3. Here ATP synthesis is only slightly less than when glucose is used, whereas the PP turnover is as low as when no oxidizable substrate is present.

Hence, although ATP synthesis can be maximal, the PP turnover rate will be diminished unless glycolysis occurs simultaneously. It is also important to know why fructose is converted to lactic acid so slowly even anaerobically. The following diagram suggests why anaerobic oxidation of glucose and fructose differs.



Glucose is converted to glucose-6-phosphate by hexokinase and then oxidised further via the pentose-phosphate pathway or converted to fructose-

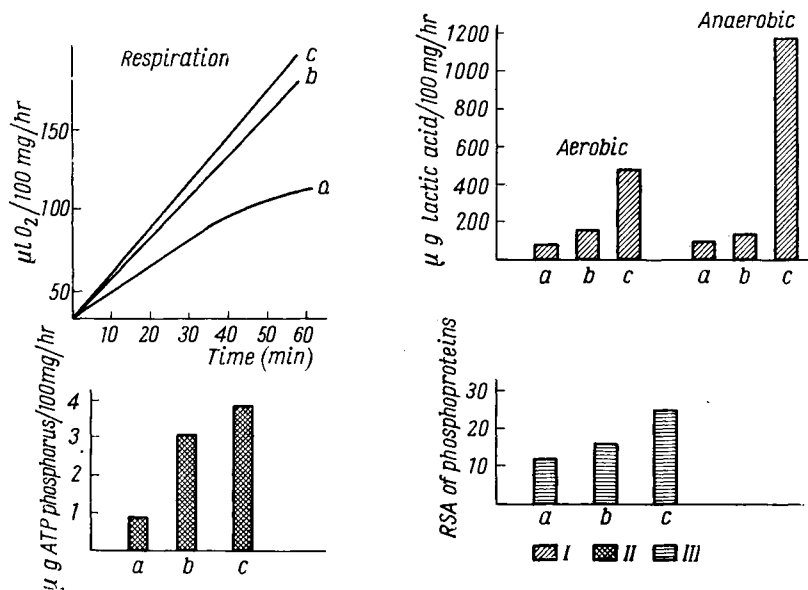


FIG. 3. Respiration, glycolysis, resynthesis of ATP and phosphoprotein metabolism in the presence of glucose and fructose: I—lactic acid; II—ATP; III—phosphoproteins; a—without substrate; b—fructose; c—glucose.

6-phosphate. Under aerobic conditions, the consumption of fructose-6-phosphate is minimized, but anaerobically it is more rapidly required to feed the glycolytic pathway via fructose-1:6-diphosphate. (6). In the brain the same hexokinase enzyme acts on glucose and fructose (unlike muscle tissue where 2 enzymes are necessary). Since the further metabolism of fructose-6-phosphate is by the same pathway, the differences observed above regarding the low rate of lactic acid formation using fructose as a respiratory substrate must be explained by a factor operating prior to the formation of fructose-6-phosphate which for fructose can only mean at the hexokinase stage. It seems likely that hexokinase has a lower affinity for fructose than for glucose. In fact Crane and Sols (7) have shown that the Michaelis constant for the phosphorylation of glucose by hexokinase is 8×10^{-6} and for fructose 1.6×10^{-3} . This provides a satisfactory explanation for the difference in the rate of glycolysis, but the question of equal rates of aerobic oxidation of fructose and glucose remains unanswered. One would expect variations in the rates of glycolysis to affect the rates of aerobic oxidation as well but since this is not so, one is inclined to believe that the aerobic oxidation of glucose and fructose is by way of different pathways from the very beginning.

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THE METABOLISM OF DIFFERENT PHOSPHOLIPIDS IN THE BRAIN

A. A. SMIRNOV AND YE. V. CHIRKOVSKAYA

Laboratory of Comparative Biochemistry of the Nervous System, Pavlov
Institute of Physiology, Soviet Academy of Sciences, Leningrad

WHEN studying the individual phospholipids of the brain, the extraction procedure is very important. The tissue can be treated with trichloroacetic acid (TCA) and the lipids extracted with boiling solvents (1) (Method I) or else the lipids can be extracted in the cold without preliminary treatment with TCA (2) (Method II).

METHODS

The rats, rabbits and dogs were injected with ^{32}P some hours before sampling. The samples were ground in a mortar with sand before treatment by one of the two methods (the sand was shown to have no effect itself). *Method I:* Acid-soluble phosphorus was removed by repeated washing with 5 per cent TCA at 0°C . Samples were taken from the first washings for specific activity (SA) measurements of the inorganic phosphorus. Lipids were extracted by mixtures of boiling 1:1 chloroform-ethanol and 3:1 ethanol-ether. *Method II:* Lipids were extracted by repeated washing with 1:2 ethanol-chloroform in the cold. The extract was freed from inorganic phosphate by washing with an equal volume of 0.1 N HCl. at 0°C . Centrifugation enabled a sharper separation of the layers but a dense white layer remained at the interface. The aqueous layer was removed, and to the remainder was added one third of the volume of the original extracting solvent, whereupon the white residue dissolved. This procedure was repeated twice with 0.1 N HCl, and twice with 0.01 N HCl, to remove virtually all contaminating inorganic phosphorus.

The specific activity of the inorganic phosphorus in Method II was measured in the 5 per cent TCA extract from the lipid-free tissue residue.

The total phosphorus content and phosphorus content of the lipid extracts was estimated and from their respective SA values the relative specific activity (RSA) was calculated. Results of these experiments are given in Table 1. These show that Methods I and II lead to marked differences

TABLE 1. QUANTITY AND RELATIVE SPECIFIC ACTIVITY OF PHOSPHOLIPIDS IN THE BRAIN OF ANIMALS

Animal	Duration of experiments (hr)	Method of extraction	P in $\mu\text{g/g}$ tissue	Specific activity of inorganic phosphate	RSA of lipids $\times 100$
Rat (cerebral hemispheres)	3	1	1710	11.1	2.2
		2	2150	10.6	3.5
Rabbit (cerebral cortex)	5	1	1010	14.3	2.3
		2	1535	14.5	2.9
Dog (cerebral cortex)	5	1	1555	32.4	2.8
		2	1855	30.3	3.5
Rat (cerebral hemisphere)	3	1	1590	—	2.1*
Dog (motor zone of the cerebral cortex)	5	1	1500	—	3.1**

* Vladimirov, Ivanova and Pravdina (3)

** Smirnov (4, 5)

in the quantity and RSA of phospholipids extracted. Method II gives a greater quantity of phospholipid with a higher RSA than Method I. This difference is due to the SA of the phospholipid itself, since the SA of the inorganic phosphorus is the same in both methods.

For comparison, we quote in Table 1 data of other authors (3, 4, 5) who used Method I. Their results which are similar to ours.

The apparent loss of phospholipids during Method I may be due either to destruction by treatment with TCA or incomplete extraction. We therefore decided to discover what phospholipids were lost during extraction by this method, since they included those with a high SA. This was done by fractionating the phospholipids derived from the two methods by the procedure of Dawson (6) following removal of inorganic phosphate.

FRACTIONATION OF PHOSPHOLIPIDS

Isolation of the Methanol-Insoluble Cephalin Fraction

A 1 per cent solution of wax in chloroform is added to the lipid extract and quickly evaporated under reduced pressure (100–200 mg Hg) in a water-bath at not more than 60°C. The residue is treated with hot methanol, cooled to 0°C for 30 min and centrifuged. The supernatant is washed three

times with methanol, made turbid with chloroform, and filtered. The residue on the paper is washed twice with chloroform, the extract containing di-phosphoinositide and probably proteolipid "C" which is incompletely broken down when the original phospholipid extract is dried (7). The residue contains phosphorus which is almost completely extracted by 0.04 N HCl-acidified chloroform-methanol. Neither treatment with the HCl nor the chloroform-methanol alone, even after 24 hr, removes this phosphorus suggesting that it is a lipoprotein.

Isolation of the Methanol-Soluble Cephalin Fraction

100 mg MgO per 100 mg of original tissue is added to the methanol soluble fraction and the mixture shaken at intervals for 30 min. The MgO with adsorbed lipids of the cephalin fraction is separated by centrifugation and washed twice with methanol, when mainly choline-containing phospholipids remain in the supernatant.

The cephalins are freed from the MgO by treatment with cold N HCl. Chloroform is next added and the mixture shaken for 2-3 min, cooled and centrifuged. The upper aqueous layer is discarded and the remaining chloroform extract contains mainly the cephalins: serine-phosphatide, ethanol-amino-phosphatide and probably acetal-phosphatides.

Isolation of Lecithin and Sphingomyelin

The methanol solution of choline-containing lipids is quickly evaporated *in vacuo* at not greater than 60°C. The dried residue is hydrolysed in 1 N NaOH for 20 hr at 37°C, and cooled and neutralized with HCl. The unsaponifiable material (sphingomyelin) is precipitated with TCA and filtered from the lecithin in solution. The residue is washed with 10 per cent TCA.

Isolation of Lipoproteins

After removal of lipids from the tissue by Method I, the residue is kept for several days in the cold with 1:2 ethanol-chloroform acidified with 0.04 N HCl (8). Under these conditions the phospholipid component of the lipoproteins separates out.

If the tissue is treated by Method II, the lipoproteins are extracted from the residue after it has been washed in 5 per cent TCA. The remaining traces of TCA are removed with alcohol. Lipoproteins are extracted for several

TABLE. 2. CONTENT AND RSA OF VARIOUS PHOSPHOLIPIDS FRACTIONS IN THE BRAIN OF ANIMALS

Animal	Duration of experiments (hr)	Method of extraction	Cephalin fraction						Lecithin	Sphingomyelin		Lipoproteins					
			Fraction insoluble in methanol		Fraction insoluble in chloroform		Fraction insoluble in methanol			$\mu\text{g P in 1 g tissue}$	RSA + 100	$\mu\text{g P in 1 g tissue}$	RSA $\times 100$				
			Fraction soluble in chloroform	$\mu\text{g P in 1 g tissue}$	RSA $\times 100$	$\mu\text{g P in 1 g tissue}$	RSA $\times 100$	$\mu\text{g P in 1 g tissue}$	RSA $\times 100$								
Rat (cerebral cortex)	3	I	61	4.3	70	7.3	395	2.5	3.1	92	0.7	162	19.5				
		II	74	7.2	42	13.2	768	4.7	3.5	92	0.8	33	46.6				
Rabbit (cerebral cortex)	5	I	—	—	—	—	312	1.8	1.8	122	0.4	120	33.0				
		II	—	—	—	—	643	3.6	2.0	143	0.9	32	51.0				
Dog (cerebral cortex)	5	I	11	3.7	106	7.0	415	3.0	2.8	150	—	140	16.5				
		II	18	5.1	30	6.8	592	4.2	3.0	153	0.5	20	38.1				

days in the cold by an acidified (0.04 N HCl) alcohol-chloroform mixture. The results of fractionating the phospholipids obtained by Methods I and II are given in Table 2.

The loss of phospholipids during Method I is manifested to some degree in almost all the fractions. The loss is, however, particularly noticeable in the methanol-soluble cephalin fraction. The RSA of all fractions obtained via Method I is lower than the values for Method II and again the difference is most marked in the methanol-soluble cephalin fraction.

These differences were found in experiments on rats, dogs and rabbits. In all cases extraction by Method II gave almost twice the amount of phospholipids in the cephalin fraction (serine- and ethanolamine-phosphatide) along with a higher RSA which suggests a loss of metabolically active phospholipids by Method I. Such a loss could be partly due to the breakdown of acetal-phosphatides by TCA treatment (9). The acetal-phosphatides are very soluble in methanol and contain no choline. They, along with ethanolamine-phosphatide and serine-phosphatide, are then adsorbed by MgO, hence Method II retains these acetal-phosphatides which constitute about 8-10 per cent of brain phospholipids (10).

To follow the fate of the phospholipid lost during extraction by Method I, we investigated the quantitative distribution of phosphorus in the cerebral cortex of rats after extraction by both methods.

If the lipids are extracted by Method I, 3 and more times phosphorus is found in the lipoprotein fraction but the RSA is only half that found when Method II is used. We conclude that the smaller amount of phosphorus found in the lipoprotein fraction by Method II is due to incomplete extraction, and even if the residue is treated either with non-acidified ethanol-chloroform or acidified solvents, no further appreciable quantities of phosphorus can be extracted. Hence we conclude that all this phosphorus is from lipoprotein.

It seems that the increase in the amount of lipoprotein phosphorus paralleled by a decrease in total phospholipid extracted by Method I is due to changes in the solubility of certain protein-lipid complexes. Proteolipids normally soluble in fat solvents, after exposure to a denaturing agent like TCA cease to remain so and remain in the residue. Since lipoproteins can be extracted only by acidified organic solvents, this explains why these proteolipids could appear in the lipoprotein fraction.

We feel that division of lipid-protein complexes by Folch into lipoproteins and proteolipids, based on organic solvent solubility, is rather arbitrary. Depending on the method of treatment, protein-lipid complexes classified according to their solvent solubility, can change from one class to another.

The lower RSA of the lipoproteins obtained after extraction by Method I can be explained by the fact that this fraction contains, along with highly active phospholipids, the less active phospholipids of the proteolipids.

TABLE 3. PHOSPHORUS BALANCE IN THE BRAIN OF RATS ESTABLISHED BY THE FIRST AND SECOND METHOD OF EXTRACTION

	³² P (in µg/g wet tissue)		
	1st method	2nd method	Difference
Acid-soluble phosphorus			
trichloroacetic acid	884	705	179
Washings (HCl)	—	16	—16
Lipids	1958	2226	—268
Lipoproteins	136	47	89
Proteins residue	314	313	1
Total	3292	3307	—15

TABLE 4. THE RSA OF PHOSPHOLIPID FRACTIONS OF THE BRAIN IN VARIOUS FUNCTIONAL STATES

Phospholipid fraction		Cerebral cortex			
		Dog		Rabbit	
		Amytal-induced sleep without hypothermia	Amytal-induced sleep + hypothermia at 26°C	Relative rest	Stimulation with desoxyephedrine
Phospholipids (total)		2.8	0.7	2.9	3.7
Cephalin fraction	Soluble in chloroform (diphosphoinositide)	5.9	3.7	—	4.6
	Fraction insoluble in methanol	6.8	4.4	—	13.0
	Fraction insoluble in chloroform (protein-lipid complexes)	2.7	0.9	3.7	4.0
	Fraction soluble in methanol	2.2	0.5	2.4	3.3
Lecithin		31.5	27.3	46.0	46.0
Lipoproteins					

The relative rates of incorporation of ³²P into various phospholipids and lipoproteins of the cerebral cortex from sleeping and conscious animals have been measured, and Table 4 gives the results of these experiments, along with data for other functional states. Depressed metabolism due to

hypothermia under narcosis is reflected in the decreased phospholipid turnover. The rate of ^{32}P incorporation into the phospholipids of dog cerebral cortex was studied under amylobarbitone narcosis at normal and lowered (26°C) body temperatures. Without hypothermia, phospholipid turnover was reduced to one-third or a quarter of the normal, the cephalin fractions and lecithin showing similar decreases, whereas lipoprotein ^{32}P incorporation was hardly affected. Hypothermia, furthermore, had little effect on the turnover of the methanol-insoluble "diphosphoinositide and proteolipid C". Thus, these phospholipids which possess the highest rate of turnover are the least affected by hypothermia, as judged by ^{32}P incorporation.

Stimulation by desoxyephedrine (7 mg per kg body weight) produces a 30 per cent increase in the general phospholipid turnover. Lecithin turnover shows a similar increase and methanol-soluble cephalin fraction less. The lipoprotein fraction is scarcely affected in its ^{32}P incorporation rate (note: the exposure period of 5 hr was too long to be conclusive).

It seems that the turnover rate of lipoproteins is unaffected by conditions that affect the turnover of other phospholipids considerably.

CONCLUSIONS

1. When phospholipids are extracted from brain tissue with a cold mixture of chloroform and ethanol, without prior treatment with TCA (Method II), more phospholipids with a higher RSA are obtained than with Method I using TCA. The phospholipids lost are found in the acid-soluble fraction.

2. The extra phospholipids extracted by Method II are especially rich in the methanol-soluble cephalin group, i.e. cephalin, serine- and ethanolamine-phosphatides. It is thought that TCA causes a loss of acetal-phosphatides from this fraction.

3. The lipoproteins extracted by Method I contain more lipid phosphorus than those extracted by Method II, but have a much lower RSA. We conclude that owing to denaturation by TCA, the solubility characteristics of brain proteolipids change so that they can only be extracted by acidified organic solvents.

4. Various physiological states influence the rate of ^{32}P incorporation into those lipids with a low turnover, like serine- and ethanolamine-phosphatides, and lecithin, but do not affect the lipoproteins with a high rate of turnover.

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UTILIZATION OF GLUCOSE AND PYRUVIC ACID BY THE BRAIN IN VARIOUS FUNCTIONAL STATES

G. KH. BUNYATYAN

Department of Biochemistry, Institute of Physiology, Armenian Academy of Sciences and Department of Biochemistry, Erevan Medical Institute

KNOWLEDGE of the biochemistry of the brain requires study of the types of control which the brain exerts on effector organs. This control is mediated through two basic nervous processes, stimulation and inhibition. It is therefore necessary to know how these two processes affect both the metabolism [A/V] of the effector organs and the metabolism of the brain itself.

Much important work can only be done *in vivo* measuring arteriovenous [A/V] differences and cerebral blood flow.

Cortical stimulation produced by conditioned reflexes and such unconditioned stimuli as adrenaline, insulin, pain, food stimulation, etc. on the metabolism of effector organs has been extensively studied on dogs at our Institute. Cortical inhibition has been similarly studied by extinction of the conditioned reflex and conditioned inhibition. These studies showed that cortical stimulation and inhibition have an opposite effect on the metabolism of the effector organ (1-7). Thus during inhibition, the levels of several substances that had become depleted in the effector organ during stimulation would be restored. Hence one expects a period of inhibition to be essential following a period of activity, not only for the brain but for other organs. It is interesting that intensive inhibition can terminate the effects of unconditioned stimuli. Adrenaline, for instance, did *not* cause hyperglycaemia, or alter blood clotting; insulin did *not* produce hypoglycaemia; pain did *not* accelerate blood clotting or alter the Ca^{++} associated with prothrombin during intense inhibition induced by extinguishing the corresponding reflexes. These stimuli had to be applied 2 or 3 times before showing normal reactions.

We studied the uptake of glucose and pyruvic acid by brain tissue during cortical stimulation and inhibition. Uptake was measured by cerebral A/V difference. Cerebral blood flow rate was measured with ^{32}P , blood samples being taken from the carotid artery in a skin flap and the external jugular

vein from which all branches were ligatured except the posterior facial vein connecting with the transverse sinus. (The method of Gärtner and Wagner (8), and Kedrov *et al.* (9).)

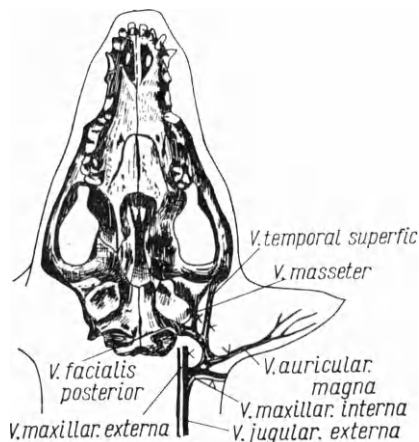


FIG. 1. Dissection to show surgical procedure.

BLOOD GLUCOSE AND PYRUVIC ACID DURING STIMULATION AND INHIBITION

Khachatryan used gastric stimuli (10–12), his dogs receiving 4–5 g meat-rusk powder and sugar in 3 doses at intervals of 5 min. Feeding was accompanied by the sound of a buzzer. Changes in blood glucose and pyruvic acid were measured after feeding sugary food, after the alimentary stimulus and at the onset of inhibition, and after the extinction of the conditioned reflex. Criteria for cortical stimulation and inhibition were judged not only by changes in the amount of blood glucose and pyruvic acid, but also by observing the animal's salivation. Seven dogs were used.

We will first discuss the pattern of changes found in the blood glucose and pyruvic acid levels following unconditioned and conditioned stimuli (buzzer). As shown in Figs. 2 and 3, the blood content of glucose and pyruvic acid increased markedly after unconditioned or conditioned stimulation. Conditioned changes occurred as follows: salivation after 20–25 stimuli, hyperglycaemia after 35–40, elevated blood pyruvate after 35–45. Changes following extinction of the conditioned reflex (i.e. onset of inhibition) are shown in Figs. 4 (glucose) and 5 (pyruvate).

Figure 4 shows that conditioned stimulation produces hyperglycaemia on the first and second day but no further change on the third. As the condi-

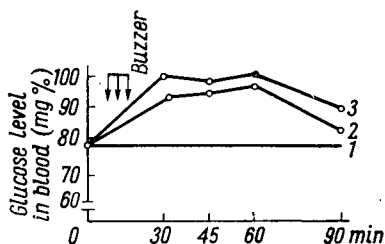


FIG. 2. Unconditioned and conditioned increase in the blood glucose level: 1—control; 2—conditioned stimulus; 3—unconditioned stimulus.

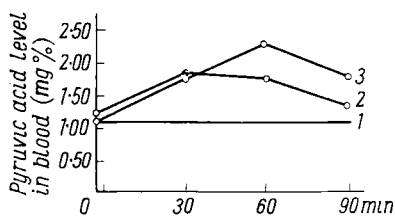


FIG. 3. Unconditioned and conditioned increase in the pyruvate level of the blood: 1—control; 2—conditioned reflex increase in the level of pyruvic acid; 3—unconditioned reflex increase in the pyruvic acid level.

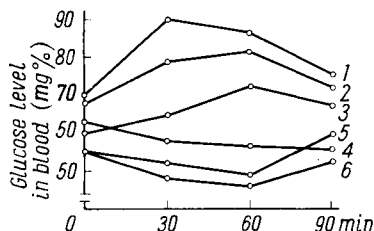


FIG. 4. Changes in the blood glucose level at the onset of cortical inhibition extinction of the conditioned reflex: 1, 2, 3, 4, 5, 6—day of experimental period.

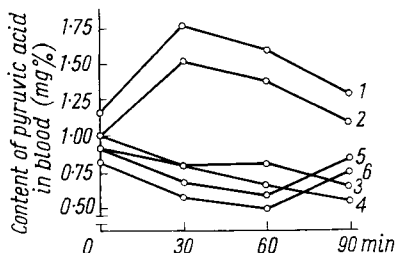


FIG. 5. Changes in the blood pyruvate level at the onset of cortical inhibition (extinction of the conditioned reflex) 1, 2, 3, 4, 5, 6—day of experimental period.

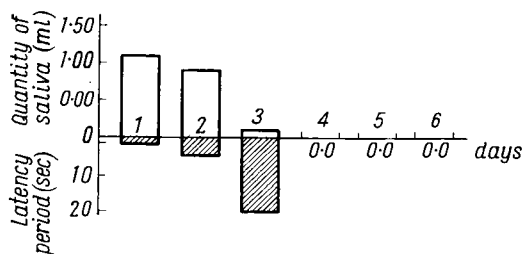


FIG. 6. Salivation at the onset of cortical inhibition.

tioned reflex weakens and inhibition strengthens, the blood glucose level drops, and on the sixth day reaches 46 mg % an hour after stimulation. Figure 5 shows that pyruvic acid follows a similar pattern of changes.

It is interesting to compare the development of inhibition with changes in the levels of blood glucose and pyruvic acid and salivation. Complete cessation of salivation occurs on the fourth day of extinction of the conditioned reflex (see Fig. 6). The blood glucose and pyruvic acid especially continue to decrease after the zero effect, indicating further intensification of inhibition.

We also observed that as the conditioned reflex weakened, the animal became stuporose and frequently fell asleep. Pavlov suggested that the zero effect could vary in intensity, depending upon some other latent process (13). Our studies support this hypothesis and give quantitative data regarding the degree of inhibition.

UPTAKE OF GLUCOSE AND PYRUVIC ACID BY THE BRAIN DURING STIMULATION AND INHIBITION

Figures 7 and 8 show that hyperglycaemia and increased uptake of glucose and pyruvate by the brain follows the application of conditioned stimuli. In these experiments blood passed through the brain in 9–10 sec. During

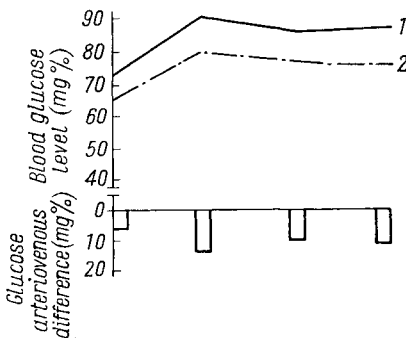


FIG. 7. Uptake of glucose by the brain during cortical (conditioned) stimulation: 1—carotid artery; 2—posterior facial vein.

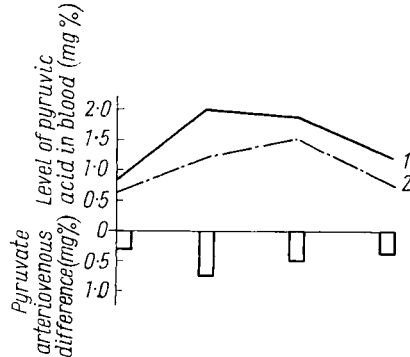


FIG. 8. Uptake of pyruvate by the brain during cortical (conditioned) stimulation: 1—carotid artery; 2—posterior facial vein.

inhibition the blood glucose level and A/V difference fell in proportion to the degree of inhibition and occasionally the A/V difference became negative (Fig. 9). Blood then took 16–17 sec to flow through the brain. Pyruvic acid levels showed similar changes during inhibition although the A/V

difference never became negative. These results again illustrate the reverse effects of stimulation and inhibition on cerebral metabolism. Decreased cerebral glucose uptake has also been observed during decreased nervous activity induced by narcosis, hypoglycaemia or hypoxia.

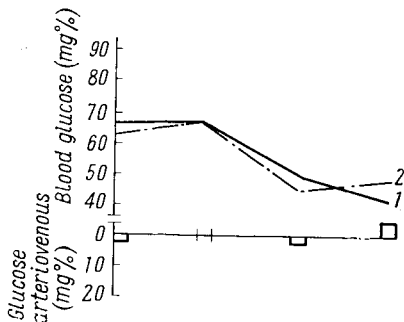


FIG. 9. Adsorption of glucose by the brain during cortical (conditioned) inhibition: 1—carotid artery; 2—posterior facial vein.

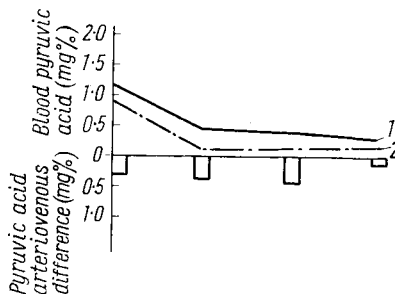


FIG. 10. Uptake of pyruvate by the brain during cortical (conditioned) inhibition: 1—carotid artery; 2—posterior facial vein.

It is interesting that the pyruvic acid A/V difference should increase during stimulation and remain positive during inhibition. Both glucose and pyruvic acid are oxidised by cerebral tissue *in vitro* (14, 15) but pyruvic acid is not easily absorbed by the brain (16, 17), and in hypoglycaemia has little effect on the oxygen A/V difference, whereas glucose greatly increases it (18, 19). In our studies we found the following pyruvic acid A/V differences: controls 0.1–0.2 mg %: unconditioned or conditioned stimulation 0.25–1.3 mg %: inhibition 0.2–0.5 mg %. Since increased blood pyruvic acid is accompanied by an increased A/V difference during stimulation, we conclude that this substance acts as an energy source for the brain. An additional fact about the blood pyruvate during inhibition is that although the A/V difference is lower than that during stimulation, it is still higher than the control. The question arises as to whether reduced glucose uptake is due to its low blood level, since it has been found that insulin-induced hypoglycaemia caused a lowered oxygen and glucose A/V difference (19,20). We showed that hypoglycaemia was not the cause of lowered glucose uptake by the brain. Khachatryan found that an auditory stimulus given to a dog initially caused an increased glucose uptake but 60–90 min later the glucose A/V difference was nil or negative in spite of a normal blood level of 80–85 mg % but arterial pyruvate and cerebral uptake had increased during this latter period.

In further experiments sugar was administered during inhibition, as it had been found that the effects of such unconditioned stimuli as adrenaline, insulin or even pain were eliminated if applied during a state of inhibition. Results are given in Figs. 11 and 12.

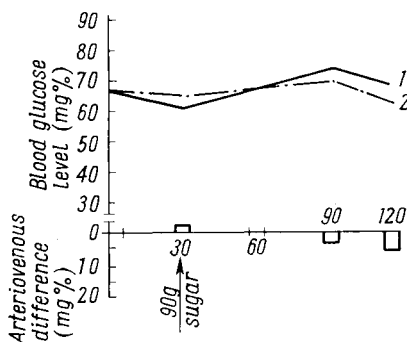


FIG. 11. Blood glucose level, and glucose uptake by the brain after ingestion of sugar during a state of inhibition: 1—carotid artery; 2—posterior facial vein.

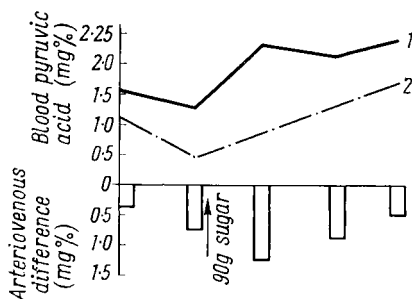


FIG. 12. Changes in blood levels of pyruvate, and its uptake by the brain after ingestion of sugar during a state of inhibition: 1—carotid artery; 2—posterior facial vein.

Eight days after inhibition had been induced, blood samples were taken 3 times at 5 min intervals, then the conditioned stimulus applied (buzzer). Thirty minutes later blood was sampled as before and 90 g of sugar administered. Figure 11 shows that the blood glucose level remained unchanged and the A/V difference did not alter until the 90th–120th minute of stimulation.

Interesting results were also obtained for pyruvic acid. Following an isolated conditioned stimulus, the level fell after 30 min but the A/V difference was still well maintained. Administration of sugar caused a marked increase in the blood level and A/V difference by 1 hr and values returned to normal by 2 hr. It appears that inhibition eliminates the effects of sugar administration normally expected and, in spite of the absence of hypoglycaemia, cerebral uptake of glucose was depressed.

Table 1 gives data for control experiments and the effects of sugar administration, conditioned stimulation, inhibition of the conditioned reflex and sugar administration during advanced inhibition.

In control experiments, blood glucose and pyruvate vary little. The glucose A/V difference ranges from 3–6 mg % and pyruvic acid from 0.09–0.25 mg %. Following sugar administration, blood glucose and pyruvic acid increase maximally at 30 min, and the A/V differences increase several

TABLE 1.

Number of experiment	Date of investigation	Experimental conditions	Time of collection of the blood (min) after the original sample	Blood glucose level (mg %)		Glucose arteriovenous difference (mg %)	Blood pyruvic acid (mg %)		Pyruvic acid arteriovenous difference (mg %)
				Carotid artery	Posterior facial vein		Carotid artery	Posterior facial vein	
2	20.II.	Control experiment (empty stomach)	Original sample	74	70	- 4	1.00	0.91	-0.09
			30	76	73	- 3	1.25	1.00	-0.25
			60	75	69	- 6	1.16	0.98	-0.18
3	26.II.	120 g sugar given in 3 doses	90	77	72	- 5	1.16	1.00	-0.16
			Original sample	72	60	-12	1.58	1.00	-0.58
			30	101	85	-16	2.00	1.25	-0.75
4	2.III.	Same	60	97	79	-18	2.67	1.83	-0.84
			90	96	79	-17	2.67	1.67	-1.00
			Original sample	81	68	-13	1.33	0.91	-0.42
8	16.III.	Same	30	108	103	- 5	2.58	1.92	-0.66
			60	86	74	-12	2.50	1.67	-0.83
			90	76	63	-13	2.50	1.92	-0.58
14	4.IV.	Same	Original sample	79	72	- 7	1.08	0.83	-0.25
			30	90	76	-14	2.33	1.16	-1.17
			60	92	88	- 4	2.41	1.25	-1.16
			90	97	83	-14	2.67	1.67	-1.00
			Original sample	85	71	-14	1.08	0.91	-0.17
			30	103	92	-11	2.50	2.00	-0.50
			60	94	83	-11	2.17	2.08	-0.09
			90	92	83	- 9	2.50	1.92	-0.58

cntd. Table 1.

Number of experiment	Date of investigation	Experimental conditions	Time of collection of the blood (min) after the original sample	Blood glucose level (mg %)		Glucose arteriovenous difference (mg %)	Blood pyruvic acid (mg %)		Pyruvic acid arteriovenous difference (mg %)
				Carotid artery	Posterior facial vein		Carotid artery	Posterior facial vein	
16	13.IV	Same	Original sample 30 60 90	57	50	- 7	0.83	0.42	-0.41
				75	68	- 7	1.92	1.50	-0.42
				95	86	- 9	2.08	1.42	-0.66
				105	84	- 21	—	1.25	—
18	20.IV.	Buzzer $\times$ 3	Original sample 30 60 90	70	66	- 4	0.83	0.68	-0.15
				92	79	- 13	2.00	1.25	-0.75
				88	79	- 9	1.87	1.50	-0.37
				88	78	- 10	1.17	0.83	-0.34
19	22.IV.	Same	Original sample 30 60 90	65	56	- 9	1.17	0.67	-0.50
				79	70	- 9	1.50	1.00	-0.50
				85	76	- 9	1.25	0.73	-0.52
				78	69	- 9	0.91	0.25	-0.66
20	25.IV	Same	Original sample 30 60 90	62	49	- 13	0.67	0.33	-0.34
				53	57	- 2	0.50	0.50	0
				58	56	- 2	0.67	0.24	-0.43
				53	62	+ 9	0.59	0.17	-0.42
21	27.IV.	Same	Original sample 30 60 90	66	64	- 2	1.17	0.91	-0.26
				66	66	0	0.42	0.16	-0.26
				48	46	- 2	0.42	0.16	-0.26
				42	46	+ 4	0.33	0.25	-0.08

23	30.IV.	Same	Original sample 30 60 90	51 48 44 51	51 46 32 46	0 -2 -12 -5	0.91 0.67 0.67 0.50	0.9 0.42 0.08 0.25	0.01 -0.25 -0.59 -0.25
24	4.V.	Same	Original sample 30 60 90	43 45 45 54	39 50 38 45	-4 +5 -7 -9	0.58 0.30 0.40 0.20	0.17 0.08 0.10 0.30	-0.41 -0.22 -0.30 +0.10
25	6.V.	Same	Original sample 30 60 90	63 54 54 47	50 39 57 48	-13 -15 +3 +1	0.50 0.67 0.57 0.25	0.67 0.50 0.38 0.17	+0.17 -0.17 -0.19 -0.08
26	9.V.	120 g sugar given in 3 doses	Original sample 30 60 90	49 47 51 35	47 51 49 40	-2 +4 -2 +5	0.57 2.42 2.42 2.00	0.57 1.50 1.67 1.58	0 -0.92 -0.75 -0.42
27	11.V.	Same	Original sample 30 60 90	57 — 69 65	58 — 67 58	+1 — -2 -7	1.00 — 2.42 3.00	0.75 — 1.41 2.25	-0.25 — -1.01 -0.75
28	13.V.	Same	Original sample 30 60 90	57 68 72 74	52 61 66 65	-5 -7 -6 -9	1.08 2.17 2.58 2.75	0.92 1.41 1.58 1.75	-0.16 -0.76 -1.00 -1.00
29	20.V.	Same	Original sample 30 60 90	66 93 94 76	55 80 76 62	-11 -13 -18 -14	1.57 2.42 2.58 2.08	1.33 1.75 1.83 1.58	-0.24 -0.67 -0.75 -0.50

fold. Thirty minutes conditioned stimulation causes similar changes, the arterial blood glucose increase reaching 22 mg % and venous blood 13 mg %. Pyruvic acid increases by 1.27 and 0.82 mg %, giving A/V differences of 9–13 mg % and 0.34–0.75 mg % for glucose and pyruvic acid respectively. Another experiment showing similar but less marked changes during conditioned stimulation was followed into the inhibition phase. Blood glucose and pyruvic acid then begin to fall and sometimes reach 42–46 mg % in the arterial blood and 32–38 mg % in venous blood for glucose, while the pyruvate falls to 0.2 and 0.1 mg % respectively. The glucose A/V difference drops to zero and sometimes becomes negative and the pyruvic acid A/V difference also decreases.

Administration of sugar during inhibition does not lead to an increased blood glucose level. The hypoglycaemia caused by inhibition persists and the glucose A/V difference remains at zero or even becomes negative. However, the pyruvate blood level and A/V difference increases. Sugar administration shows its usual effects only after the 4th application.

After alimentary and conditioned alimentary stimulation, blood takes 9–10 sec to pass through the brain; as inhibition develops, this period increases to 17 sec and remains at this value after the 1st and 2nd administration of sugar, dropping to 12 and 9 sec after the 3rd and 4th administration.

The above data show that inhibition is reversed in stages as assessed by several criteria. Changes in the blood level and A/V difference of pyruvate after sugar administration during inhibition parallel the changes observed after stimulation, whereas the glucose blood level and A/V difference do not respond to sugar administration.

In order to find out why sugar administration during inhibition fails to produce a hyperglycaemic effect, experiments were conducted on dogs with exposed ureters. Under these conditions, it was found that along with increased blood pyruvic acid levels (i.e. increased glucose breakdown), glycosuria occurred and continued, in spite of the low blood glucose. Even without sugar administration glycosuria occurred during inhibition while the blood glucose was still low. It thus appears that elimination of the effect of sugar administration on the blood glucose level is due to an increased decomposition of glucose, renal glycosuria and maybe other unknown mechanisms.

Administration of sugar or conditioned stimulation causes increased pyruvate and glucose levels, increased uptake of these substances by the brain and quicker blood circulation through the brain. Inhibition produces the reverse effects and therefore probably acts by suppressing the functional activity of the sympathetic-adrenaline system and stimulating the “insulin apparatus”

which, in turn, inhibits the sympathetic adrenaline system. To examine this theory, we tried to see if one could obtain the same effects during inhibition with insulin.

Insulin was injected (0.5 units per kg) and blood samples removed before injection and 20 and 40 min after. The resultant hypoglycaemia caused the glucose A/V difference to fall, reach zero and even become negative when the blood sugar reached 30 mg %. We have previously observed that during inhibition these effects could be obtained with a blood glucose level of 40 mg % and higher, but again after the 6th–7th insulin injection, these effects could also be achieved with a blood glucose of 40 mg %.

Insulin lowered the blood pyruvate but the A/V difference varied with different dogs, mostly showing a decrease. 20–40 minutes after the insulin injection it took 3–4 sec longer for the blood to circulate through the brain.

Thus the effects of insulin injection are similar to those observed after sugar administration during inhibition. It can be assumed that inhibition of the insulin conditioned hypoglycaemia leads to changes characteristic of sugar administration or of a positive conditioned reflex based on sugar administration. The extinction of the insulin-conditioned reflex leads to hyperglycaemia (2, 18), i.e. as does sugar administration.

It seems that one type of stimulus—insulin hypoglycaemia—produces a conditioned reflex whose effects are similar to those produced by a stimulus of an opposite character—sugar administration. This makes interpretation of the effects of stimulation and inhibition on the metabolism of the brain and effector organs very difficult.

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METABOLISM AND CHEMICAL STRUCTURE OF GLYCOGEN FRACTIONS IN THE BRAIN

B. I. KHAIKINA AND YE. YE. GONCHAROVA

Institute of Biochemistry,
Ukrainian Academy of Sciences, Kiev

PRZYLECKI and co-workers showed in 1930 that glycogen was capable of forming complexes with various proteins and lipids (1-3). Later, Willstätter and Rohdewald (4) showed that liver, muscle and leucocyte glycogen occurred in two forms: a free or lyoglycogen extractable with hot water or dilute TCA, and a desmoglycogen which was bound. Other workers (5-10) showed that in muscle and liver tissue the rate of glycogenolysis depended not so much on the total amount of glycogen present, but on the ratio of free glycogen to bound glycogen. This ratio was found to vary with the physiological state of the body. Previous studies (11-13) indicated that the ratio of free to bound glycogen in the brain changed according to its physiological state.

Although the existence of two forms of glycogen is well established, their metabolic role and character are still obscure. Protein-bound glycogen was considered to be a better substrate for enzyme action than the free form (14), but this was disputed by Przylecki (15) and Breitburg (16), who found the free form more active. These divergent results are now known to be due to different extraction techniques.

In our present work we have endeavoured to characterize the various chemical forms of glycogen and determine their metabolic roles.

Khaikina (17) developed a method for fractionating glycogen which was estimated colorimetrically by reaction with anthrone. Using this method, it was shown that only 20 per cent of brain glycogen was in the free form, 60 per cent bound to proteins of which 15 per cent could be extracted with water or dilute saline; and the rest bound to lipids (see Fig. 1). Lipid-bound glycogen extracted from rabbit brain was found to be complexed with phosphatide and not cholesterol. This latter form also could not be detected *in vitro* by Przylecki.

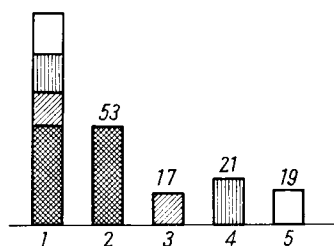


FIG. 1. Content of various glycogen fractions in rabbit brain (mg %): 1—total glycogen; 2—glycogen combined with insoluble proteins; 3—glycogen combined with soluble proteins; 4—glycogen combined with lipids; 5—free glycogen.

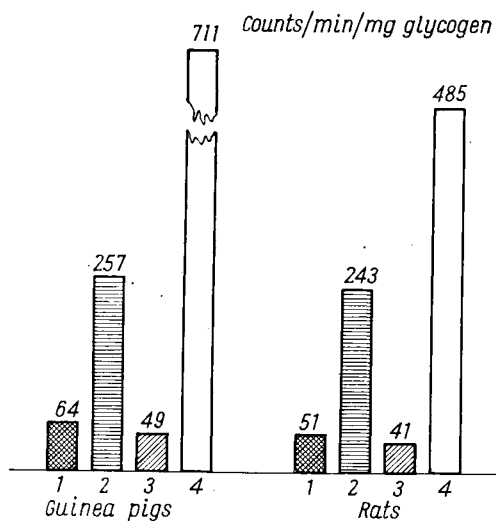
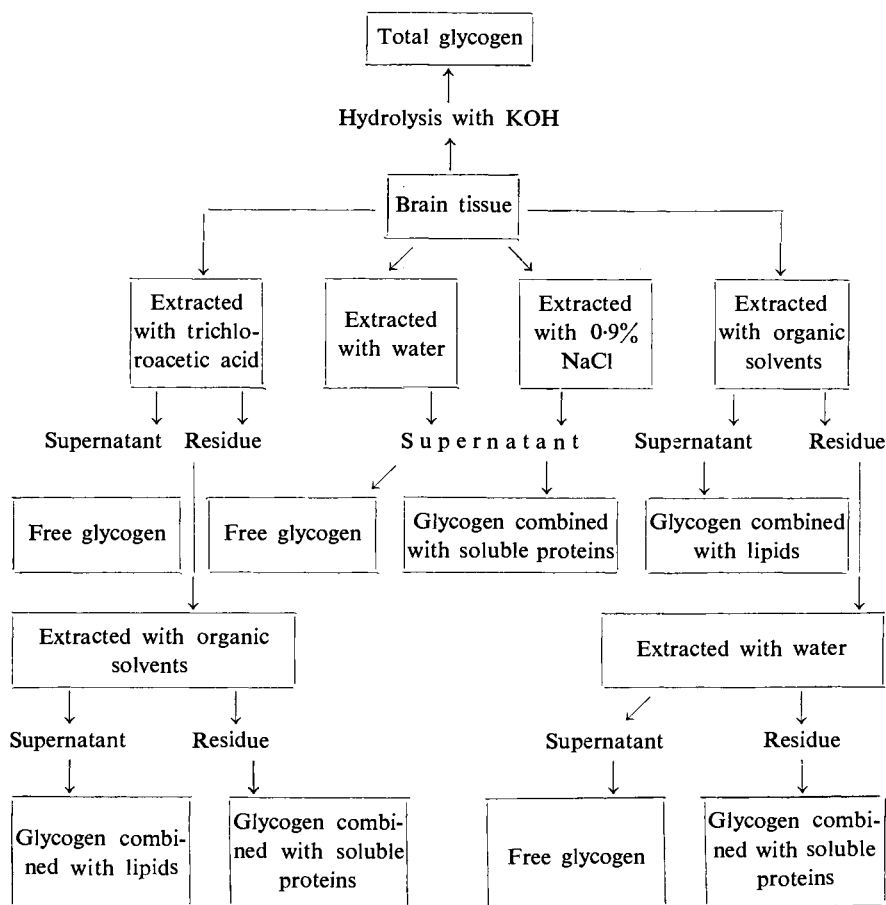


FIG. 2. Specific activity of various glycogen fractions in rat and guinea pigs brain: 1—total glycogen; 2—glycogen combined with lipids; 3—glycogen combined with proteins; 4—free glycogen.



THE RATE OF TURNOVER OF GLYCOGEN IN THE BRAIN

Prokhorova (18) had previously shown that the rate of glycogen turnover in brain tissue was as high as in liver. For our studies we injected ^{14}C -labelled glucose, prepared photosynthetically, into guinea pigs and killed them $1\frac{1}{2}$ hr later. The highest specific activity (Fig. 2) was subsequently found in the free glycogen being much greater than either protein or lipid-bound glycogen (19).

The fact that we found different rates of ^{14}C -glucose incorporation in to glycogen seems to demonstrate the metabolic significance of more than one glycogen form.

We also tried using carboxyl ^{14}C -labelled acetic acid since White and Werkmann (20) and Lifson *et al.* (21) had shown that radioactive acetic

acid was incorporated into the polysaccharides of yeast and muscle. They used carboxyl ^{14}C -labelled acetic acid which labelled glucose in the 3 and 4 positions. By using methyl ^{14}C and both methyl ^{14}C - and carboxyl ^{14}C -labelled acetic acid, they further showed how the acetate fragments were synthesized into the hexose molecule. Topper and Hastings (22) confirmed these results, and demonstrated the synthesis of glycogen from acetate fragments.

Procedure using Radioactive Acetic Acid

Rats were injected subcutaneously with carboxyl ^{14}C -labelled acetic acid at a dose of 30,000 counts per gram body weight followed by a similar dose $1\frac{1}{2}$ hr later, and then decapitated into liquid air $1\frac{1}{2}$ hr after the 2nd injection. The glycogen was extracted, fractionated and estimated by the method of Khaikina (19). The radioactivity of cerebral glycogen and glucose, of blood glucose and of liver glycogen was measured.

The results in guinea pigs were similar to those in rats. After $1\frac{1}{2}$ hr exposure to radioactive acetic acid, the highest specific activity (SA) was found in free glycogen, then lipid-bound glycogen, then protein-bound glycogen (see Fig. 2). After 3 hr exposure, however, the free glycogen ceased to have the highest SA being proportionately greater in the total glycogen, protein-bound and lipid-bound glycogen (Fig. 3).

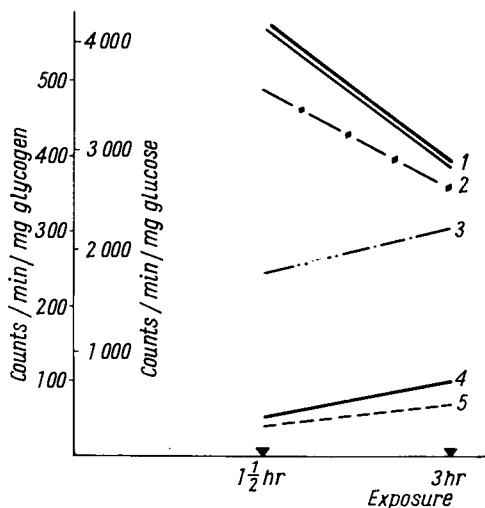


FIG. 3. Specific activity of the glycogen fractions and glucose in normal brain $1\frac{1}{2}$ and 3 hr after administration of the radioactive isotope: 1—brain glucose; 2—free glycogen; 3—glycogen combined with lipids; 4—total glycogen; 5—glycogen combined with proteins.

Rate of Turnover of Glycogen Fractions and Glucose of the Brain during Amphetamine Stimulation

Rats were injected with 5 mg per kg body weight amphetamine and killed 3 hr later. Resting animals served as controls.

Stimulated animals showed a decrease of free glycogen and an increase in protein-bound glycogen. The lipid-bound glycogen remained unchanged. Brain glucose fell 50 per cent (see Figs. 4 and 5).

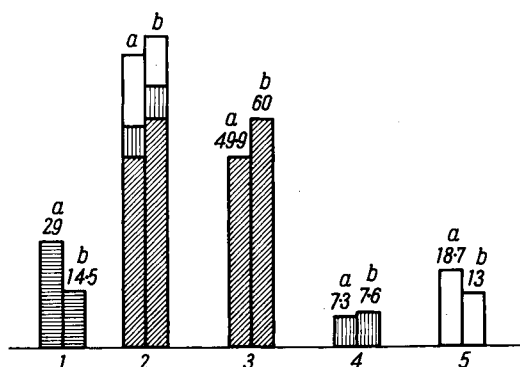


FIG. 4. Content of the glycogen fractions and glucose in brain tissue under normal conditions and during stimulation (mg %): 1—brain glucose; 2—total glycogen; 3—glycogen combined with proteins; 4—glycogen combined with lipids; 5—free glycogen; a—normal state; b—stimulated.

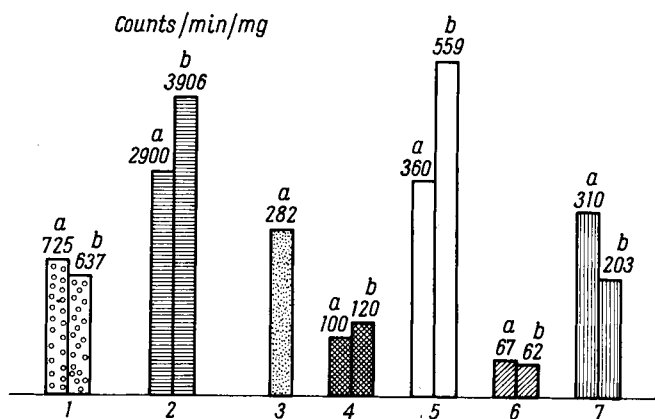


FIG. 5. Specific activity of the glycogen fractions in brain and liver, and the glucose content of brain and blood in the normal and stimulated state: 1—blood glucose; 2—brain glucose; 3—liver glucose; 4—total brain glycogen; 5—free glycogen; 6—glycogen combined with proteins; 7—glycogen combined with lipids; a—normal state; b—stimulated.

Stimulation also caused marked changes in the rates of ^{14}C incorporation into glycogen fractions. The SA of free glycogen increased by 50 per cent and total glycogen by 25 per cent, whereas the SA of the protein-bound glycogen remained the same and the lipid-bound glycogen decreased. During excitation the SA of cerebral glucose increased and that of the blood glucose fell. No variations in liver glycogen turnover could be correlated with amphetamine stimulation.

A more accurate impression of the incorporation rates can be obtained by expressing the results as relative specific activity (RSA). These are defined as the ratio of the SA of the brain fraction to the blood glucose SA.

During stimulation the protein-bound glycogen RSA was least affected and free glycogen showed a sharp increase. From these results we concluded that stimulation caused an increase in the utilisation and rate of resynthesis of energy resources, e.g. glycogen.

Next we examined the glycogen bound to protein. Although the chemistry of liver and muscle glycogen has been extensively studied, our knowledge of brain glycogen is comparatively small. It resembles liver glycogen in its optical rotation, iodine-staining properties, formation of opalescent solutions and the number of glucose units in the molecule.

It has been shown that the ability of polysaccharides to form complexes with proteins depends on their molecular structure (24). Polysaccharides with long non-branching glycopyran chains, e.g. starch and inulin, do not form complexes with proteins, neither do those with very short terminal branches such as β - or Φ -dextrins. Complexing occurs with side chains of at least 5 or 6 glucose units.

Liver glycogen forms complexes with proteins more readily than muscle glycogen. Goncharova (25) studied brain and liver glycogen for comparison. She compared their colour reaction with iodine, assessed from absorption curves (2) their degree of cleavage by β -amylase (3) and their average chain length measured by periodate oxidation.

The glycogen was extracted by alkaline hydrolysis and purified by the method of Kerr and Somogyi (26, 27). This removed cerebrosides, dextrins and other impurities. The absorption curves for liver and brain glycogen are given in Fig. 7. The absorption maxima of 500 $\text{m}\mu$ is the same for both types but the extinction value for liver is 0.43 and for brain 0.30.

Stepanenko (28) and others have shown that as the side chains of the glycogen molecule become shorter, the iodine absorption curve maxima shift to a shorter wavelength and the extinction coefficient decreases. The data in Fig. 7 indicate that brain glycogen has shorter side chains than liver

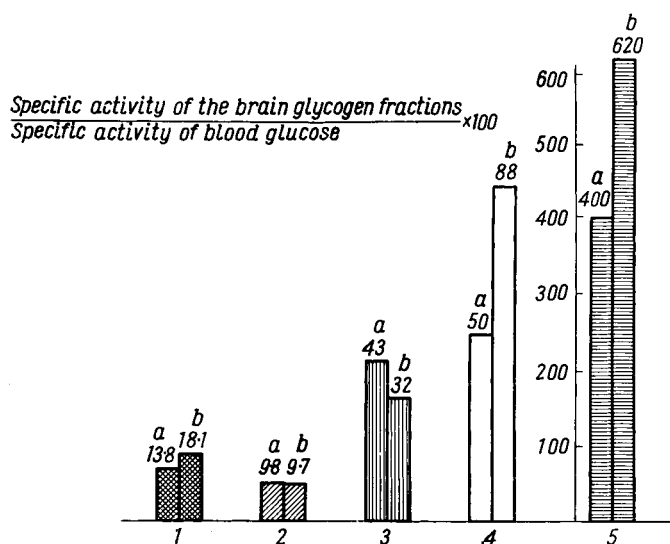


FIG. 6. Relative specific activity of brain glucose and glycogen fractions in the normal and stimulated state: 1—total glycogen; 2—glycogen combined with proteins; 3—glycogen combined with lipids; 4—free glycogen; 5—brain glucose; a—normal state; b—stimulated.

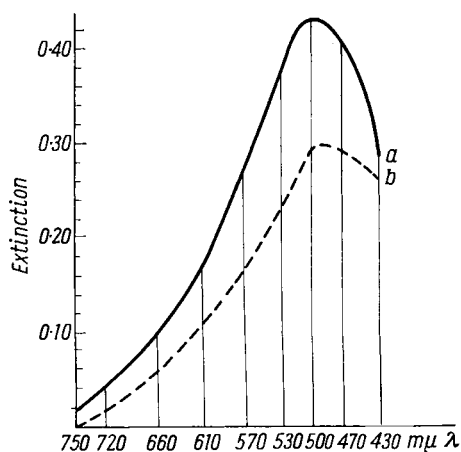


FIG. 7. Absorption curves of iodine combined with brain and liver glycogen of rabbit: a—liver glycogen; b—brain glycogen.

glycogen and this has been confirmed also by the fact that β -amylase splits brain glycogen less easily than liver glycogen.

TABLE 1. THE DEGREE OF BREAKDOWN OF BRAIN AND LIVER GLYCOGEN IN RABBITS BY β -AMYLASE AND THE AVERAGE CHAIN LENGTH OF THE GLYCOGEN

Glycogen	Degree of breakdown of glycogen (%)		Average chain length (glucose units)
	Wheat β -amylase	Soya bean β -amylase	
Brain	37.2	39.2	12
Liver	48.9	45.5	16

Finally, by periodate oxidation, we showed that the length of the liver glycogen chains was 16 glucose units whereas the figure for brain glycogen was 12. These results confirm the shorter side chain length of brain glycogen and its greater degree of branching (see (29)).

It is intended to carry out further studies of the chemical properties of glycogen in the brain, its ability to bind other molecules, and rate of turnover during excitation and inhibition of the nervous system.

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THE RATE OF TURNOVER OF CARBOHYDRATES AND LIPIDS IN THE BRAIN AND LIVER DURING EXCITATION AND DRUG-INDUCED SLEEP

M. I. PROKHOROVA AND Z. N. TUPIKOVA

The Metabolic Laboratory, Department of Biochemistry, University of Leningrad

THE relation between the function and biochemistry of the brain and other organs is extremely interesting, particularly during excitation and inhibition (1, 2). In the past these processes were studied by gross measurements of different constituents, but these did not always reflect changes in their rate of metabolism; for example, they did not give information on the relative rates of breakdown and resynthesis. The recent use of radioactive tracers, however, has now enabled us to make a more dynamic study of the biochemical processes involved. We have used them to study the metabolism of carbohydrates and lipids of the brain and liver.

METHOD

These experiments were mainly carried out on white rats, but, in a few cases, dogs with severed sinus nerves were used. Caffeine and amphetamine were used as cortical stimulants, and chloral hydrate and morphia were administered as depressants. The quantities and relative activities of glycogen, glucose, lipids and cholesterol of the brain and liver, and the expired carbon dioxide, were measured. Radioactive carbon-labelled glucose or acetic acid was injected into the animals. Glucose obtained by photosynthesis was injected at a dose of 4000–6000 or 8000–10,000 counts/min per g tissue or acetic acid at a dose of 2000–2500 counts/min per g tissue.

The rat was placed in a desiccator with a carbon dioxide absorbent. The experiment was continued for 1 hr and then the rats were submerged in liquid oxygen.

Glucose was estimated in a cadmium filtrate by the method of Fuyita and Iwatake. Glucose radioactivity was estimated after precipitation as glucosazone. Using E. Fischer's principle, we developed a method for isolation of these glucosazones. Brain glycogen was precipitated and purified by the

method of Kerr (3). Its activity was measured by transferring the precipitate on to metal plates and after the radioactive counting, the precipitate was hydrolysed in 2 N HCl. Liver glycogen activity was measured in the same way. Liver glycogen was estimated chemically by the method of Kemp and Kits van Heijningen (4). The expired carbon dioxide was absorbed in 5 N sodium hydroxide and precipitated as barium carbonate; the precipitate was washed with distilled water and alcohol several times, dried, and the specific activity (SA) of its carbon then estimated. The specific activity of the carbon in the whole homogenate was also measured. Lipid estimation in the brain and liver was carried out by the methods of Folch (5) and Sperry (6) and the cholesterol estimated by the method of Engelhardt and Smirnova. The SA of the cholesterol was measured in a digitonin-cholesterol precipitate isolated by the method of Windaus.

RESULTS

Metabolism in the whole Animal during Excitation and Induced Sleep

Analysis of the CO_2 and its specific activity in the expired air shows that it is very different in different functional states (Fig. 1). The average amount of CO_2 expired per hour was 363 mg; it decreased during narcotic sleep to 318 mg and increased during excitation to 614 mg. The average specific activity of the expired CO_2 was 4180 counts/min/mg; it fell to 1400 counts/min during induced sleep and during excitation rose to 8940.

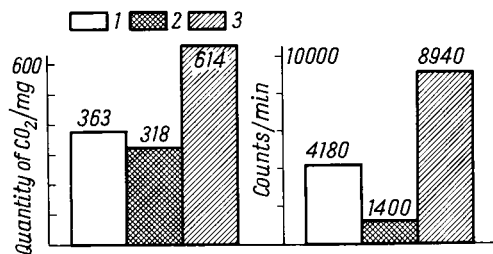


FIG. 1. Total quantity of CO_2 expired and the degree of its radioactivity:
1—normal state; 2—narcosis; 3—excitation.

In dogs with severed sinus nerves there was an increase in uptake by the brain of glucose from the blood during excitation, while it decreased during narcotic sleep (7). To study the rate at which injected glucose and acetic acid were oxidized, we had a look at the specific activity of the expired CO_2 . Oxidation of radioactive glucose began 30–40 min after its injection and reached a maximum by 60 min; during excitation the utilization of

injected glucose occurred after only 10–20 min, whereas in narcotic sleep it began later. A similar picture was seen with injected radioactive acetic acid except that this compound is, as a rule, utilized more quickly than glucose.

Lipid Metabolism

The lipid content of the brain does not vary much, as can be seen from the analyses of lipid and cholesterol in the brain and liver in Table 1. Neither excitation nor sleep altered the lipid or cholesterol in either the brain or the liver.

TABLE 1. TOTAL LIPID CONTENT AND CHOLESTEROL CONTENT OF THE BRAIN AND THE LIVER
(in per cent)

Experimental condition	Brain		Liver	
	Total lipids	Cholesterol	Total lipids	Cholesterol
Normal state	9.2 ± 0.7	1.67 ± 0.09	5.2 ± 0.7	0.325 ± 0.014
Excitation	9.3 ± 0.4	1.64 ± 0.06	5.7 ± 0.4	0.335 ± 0.028
Narcosis	9.0 ± 0.3	—	5.0 ± 0.6	0.340 ± 0.024

Since a number of authors have shown that acetic acid and glucose are involved in the synthesis of cholesterol, fatty acids and glycogen—as well as being rapidly oxidized to CO_2 and water (8, 14)—we studied the turnover of lipids in the brain and liver, using radioactive, carbon-labelled acetic acid and glucose.

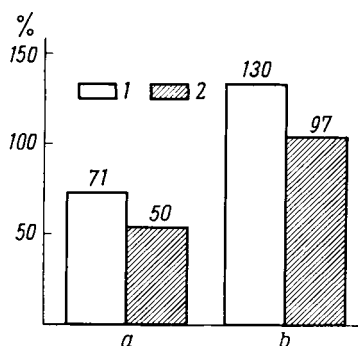


FIG. 2. The relative specific activity of the brain and liver lipids:
a—brain; b—liver; 1—normal state; 2—excitation.

One hour after injection of the acetic acid or glucose, radioactive carbon was found in the lipids of the brain and liver (Fig. 2).

If one takes the specific activity of carbon in the brain homogenates as 100 per cent, its relative activity in the brain lipids was 71 per cent falling to 50 per cent during narcotic sleep. The relative specific activity (RSA) of carbon of liver lipids under normal conditions is 130 per cent: during excitation it rises to 160 per cent and during narcotic sleep it falls to 97 per cent.

In spite of the high glucose activity (10,000 counts/min/g) and of acetic acid (20,000–25,000 counts/min), we were unable to obtain radioactive carbon incorporation of more than 10 per cent in the brain cholesterol. This difficulty has been met with by other authors (15).

Glycogen Metabolism

Until recently brain glycogen was regarded only as a structural substance, with very little participation in metabolic processes. However, since Kerr described an accurate method for estimating brain glycogen, it has been possible to investigate its metabolism in different functional states (7, 16–19). Our radioactive carbon studies show that the rate of glycogen metabolism in the brain is 50–100 times greater than the turnover of lipids, cholesterol, or carbon (Fig. 3).

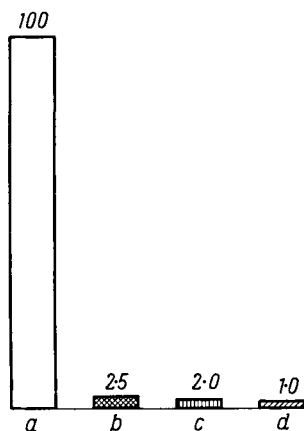


FIG. 3. Relative specific activity of carbon in glycogen, lipids, cholesterol and of total carbon: a—glycogen carbon; b—total carbon; c—lipid carbon; d—cholesterol carbon.

We further investigated the rate of glycogen turnover in various organs, and showed that the rate of incorporation of glucose into glycogen in the hemispheres is greater than the cerebellum, liver or muscles. The RSA of liver glycogen depends upon the amount of glycogen present in that organ.

If the liver contains 2–2.5 per cent glycogen, the RSA is 16 per cent, but if the original glycogen in the liver is only 0.2–0.3 per cent the RSA reaches 68 per cent. Differences in the glycogen SA occur under normal conditions and in different functional states (Fig. 4).

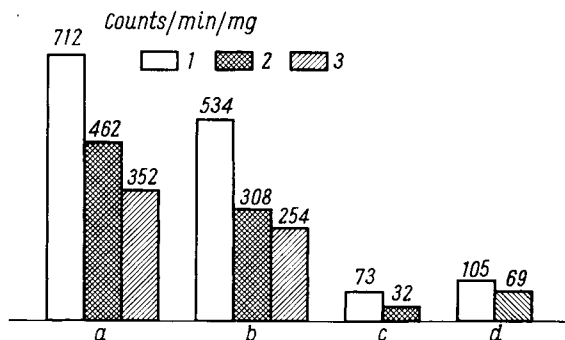


FIG. 4. Specific activity of glycogen in the brain, cerebellum, liver and muscles: a—brain; b—cerebellum; c—liver (glycogen content 2–2.5%); d—muscles; 1—normal state; 2—narcosis; 3—excitation.

Rate of Glycogen and Glucose Metabolism during Excitation and Drug-Induced Sleep

It has been previously shown (7) that stimulating doses of caffeine cause an increased breakdown of carbohydrates in the brain. This is reflected in the decreased glycogen content of the brain. Induced sleep, on the other

TABLE 2. TURNOVER RATE OF BRAIN GLYCOGEN IN THE NORMAL STATE AND IN NARCOSIS

Experimental condition	Carbohydrate content of the brain (mg %)		Glucose arteriovenous difference (mg %)
	Glycogen	Glucose	
Normal state	72±10	45±5	10
Excitation	56±7	48±9	15
Narcosis	84±12	84±12	4

hand, is accompanied by inhibition of carbohydrate breakdown, as shown by an increase in the glycogen and glucose of the brain, despite the decreased supply of glucose from the blood (Table 2). From this table also it can be seen that during excitation, the glucose utilization by the brain—as judged

by the arteriovenous difference—is 10–15 mg per 100 ml cerebral blood flow higher than normal.

These studies do not give us any information about the relative rates of anabolism and catabolism during stimulation or sedation. Thus the increased glycogen in the brain during narcosis could be due to inhibited breakdown or increased synthesis, or both. Similarly, the decreased glycogen during excitation could be due to increased breakdown or lowered rate of synthesis. To find out which process predominates, we used radioactive tracers. We estimated the rate of glycogen turnover, glucose consumption and oxidation in the animal.

Caffeine stimulation caused a marked fall in the SA of the glucose in the brain to 50 per cent of normal (Fig. 5). The increased utilization of carbohydrate observed by us in the brain (Table 2) is reflected in the whole body, as shown by the analysis of the radioactivity of the CO_2 (Fig. 1). It thus appears that stimulation causes an intense breakdown of carbohydrates which leads to an exhaustion of the body's energy resources.

The opposite effect is seen during drug-induced sleep. Table 2 shows that the glycogen content increases from 72 to 85 mg % and the glucose from 45 to 84 mg %. Whereas under normal conditions the arteriovenous glucose difference is 10 mg %, during narcosis it is only 4 mg %. Thus during narcotic sleep the brain uses up less carbohydrates.

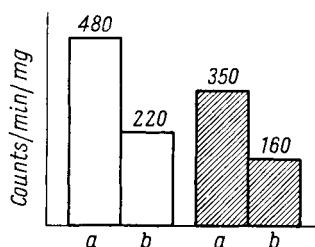


FIG. 5. Specific activity of brain glucose and glycogen in the normal state and during excitation: a—glucose; b—glycogen.

We studied the rate of synthetic processes in the brains of narcotized animals by estimating the rate of glycogen turnover in the brain. The SA of the glycogen during sleep falls from 722 counts/min/mg to 462 counts/min/mg. At the same time the SA of the brain glucose increases almost $1\frac{1}{2}$ times during sleep (Fig. 6). The proportion of radioactive carbon of glucose in the total activity of the tissue homogenates increases from 19 to 35

per cent (Table 3). It thus appears that the amount of radioactive glucose *increases* but its incorporation into glycogen *decreases*. This is brought out more clearly by using the ratio $\frac{\text{SA of glycogen}}{\text{SA of glucose}} \times 100$ in the brain, which gives 46 per cent under normal conditions and 32 per cent during sleep.

TABLE 3. TURNOVER RATE OF BRAIN GLYCOGEN IN THE NORMAL STATE AND DURING NARCOTIC SLEEP

Experimental condition	Number of experiments	Ratio of the activity of glycogen C and glucose C to the activity of total C		Relative specific activity of glycogen	
		Glycogen	Glucose	Per mg	Per g tissue
Normal state	14	11	19	46	66
Narcosis	15	10	35	32	32

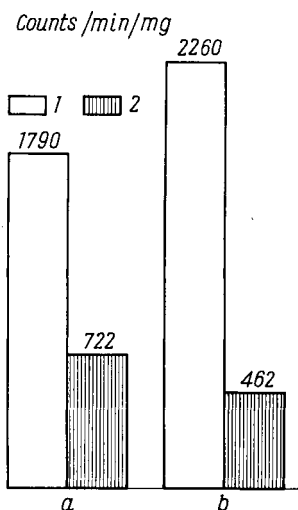


FIG. 6. Specific activity of brain glucose and glycogen in the normal state and during narcosis: a—normal state; b—narcosis; 1—glucose; 2—glycogen.

If one compares the SA of carbon in brain glycogen with the SA of total carbon in brain tissue, one sees again that the rate of brain glycogen turnover decreases during sleep. One can calculate that under normal conditions the radioactive carbon of brain glycogen averages 11 per cent of the total

brain radioactivity, whereas in narcotic sleep it is 10 per cent. If one takes into account, however, that the glycogen content of the brain increases in narcotic sleep while the amount of radioactive carbon does not increase, these results indicate a decrease in the SA of the glycogen. We conclude that the increase in glycogen content in the brain during narcotic sleep is a consequence of inhibition of carbohydrate breakdown and not of increased synthesis, because the rate of synthesis is considerably lowered. This shows that in nerve cells of anaesthetized animals metabolism takes place at a lower rate.

Our data are consistent with the result of Vladimirov (1) and his co-workers, who showed that narcotics decrease the ^{32}P turnover of phospholipids. The slowing of metabolic processes in nerve cells during sleep seems to be important in the role played by the inhibitory process. A high energy expenditure is characteristic of the normal function of the brain, and prolonged administration of narcotics, which probably decreases the rate of metabolism, therefore damages the central nervous system.

In summary, it should be emphasized that the intensity of metabolic processes in the brain is higher during excitation than during narcotic sleep, and that both breakdown *and* synthesis of carbohydrates take place at a higher rate during excitation than under narcosis. The intensity of breakdown, however, exceeds that of synthesis and, as a result, the energy resources of the nerve cell are exhausted. During sleep the opposite changes occur.

SUMMARY AND CONCLUSIONS

1. Excitation increases and narcotic sleep decreases, the amount and specific activity of expired CO_2 in animals.
2. Considerable amounts of carbon-labelled radioactive glucose and acetic acid are incorporated into the lipids of brain and liver.
3. Glycogen, lipid and cholesterol turnover rates are at their highest in the cerebral hemispheres compared with cerebellum, liver and muscle, in both normal and excited states.
4. Excitation raises the level of carbohydrate metabolism, breakdown processes predominate, and lead to exhaustion of the energy resources of the nerve cells.
5. During narcotic sleep the rate of glycogen turnover in the brain is considerably lower than normal. Breakdown occurs more slowly and this leads to accumulation of carbohydrates in the brain. Thus narcotic sleep is characterized by a low level of metabolism in the nerve cells.

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ADRENALINE AND NORADRENALINE IN NERVOUS TISSUES AND EFFECTOR ORGANS

A. M. UTEVSKII

Department of Biochemistry, Ukrainian Institute of Experimental Endocrinology,
and Department of Biochemistry, Khar'kov Medical Institute

THIS paper deals with adrenaline-like substances in the brain during rest, during muscle function, the effect of hyperthyroidism, of 6-methyl-thiouracil, and of castration. Changes in these substances in the brain, and nervous influences on adrenaline and noradrenaline metabolism in the heart are discussed.

We had previously shown that electrical stimulation of thigh muscles in rabbits affected the catecholamines in the same muscles of the opposite limb, which would prepare them for work. In the present paper we have studied the behaviour of adrenaline-like substances in the motor area of the rabbit during thyroid dysfunction (1, 2).

METHODS

Catecholamines were studied by two methods: (i) Shaw's method of adsorption photometry which gives one a coefficient of specificity (CS). This is defined as the ratio of the capacity of the material in question to reduce arsenious molybdate on addition of alkali, to the capacity of the same material *without* the addition of alkali; for adrenaline it should be at least 2 (3-6): (ii). The method of differential fluorescence developed in our laboratory by Osinskaya (4, 5) which estimates adrenaline, noradrenaline and their oxidation products. The majority of experiments were carried out in rabbits, but we also used rats, guinea pigs and Siberian marmots.

THE LEVELS OF ADRENALINE-LIKE SUBSTANCES IN THE BRAIN

Euler (7), Bacq and Fisher (8), Holtz (9), Lissak (10) and Vogt (11) have found wide variations in the amount of noradrenaline in different parts of the nervous system. Vogt found that in dogs the highest level of noradrenaline (100-200 $\mu\text{g}\%$) was found in the hypothalamus. Holtz found 20 $\mu\text{g}\%$

in the whole brain of rabbits. Of the nerves, the upper cervical sympathetic ganglia of cat and rabbit are particularly rich in noradrenaline (10).

We found that the CS of the catecholamines in the brain was frequently higher than 2, i.e. corresponded to the CS of adrenaline (12). This was using photometric measurements, but by the more specific differential fluorescence method we did not usually find any adrenaline in the brain, but there was a considerable quantity of noradrenaline and other catecholamines (4). As noradrenaline is unaffected by alkali, its CS is 1, and the higher values we usually found were probably due to oxidation products of catecholamines of the leucoadrenochrome or leucooxyadrenochrome type, which is consistent with results obtained from fluorescence studies. We are going into this question further, using spectrophotometric methods.

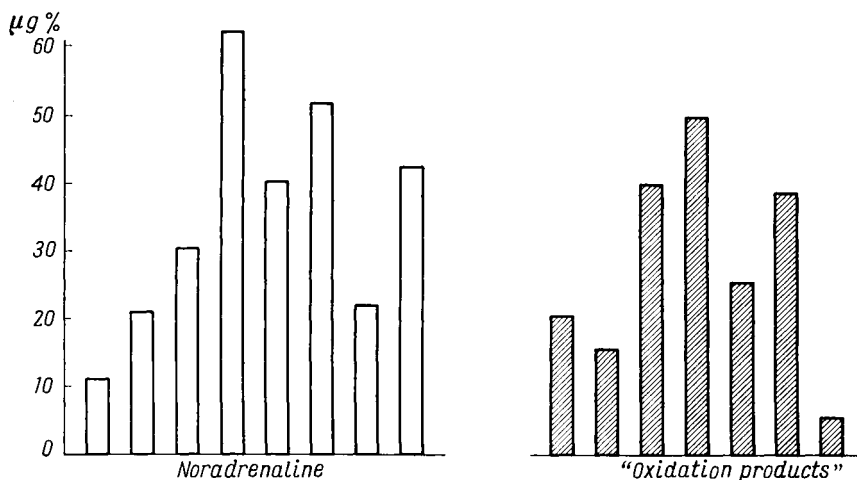


FIG. 1. The content of noradrenaline and adrenaline-like oxidation products in rabbit brain.

Figure 1 shows the content of these substances in rabbit brain estimated by fluorescence analysis (Osinskaya). Each column represents a different experiment, and it can be seen that rabbit brain contains a considerable amount of noradrenaline and substances similar to oxidation products of adrenaline and noradrenaline. If we compare these figures with our photometric data in Fig. 2, we see that the CS of the adrenergic substances is frequently higher than 2, so that it cannot possibly be due to noradrenaline alone, but must also include some oxidation products.

According to Osinskaya, the brains of some rodents do not contain adrenaline, but considerable quantities of noradrenaline and oxidation

products of adrenaline-like substances. Lipovetskaya finds that guinea pigs have substances whose CS is usually equal to 1. Similar findings are obtained in rats. In some cases, however, guinea pig and rat brains contain adrenaline-like substances with a higher CS; this will require further investigation.

THE EFFECT OF STIMULATION OF MUSCLES ON THE ADRENALINE-LIKE SUBSTANCES IN RABBIT BRAIN

Stimulation was carried out for 10 min, using an induction coil and a step-down transformer. In intact controls and in stimulated animals, areas roughly corresponding to the right and left motor areas were investigated.

In Fig. 2 the average result of 12-15 readings showed that the motor area of the cortex on both sides in intact animals usually contained an equal and appreciable amount of catecholamines. Their CS usually exceeded 2. Electrical stimulation of the right thigh muscles usually caused some contralateral increase of adrenaline-like substances. Their CS on the contralateral side fell to 1 in most experiments, whereas at the site of stimulation it remained nearer 2. This indicated that qualitative changes were taking place in the brain when functional changes only were occurring in the effector organ.

ENDOCRINE INFLUENCES ON CATECHOLAMINES IN THE BRAIN

Some animals had been given 0.25-0.30 g per kg of thyroid for 3 months, while in others the rabbits had been given 0.25 g per kg of 6-methyl-thiouracil for 25 days.

The means from a number of experiments are shown in Fig. 2. Both thyroid and 6-methyl-thiouracil administration caused an increase in the adrenaline-like substances in the brain and changes of the CS of the substances. The exact interpretation of these changes is difficult, due to their variability and the difficulty of reproducing them. However, one reproducible result was of great interest. Animals not given thyroid or 6-methyl-thiouracil showed differences of CS in the two motor areas on the opposite sides of the brain on stimulation, as well as in the contralateral muscles. However, those pretreated with these drugs did not show the characteristic differences between the two sides on stimulation. Under such conditions the subtle changes which took place in the catecholamines of the motor areas seemed to be "evened out".

It is interesting that both excess of thyroid hormones and their blockage should produce a similar effect. It is possible that this is because injection of thyroid suppresses the thyroid gland of the animal, as does 6-methylthiouracil. It is also possible that the functional state of the gland and perhaps interoceptive impulses from hormone-producing cells of this gland play a role in the metabolism of adrenaline-like substances in the brain.

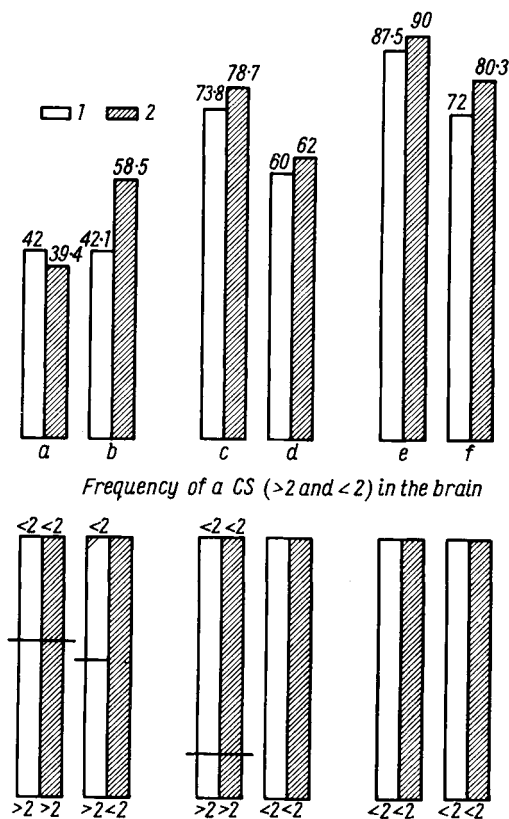


FIG. 2. Changes in the content of adrenaline-like substances in the motor regions of the left and right cerebral hemispheres of rabbits with various functional states in the muscles, after hyperthyroidization and treatment with 6-methylthiouracil: 1—right hemisphere; 2—left hemisphere; a—control; b—stimulation; c—hyperthyroidization; d—hyperthyroidization + stimulation; e—treatment with 6-methylthiouracil; f—treatment with 6-methylthiouracil + stimulation.

Butom and Vartapetov showed the importance of endocrine factors for the metabolism of mediator systems of the brain. They studied the effect of castration on the catecholamines of the brain. The effect of cessation of

the endocrine function of the sexual glands on the whole body, and particularly on higher nervous activity, is well-known (Pavlov and Petrova).

Sexually mature male rabbits were divided into several groups. Some of them were killed 10 days after castration, others at 45 and 90 days. To exclude the effect of the operation itself, control animals had laparotomies and were killed at the same intervals. Figure 3 shows results of these experiments. Each column is a single experiment. It can be seen that castration led to a prolonged increase in the content of adrenaline-like substances and marked qualitative changes in these substances in the brain. Bromide, which Pavlov and Petrova had shown to have a favourable effect on the higher nervous activity disordered by castration, led to some restoration of these substances in the brains of the castrated animals.

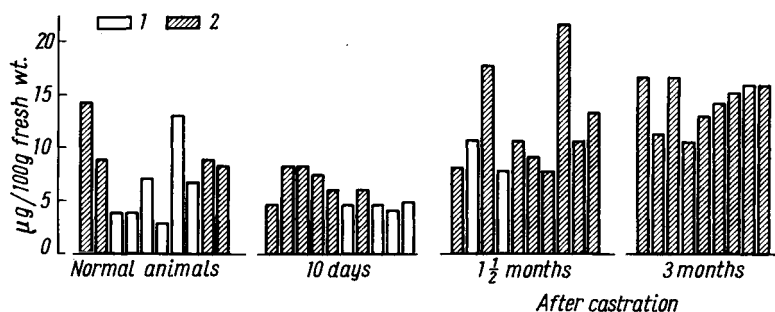


FIG. 3. The influence of castration upon the content of adrenaline-like substances in the brain of rabbits: 1—CS > 2; 2—CS < 2.

CHANGES IN THE CONTENT OF CATECHOLAMINES IN THE HEART DURING NERVE STIMULATION

According to Osinskaya, the hearts of rabbits contain mostly noradrenaline with a small amount of adrenaline. The question of whether the noradrenaline is localized mainly in the nerve endings or in the heart muscle cannot be regarded as settled. Barts, in our laboratory, using TCA, ammonium sulphate and 30 per cent urea as precipitating agents, showed that part of the noradrenaline in rabbit heart is present in a bound state. These experiments are shown in Fig. 4 in which each column represents a single experiment.

In an animal under sodium amylobarbitone and urethane intravenous anaesthesia, stimulation of the peripheral end of the cut cervical sympathetic nerve was carried out with 4 V pulses for 3 periods of 30 sec at 5 min intervals. When stimulation was accompanied by an inotropic effect, regular changes in the amount of noradrenaline in the heart occurred, as well as changes in the relative proportion of the free- and protein-bound fractions.

Usually the total noradrenaline increased, mostly in the protein-bound fraction. This indicates the functional importance of protein-binding which the catecholamines seem to undergo (Fig. 4).

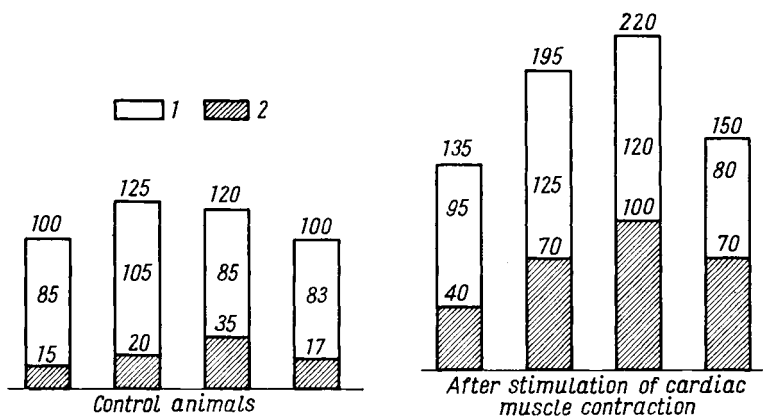


FIG. 4. Influence of stimulation of the cervical sympathetic nerve upon the content of combined and free noradrenaline in the heart of rabbits ($\mu\text{g}/100\text{ g}$ tissue): 1—free noradrenaline; 2—combined noradrenaline.

It should be emphasized that those substances found in nervous tissue and the effector organs which have similar properties to the oxidation products of adrenaline and noradrenaline are also to some extent present in the bound state (Osinskaya). What is the functional significance of the combination of these substances and their intermediates with proteins in the central nervous system?

DISCUSSION

It can be regarded as established that it is the noradrenaline, and not the adrenaline, which is the important catecholamine at least in some animals. It is, of course, likely that in the brain adrenaline intermediates and other adrenaline-like substances are also present. This idea should be viewed in the context of the histochemical studies of the brain developed by Palladin and his co-workers.

Stimulation of the muscles causes similar changes in the brain and effector organ catecholamines. These changes are disturbed by changes in the state of the thyroid gland, and castration also exerts a marked influence on these substances in the brain of rabbits. Nervous stimulation to the heart of rabbits is accompanied by changes in their metabolism, and an increase in the fraction of protein-bound noradrenaline. We have often stated our view that catecholamines and their intermediates play an important part in the formation

and function of mediator substances (14). However, we do not know what the differences are in the metabolism and function of catecholamines between the central and peripheral nervous systems, and between conduction and transmission. Dale, one of the chief advocates of the chemical transmission theory, said (15), "If secretion of a chemical mediator at a nerve ending should prove to be a process not peculiar and limited to that ending... but merely a local intensification to ensure transmission to a contiguous cell of a process which actually figures in the propagation of the impulse along the nerve fibre, we should have to make yet a further revision of our existing conceptions." Dale believed that the same kind of mediator processes occurred both during conduction and transmission. Feldberg (16) underlines the similar character of these processes in the central nervous system and the peripheral nerves. He does not regard acetylcholine as the only mediator in the central nervous system, as many authors do, but considers a possible role for histamine, adenosinetriphosphate and other physiologically active substances, to which 5-hydroxytryptamine could now be added. He believes that adrenergic substances in the central nervous system are not mediators themselves, but act on the sympathetic centre of the hypothalamic region.

In our opinion, the reaction of the brain catecholamines to the state of the heart points to the functional importance of those substances in the processes taking place centrally. Such studies cannot, however, differentiate between a direct mediator effect and an effect via a central neural mechanism. Whether or not the mediator function is the same during conduction as transmission, or whether the latter only represents an intensification of the former, they are both probably based on adrenergic mechanisms different in their intensity and character. The same applies to their metabolism in the peripheral and central nervous system.

Further investigation will be required on adrenaline and noradrenaline intermediates in central nervous structures and their relation to cholinergic and histaminergic factors. The protein binding of catecholamines in the central nervous system, and functional and biochemical studies of the catecholamine rich hypothalamic region will be of value, and they are at present in progress in our laboratory.

SUMMARY AND CONCLUSIONS

1. The brain of rabbits contains adrenaline and the intermediate products of oxidation of adrenaline and noradrenaline. Pilot studies of the level and character of these substances in the brains of Siberian marmots, guinea pigs, and rats have been done.

2. Electrical stimulation of the thigh muscles of rabbits causes changes in the catecholamines of the brain which are different in the two motor areas. Administration of thyroid compounds and 6-methyl-thiouracil to animals affects the reaction of these substances in the brain to muscle stimulation.

3. Nerve stimulation of the heart in rabbits is accompanied by changes in their noradrenaline content, usually with an increase in the protein bound noradrenaline.

4. The metabolism of adrenergic substances in the nervous centres during conduction and transmission is discussed.

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SOURCES OF AMMONIA IN BRAIN HOMOGENATES

YE. E. KLEIN

Department of Biochemistry, Beritashvili Institute of Physiology,
Georgian Academy of Sciences, Tiflis

ALTHOUGH the metabolism of ammonia has been studied for a very long time, its origin has always been rather obscure; it is not known whether it is a waste product or one of the necessary links in the biochemical chain of stimulation.

Some authors regard substances containing amido- or amino-nitrogen, like adenylic acid, glutamine, glutamic acid, or protein, as possible sources of ammonia in the brain. Ferdman (1) and Vladimirova (2), for example, have suggested adenylic acid and glutamine. Takagaki (3) regards glutamic acid as the main source, while Weil-Malherbe (4) and Vrba (5) throw doubt on this, and regard protein as the only source of ammonia during nervous activity. In view of these divergent views we decided to investigate this problem in a very simple system—brain homogenates.

METHOD

Ammonia formation was studied by incubation of rat brain homogenate under various conditions, while at the same time estimating changes in levels of suspected ammonia sources in the tissue. The ammonia was estimated by vacuum distillation and Nessler's reagent; the amido-nitrogen was estimated after hydrolysis for 2 hr in 1 N HCl by Conway's diffusion method (6) and Nessler's reagent. Adenylic acid was isolated from alcohol extract of brain by paper electrophoresis in 1 N acetic acid at pH 2.4, with a potential gradient of 10–12 V/cm for 10 hr, and subsequent spectro-photometry of eluates of the spots. Glutamine and amino acids were estimated by paper electrophoresis, development with ninhydrin, and photometric estimation of the eluates of the spots; electrophoresis was carried out in 0.5 M pyridine-acetate buffer at pH 4.0 at a potential gradient of 10 V/cm for 4–5 hr. The following amino acids were isolated: glutamic acid, aspartic acid, γ -amino-butyric acid, and a group of neutral amino acids. Glutamine

was then isolated in 1 N acetic acid (pH 2.4) at the same voltage and the same time for the electrophoresis of the eluate of the spots corresponding to the neutral amino acids.

FORMATION OF AMMONIA

Incubation of the homogenates was carried out in 0.05 M potassium phosphate buffer at a pH of 7.5 and in Krebs-Ringer bicarbonate solution (7). In both cases there was a rapid increase in the ammonia content in the first 15 min followed by a period of slower increase. The original increase was more marked in the phosphate buffer, and the total accumulation of ammonia was higher than in bicarbonate solution (Fig. 1). In the phosphate buffer 11.4 mg % of ammonia-N accumulated within an hour, compared with only 8.0 mg % in bicarbonate buffer. Under anaerobic conditions, somewhat less ammonia accumulated than under aerobic conditions. These findings are in agreement with those of other authors who, however, did not use such high concentrations of phosphate (3, 4).

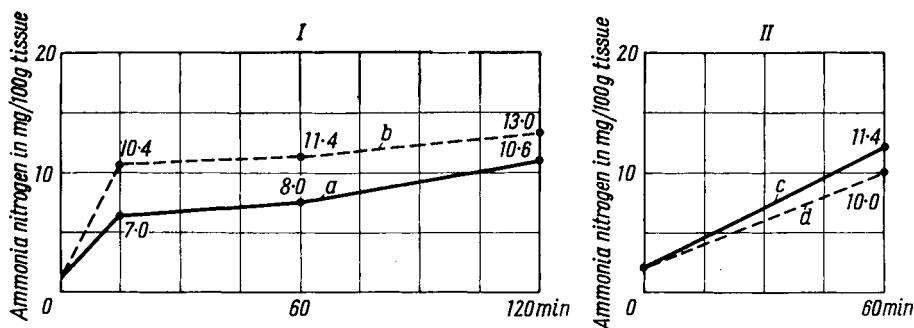


FIG. 1. The accumulation of ammonia during incubation of brain homogenates, with shaking, at 37°C: I—under aerobic conditions in Krebs-Ringer medium and 0.05 M phosphate buffer; II—under aerobic and anaerobic conditions in 0.05 M phosphate buffer; a—Krebs-Ringer medium; b—0.05 M phosphate buffer; c—aerobic conditions; d—anaerobic conditions.

Where does this ammonia come from? Adenylic acid disappears from the homogenate within 20 min of incubation. This is shown by a fall of ultra-violet absorption at a wavelength of 260 m μ . Within an hour 0.1 mMoles/100 mg of adenylic acid added to the tissue also disappears (Fig. 2). This adenylic acid is de-aminated, as is evidenced by an equivalent increase of ammonia (Fig. 3). Adenylic acid would then appear to account for about

3 mg % ammonia-N of the total ammonia which accumulates during incubation.

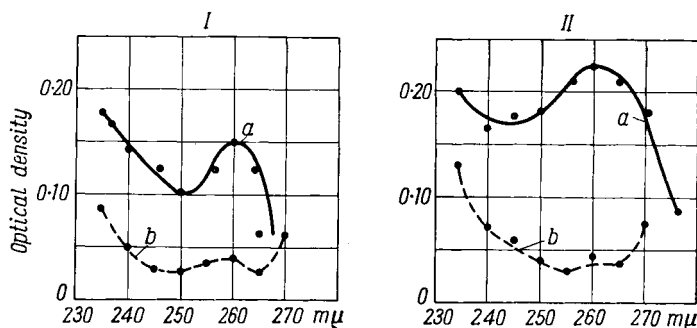


FIG. 2. Ultraviolet absorption curves before and after incubation. Spectrophotometry of eluates from electrophorogram spots containing adenylic acid: I—extract of rat brain homogenate without addition of adenylic acid, incubation 20 min; II—extract of brain homogenate with addition of 0.1 μ Moles adenylic acid per 100 mg tissue, incubation one hour; a—before incubation; b—after incubation.

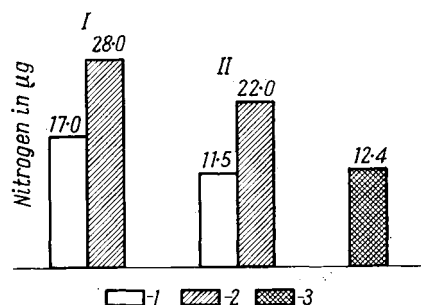


FIG. 3. The influence of added adenylic acid (12.4 μ g nitrogen per 100 mg tissue) upon the formation of ammonia by rat brain homogenate, incubation for one hour under aerobic conditions: I—in 0.05 M phosphate buffer; II—in Krebs-Ringer solution; 1—ammonia-N without adenylic acid; 2—ammonia-N with added adenylic acid; 3—amino-N with added adenylic acid.

A number of enzyme systems present in brain tissue are known to de-aminates adenylic compounds; for example, Schmidt's de-aminase, adenosine de-aminase and a specific adenylic acid de-aminase recently reported by Muntz (8). Doubts about adenylic acid as a source of ammonia formation *in vivo* had been expressed before the papers of Embden, Parnas, Ferdman and Vladimirova, because the amount in brain tissue is only 50–100 mg %

and its cyclical re-amination could not be demonstrated. For this reason, some authors had categorically denied its participation in ammonia formation. However, re-amination of adenylic acid has been demonstrated by Kometiani and Klein (9), and has been confirmed by American authors who showed this reaction with an enzyme isolated from the bone marrow (10).

GLUTAMINE AND GLUTAMIC ACID

If the homogenate is incubated in Krebs-Ringer bicarbonate buffer saline, the glutamine remains at a level of about 5 mg % of amido-nitrogen. If, however, 0.05 M phosphate buffer is used instead, glutamine quickly disappears and an almost equivalent amount of glutamic acid accumulates; such an increase in glutamic acid was not observed with bicarbonate buffer (Fig. 4). In phosphate buffer, the addition of glutamine to the reaction medium increases ammonia formation to an extent almost equivalent to the glutamine added, whereas addition of glutamine to the bicarbonate buffered solution causes very little increase in ammonia formation (Fig. 5).

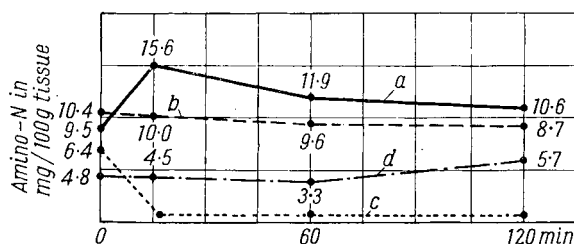


FIG. 4. Changes in levels of glutamic acid and glutamine after incubation of rat brain homogenate under various conditions: a—glutamic acid in phosphate buffer; b—glutamic acid in Krebs-Ringer solution; c—glutamine in phosphate buffer; d—glutamine in Krebs-Ringer solution.

It thus appears that glutamine in phosphate medium is quickly de-aminated to produce 6–7 mg % ammonia. If the concentration of phosphate is low, as in bicarbonate solution, this ammonia production is suppressed, but as experiments with homogenates are normally carried out in Krebs-Ringer bicarbonate buffered solution, de-amination of glutamine has not previously been observed. It is known that the pH optimum of brain glutaminase (glutaminase II) is 9, and it has very little activity at pH 7.5, but it is usually forgotten that this enzyme is activated by phosphates. Thus this path of de-amination could become important if the phosphates in the living cell were to undergo a localized increase. Experiments on living animals

have added some indirect evidence for the derivation of ammonia from glutamine during excitation in brain (11, 12) and in muscle (13).

Incubation of the homogenate leads to a fall in glutamic acid (Fig. 4); in phosphate solution this decrease occurs after a preliminary rise caused by de-amination of glutamine, whereas in Ringer solution the same increase occurs without preliminary rise. Is this increase due to the formation of ammonia? A decrease in glutamic acid was observed among others by Takagaki (3) who concluded—in our opinion, wrongly—that this was mainly due to its oxidative de-amination and that glutamic acid represents the main source of ammonia in brain homogenates, and possibly also in the living brain.

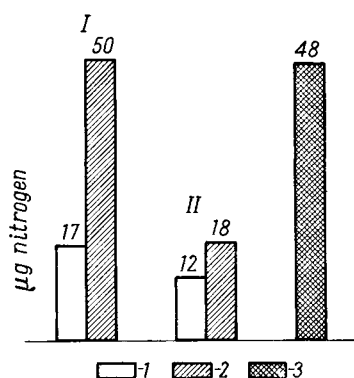


FIG. 5. The effect of adding glutamine ($48\mu\text{g CONH}_2\text{-nitrogen per } 100\text{ mg tissue}$) to rat brain suspension, on the formation of ammonia. Incubation for 1 hr: I—in 0.05 M phosphate buffer; II—in Krebs-Ringer solution; 1—ammonia nitrogen without glutamine; 2—ammonia nitrogen with glutamine; 3— $\text{CONH}_2\text{-nitrogen}$ of the added glutamine.

We investigated the transformation of the main amino acids of the brain, glutamic acid, aspartic acid and γ -amino-butyric acid, during incubation of homogenates under our experimental conditions. Some of these findings have already been published (14). We showed that oxidative de-amination of glutamic acid occurred, de-carboxylation of both two carboxyl amino-acids took place, and there were also a number of transaminating systems. The transformation of these amino acids were closely related, and changes in one led to changes in others. Decarboxylation and transamination of glutamic acid took place at a very fast rate, which was shown by a simultaneous increase in γ -amino-butyric acid and aspartic acid (Fig. 6). If glutamic acid was added to the medium during incubation, there was a greater increase in the γ -amino-butyric acid and aspartic acid (Fig. 6).

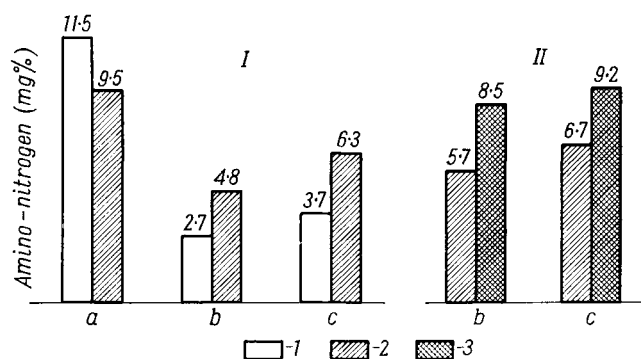


FIG. 6. Changes in the levels of glutamic acid, aspartic acid and γ -amino-butyric acid after incubation of rat brain homogenate: I—without addition; II—after addition of glutamic acid (50 μ g as NH_2 -nitrogen per 100 mg tissue). Incubation for one hour under anaerobic conditions in 0.05 M phosphate buffer; 1—without incubation; 2—incubation without addition of glutamic acid; 3—incubation with glutamic acid; a—glutamic acid; b— γ -amino-butyric acid; c—aspartic acid.

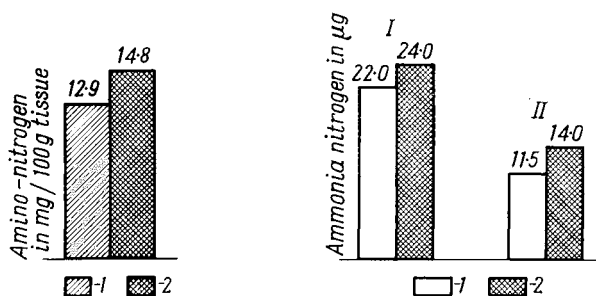


FIG. 7. The content of glutamic acid after one hour's incubation of rat brain homogenate under aerobic and anaerobic conditions in 0.05 M phosphate buffer: I—under aerobic conditions; II—under anaerobic conditions.

FIG. 8. Formation of ammonia in rat brain homogenate in the presence of glutamic acid under aerobic conditions in Krebs-Ringer medium and in 0.05 M phosphate buffer. Glutamic acid was added at 50 μ g amino-nitrogen per 100 mg tissue: I—phosphate buffer; II—Krebs-Ringer medium; 1—without glutamic acid; 2—with glutamic acid.

We therefore conclude that the decrease in glutamic acid during incubation is mainly due to decarboxylation and transamination. Oxidative de-amination takes place in that part of the glutamic acid which corresponds to the difference found in aerobic and anaerobic conditions, which amounts to only about 2 mg % of amino-N (Fig. 7). The opinion of other authors that glutamic acid oxidation hardly ever yields ammonia is confirmed by our experiments (Fig. 8).

PROTEIN

Protein has recently been suggested as a metabolic relation of ammonia. Weil-Malherbe (4) suggests that the action of proteases in the brain may lead to the formation of ammonia. Vrba has recently produced evidence for liberation of ammonia from the amino groups of protein (5). Ferdman and Epstein (6) found that muscle proteins eliminate ammonia by aminating the terminal carboxyl groups.

We next studied the role of proteins in brain homogenates. Within 15 min of incubation the amino-N of protein begins to decrease gradually until by 2 hr it is 7–10 mg % (Fig. 9). There is no difference in this effect between incubation in phosphate or bicarbonate buffered media, or between aerobic and anaerobic conditions.

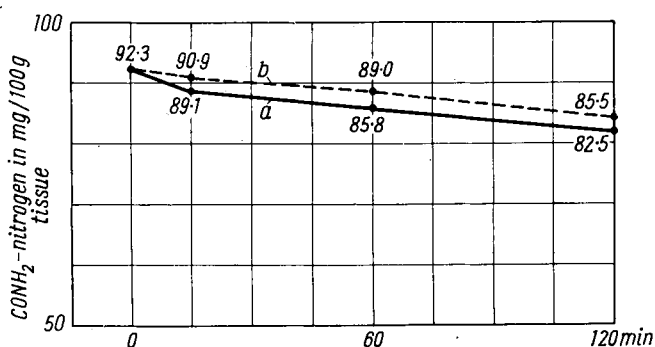


FIG. 9. Changes in the content of protein amino-nitrogen during aerobic incubation of rat brain homogenate: a—in Krebs-Ringer solution; b—in 0.05 M phosphate buffer.

The question arises whether the decrease in amino groups of protein is due to proteolysis, i.e. a decrease in the total amount of protein present. If this were the case, the amino-N of free amino acids should show a corresponding increase of about 70–100 mg % within 2 hr—amino-N in protein

is about 10 per cent of the total—whereas the amino-N actually increases by only 13–14 mg % in 2 hr (Fig. 10). It would appear that liberation of amino-N from glutamine linked to the protein molecule does take place to some extent. In this respect we agree with Vrba. We do not think, however, that there is sufficient evidence for the statements of Weil-Malherbe (4) and Vrba (5) that protein represents the *only* source of ammonia.

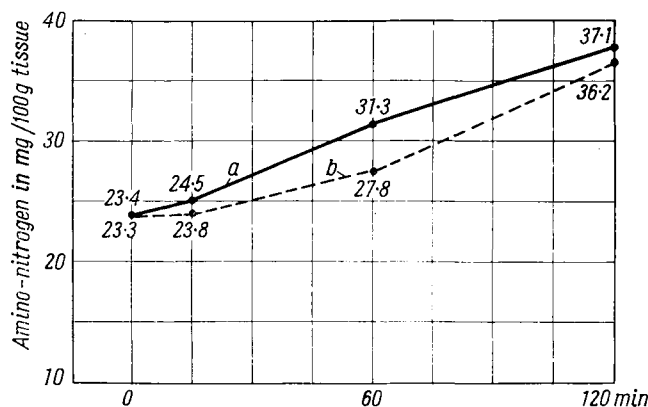


FIG. 10. Changes in the content of amino acid nitrogen (as NH_2) in brain homogenate during incubation: a—in Krebs-Ringer medium; b—in 0.05 M phosphate buffer.

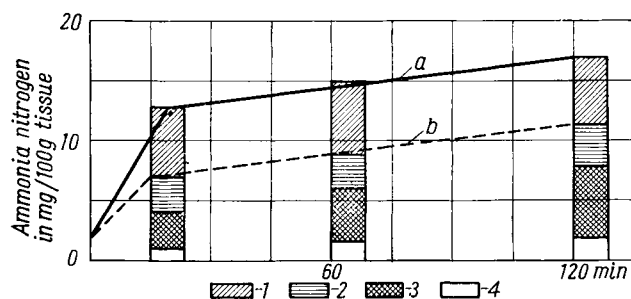


FIG. 11. Diagram to show the degree of participation of various sources of ammonia in incubated rat brain homogenate: I—glutamine; II—adenylic acid; III—protein amino-nitrogen; IV—glutamic acid; a—phosphate buffer; b—Krebs-Ringer medium.

SUMMARY

Ammonia in rat brain homogenates derives from several sources which liberate it at different rates, as shown schematically in Fig. 11. Glutamine and adenylic acid liberate it at a fast rate; glutamine produces it only in the presence of high concentrations of phosphate. Both sources are exhausted after 15–20 min incubation. Liberation at a slower rate takes place from the amino groups of proteins. A small part may be liberated from glutamic acid.

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FORMATION OF AMMONIA IN BRAIN SLICES

R. VRBA, YA. FOLBERGER AND V. KANTŮREK

Institute of Occupational Hygiene and Occupational Diseases,
Prague, Czechoslovakia

AMMONIA formation greatly influences the functioning of the nervous system. Tashiro (1) was the first to show that electrical stimulation of nerve (*in vitro*) causes increased ammonia formation. These results have been verified since, and heat, light, chemical agents and even mechanical stimulation have since been found to produce similar effects (2-7).

Similarly, in the central nervous system, several authors (8-14) have shown increased free ammonia in the brain after stimulation by electric shock, hypoxia, convulsants, conditioned and unconditioned reflexes and other means. Thus any attempt to explain the activity of the nervous system on a biochemical basis must consider the role of ammonia.

Using cerebral slices, which have been shown to produce large amounts of free ammonia when incubated (15-19), we attempted to consider the following questions.

1. *In vivo* experiments led us to believe that increased ammonia formation following increased functional activity was due to de-amination of brain proteins (20-22) and this needed testing in our *in vitro* system.

2. Weil-Malherbe and Green (19) had shown that de-aminases were the main cause of cerebral ammonia formation. They suggested that ammonia formation in slices was due to proteolysis, a theory which we have tested.

3. We also decided to test the theory (Weil-Malherbe (23)) that ammonia formation in brain slices is connected with lipid metabolism.

4. The functional activity of nervous tissue is related to the transport of intracellular potassium (24-26) which is related, in turn, to ammonia formation (1-7): such relationships have been established *in vivo* and *in vitro*. Potassium content falls with increased functional activity *in vivo* (27, 28) and lowered potassium content is accompanied by increased ammonia (10, 20-22, 29). Similar relationships may be observed in brain slices, for instance incubation without substrates causes accumulation of ammonia in the slice and leakage of potassium into the medium. We tried to elucidate the mechanisms underlying these relationships.

METHODS

Usually 36 slices were prepared from the cerebral cortex of 6 guinea pigs, and 12 of them randomly selected and incubated in two Warburg vessels. In one vessel trichloroacetic acid (TCA), was added at the beginning of the experiment and the other was incubated 4–6 hr before fixation. The medium was Krebs-Ringer (phosphate buffered) and the gaseous phase air. Slices were analysed for free ammonia and non-protein nitrogen (TCA-soluble nitrogen). The TCA extracted residue was hydrolysed for 3 hr in 20 per cent HCl and an aliquot removed for total nitrogen estimation by Kjeldahl's method (this included the lipid nitrogen). Free ammonia was measured in the TCA-soluble fraction which included that released from amide groups (33, 34).

Results are given in Fig. 1a and show that after 4 hr incubation there was an average increase of ammonia of $169 \mu\text{moles/g}$ fresh weight. Protein nitrogen decreased by $3.8 \mu\text{moles/g}$ ($p < 0.02$), i.e. 22 per cent of the ammonia was derived from the breakdown of protein-amide bonds.

Figure 1b gives the results of a second series of experiments where 6 hours' incubation produced an increase of $20.1 \mu\text{moles/g}$ of ammonia and protein nitrogen decreased by $5.6 \mu\text{moles/g}$ so that protein-amide bond breakdown was responsible for 28 per cent of the ammonia increase. These results show that about 25 per cent of the ammonia formed during incubation is derived from the breakdown of functional protein amino groups.

The observed fall in the concentration of protein functional amino groups is caused by the breakdown of protein-bound amide groups and is not due to glutamine released by proteolysis. Slices incubated without a substrate do not accumulate glutamine in the medium and free glutamine does not act as a source of ammonia in brain slices (19). From this it follows that accumulation of ammonia in the medium is not due to its release from glutamine resulting from proteolysis.

Figure 1a also shows that the increase in non-protein nitrogen (excluding ammonia) is $15.4 \mu\text{moles/g}$ after 4 hr and $29.8 \mu\text{moles/g}$ after 6 hours' incubation. Both increases are statistically significant, although small, being only 1.5 and 3.0 per cent of the total TCA-soluble nitrogen respectively. From this it follows that during incubation, certain high molecular weight nitrogenous substances are liberated although the process is slow. The chemical nature of these substances is as yet unknown and even if one assumes they are amino-acids or soluble peptides, proteolysis could still not be the cause of their release.

In subsequent experiments we noted that "lipid nitrogen" of brain slices increased during incubation. Six slices were fixed immediately after placing

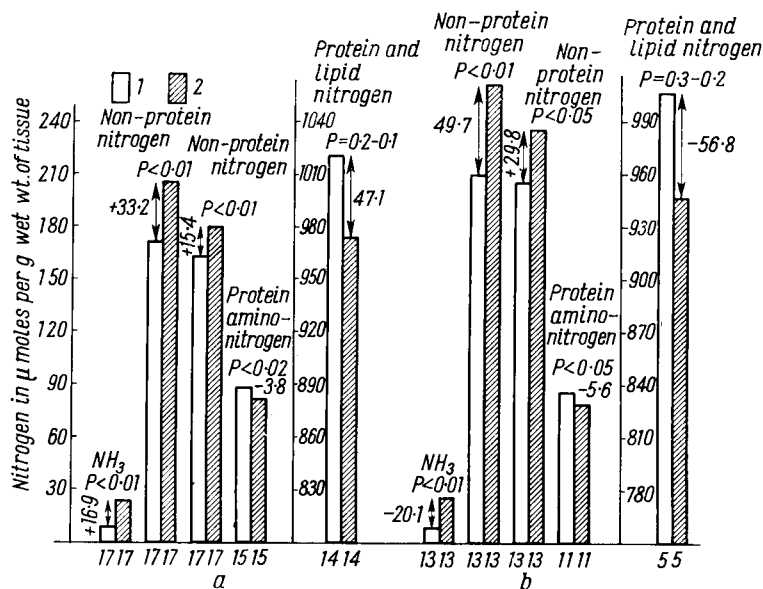


FIG. 1. a—changes in the content of ammonia nitrogen, non-protein nitrogen, amino-nitrogen of proteins and protein nitrogen in guinea pig brain slices after four hours' incubation in a Krebs-Ringer phosphate medium without oxidizable substrate; b—changes in the content of ammonia nitrogen, non-protein nitrogen, protein amino-nitrogen and protein nitrogen in guinea pig brain slices after six hours' incubation in Krebs-Ringer phosphate medium without oxidizable substrate; 1—before incubation; 2—after incubation. The numbers below the columns indicate the number of experiments.

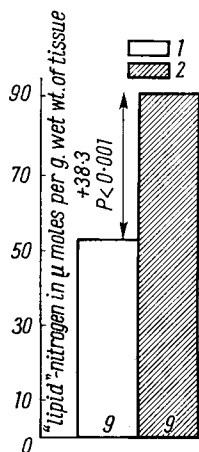


FIG. 2. Changes in the content of "lipid nitrogen" in guinea pig brain slices after four hours' incubation in Krebs-Ringer phosphate medium without oxidizable substrate: 1—before incubation; 2—after incubation.

in 4 ml Krebs-Ringer phosphate saline and 6 others from the same batch incubated for 4 hr before fixation by TCA. After extraction by TCA, 8 ml acetone were added to the residue and shaken vigorously at room temperature. After centrifugation the residue was removed and extracted with 3:1 alcohol-ether, then with 2:1 chloroform-methanol, and finally with ether. The solvent extracts were pooled and 25 ml methanol added. A 10 ml sample was evaporated to dryness and total nitrogen measured in the residue. The results are given in Fig. 2, and show that the concentration of "lipid nitrogen" increases markedly during incubation.

We conclude that during incubation protein-lipid complexes undergo enzymatic breakdown resulting in free ammonia and nitrogen extractable by lipid solvents. In spite of these observations, however, the major source of ammonia release remains unknown.

Further studies made by us throw additional light on the mechanism of ammonia formation. Formation of ammonia in brain slices is influenced by the ionic composition of the medium although de-amination of proteins seems to be insensitive in this respect. Six slices were placed in each of 6 Warburg vessels containing 4 ml of Krebs-Ringer phosphate saline. One vessel was fixed immediately with TCA and the other vessels containing media with different concentrations of sodium and potassium ions were incubated for 4 hr.

The results given in Fig. 3 show that the higher the extracellular potassium ion concentration the lower the rate of ammonia accumulation and vice versa. The lower the potassium ion concentration, the quicker the ammonia accumulated.

In other experiments we found that although potassium ions partially inhibit ammonia formation, they do not affect accumulation of non-protein nitrogen which again shows that ammonia formation is not coupled with proteolysis in spite of the fact that it is derived from protein-like substances.

DISCUSSION

Weil-Malherbe and Green (23) have shown that the ammonia of incubated brain slices is derived from a variety of sources. They emphasized two important points, namely the existence of an autolytic system and the preservation of the morphological structure and functional activity of the tissue. Our studies have shown that some of the ammonia is complexly derived from protein deamination and possibly more from lipoprotein degradation, although the main source of ammonia formation is unknown. Our results do not support the theory of Weil-Malherbe and Green that proteolysis is a major source.

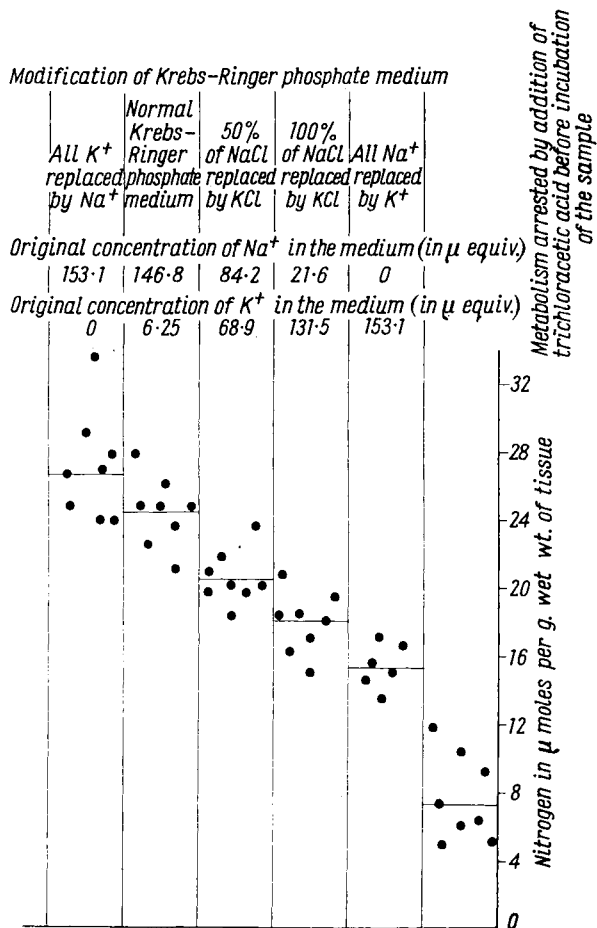
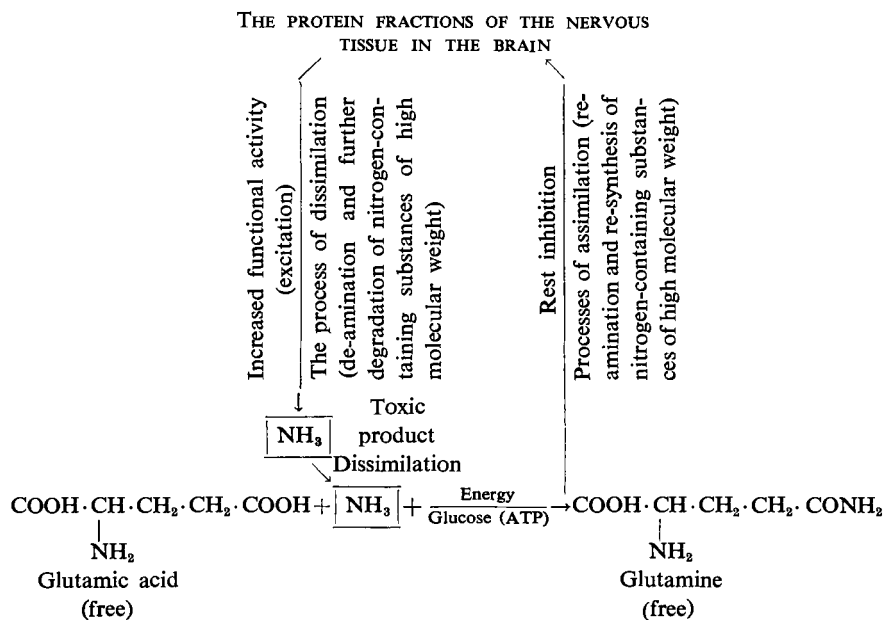


FIG. 3. The influence of various concentrations of K^+ upon the accumulation of ammonia in guinea pig brain slices.

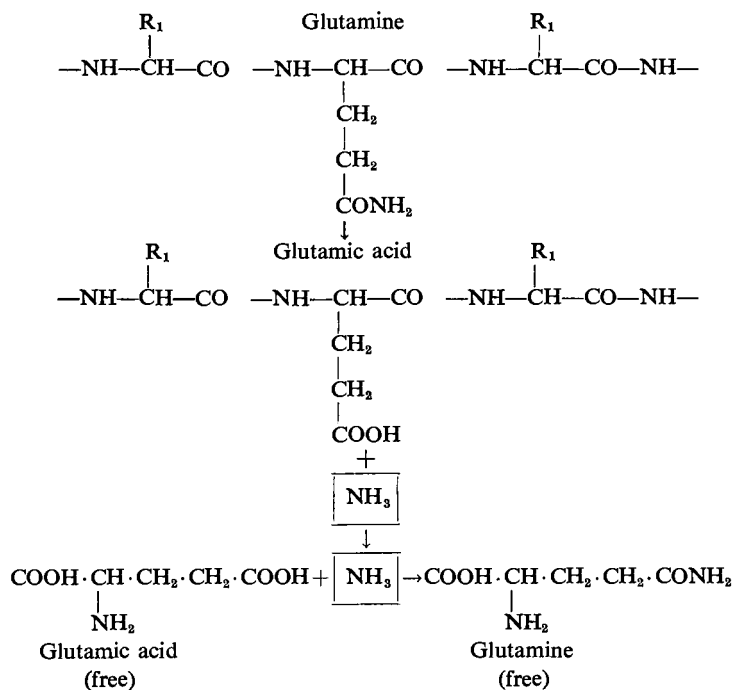
Bearing in mind our *in vivo* studies, we present the following hypothesis:

A cyclic process of protein synthesis and degradation constitutes one of the functional activities of the brain. Degradation is characterized by de-amination and further breakdown resulting in ammonia accumulation. Protein synthesis draws on this pool of free ammonia via the glutamic acid-glutamine system. This hypothesis (see also 20-22, 29, 36-38) is illustrated below.

Figure 1a and 1b show that incubation without a substrate produces protein breakdown and release of ammonia. Ammonia formed *in vivo* combines with glutamic acid to give glutamine (29, 31) and the same process occurs *in vitro* when glucose is added to the medium (17-19, 39).



SCHEME OF FORMATION AND DETOXICATION OF AMMONIA
IN THE BRAIN OF RATS UNDER PHYSICAL STRESS



Our hypothesis, presented in the schemes printed below, is based on *in vivo* experiments, but the effects can often be demonstrated *in vitro*.

In vitro the following processes have been observed:

- a. De-amination of brain proteins
- b. Further degradation of high-molecular weight nitrogenous substances
- c. Ammonia formation
- d. Synthesis of glutamine from glutamic acid and ammonia

Previous reference has been made to the extensive data demonstrating the relationship between potassium transport across the cellular membrane and ammonia formation in nervous tissue. This relationship can be observed in isolated nerves *in vivo* and in incubated brain slices. If these two processes are truly related, one would expect that with the incubated brain slice preparation an increased concentration of potassium ions in the medium would not only lower its diffusion extracellularly, but retard ammonia formation which has been demonstrated by our experiments.

Another explanation is, however, possible. Ammonia can be formed by several other tissues and Warburg *et al.* (15) observed that capacities for glycolysis and ammonia formation were directly proportional. This fact is very relevant to our work, since potassium ions are capable of suppressing anaerobic glycolysis in brain slices.

We have already referred to the fact that increased functional activity of the nervous system is accompanied by increased ammonia formation, hence inhibition of functional activity will accordingly suppress ammonia formation. One would, therefore, expect potassium ions to have an inhibitory effect on the nervous system. Such effects have already been found: for example, Gore and McIlwain (40) showed that high concentrations of potassium in the medium inhibits the increased oxygen uptake of electrically stimulated slices. Furthermore, potassium chloride applied to the brain causes the reversible disappearance of electro-encephalographic records and certain conditioned reflexes (1, 41, 42).

CONCLUSIONS

1. Ammonia accumulates in brain slices incubated in a saline containing no substrate. About 25 per cent of the ammonia is formed by the partial breakdown of protein amino groups.

2. Non-protein nitrogen accumulates in the medium in which brain slices are incubated. In 4 hr this reaches 1.5 per cent of the total TCA insoluble nitrogen. Hence it can be assumed that proteolysis is not a major source of ammonia formation.

3. During incubation the lipid-nitrogen of brain slices increases.
4. The higher the medium concentration of potassium, the slower the rate of ammonia formation: the lower the concentration, the more rapid the rate of ammonia accumulation. This points to a close relationship between potassium and ammonia metabolism in the brain.

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THE RELATIONSHIP BETWEEN DIFFERENT STAGES OF CONDITIONING AND THE AMMONIA CONTENT OF THE BRAIN

YE. A. VLADIMIROVA

Laboratory of Biochemistry of the Nervous System, Pavlov Institute
of Physiology, Soviet Academy of Sciences, Leningrad

THE relationship of behavioural physiology to metabolic processes in the brain is a very important aspect of biochemistry. While inhibition is known to occur in the same structures as excitation, the nature of the mechanisms of inhibition has received scant study. Pavlov wondered whether there was a minimum time course for an isolated conditioned stimulus, which could be sub-threshold, but which would, if prolonged, produce an increasing tendency to an inhibitory state in the cell (1).

Our experiments using the motor-defence reflex suggested that to understand the inhibitory process one has to examine biochemical changes in the brain, not only during the development of inhibition, but also with the different varieties of inhibition of Pavlov's classification (2).

We constructed a chamber to study motor alimentary reflexes in rats, which was designed so that rapid fixation of the brain could be carried out. With this we were able to demonstrate that changes in the ammonia level during conditioned and unconditioned alimentary reflexes were similar to changes in motor defence reactions (3). This latter observation was further evidence for the view we expressed in 1958, that the increase in brain ammonia during excitation was the result of metabolic processes in the functionally changed nervous tissue, and not secondary to work done by the muscles (4).

Experiments carried out with motor defence reflexes and later with motor alimentary reflexes showed that the ammonia content in rat cerebral hemispheres in differentiating inhibition not accompanied by an appreciable orientation reaction, remained within the normal range, whereas incomplete differentiation accompanied by an orientation reaction led to a considerable increase in the concentration of brain ammonia. The fact that during dif-

ferentiation uncomplicated by orientation the ammonia content showed no increase seemed to be evidence of a dynamic balance in this case between the excitatory process and the inhibitory process, similar to the state of "rest" in control rats (5, 6).

To test this interpretation, we recorded motor alimentary reactions on a kymograph, and subsequently estimated the amount of ammonia in the brain under the same conditions. We also studied the relation between changes in the ammonia present in the cerebral hemispheres of rats and the functional state of the central nervous system during the development of differentiation in the case of extinctive inhibition.

METHOD

For the development of differentiation in rats, we used the following routine stimuli: a bell followed by feeding as positive conditioned and test stimulus; there was a two minutes' interval between the end of feeding and the next ringing of the bell, and 2 min elapsed before a buzzer was sounded as a differentiating stimulus. The positive conditioned stimulus alone lasted 5-7 sec, the conditioned and unconditioned stimulus (food) together lasted 10 sec. Alimentary reinforcement, consisting of porridge and milk, was used as the unconditioned stimulus. The sound of the buzzer lasted 1 min every other minute, alternating with the sound of the bell reinforced by feeding. The positive conditioned stimulus was used 5 times in each experiment and the negative twice.

The positive motor alimentary reflex became established after 20-30 conditioned stimuli with reinforcement, and differentiation after 30-40 buzzes not followed by alimentary reinforcement. The speed of consolidation of conditioned reflexes to positive and negative stimuli, the time for animals to cease running for the food between signals, and the speed of disappearance of the orientation reaction depended mainly upon the type of nervous system of the animal in question. In this respect we did not, in fact, investigate the different species, but confined ourselves to selection of the most suitable animals according to age, rate of conditioning, rate of deconditioning, and their particular susceptibilities to these treatments.

Within the first 15 sec of a positive conditioned alimentary stimulus the content of ammonia in the brain increased on an average up to 0.52 mg %, which is 44.7 per cent above normal, and within 30-45 sec it reached 0.5 mg %: these changes were statistically significant (Table 1). Similar changes in the ammonia content of the brain took place at the same intervals when the animals were fed in the usual way.

In those cases where the differentiating stimulus was not followed by a positive motor alimentary reaction, some animals showed signs of excitation, listening with great attention to the buzzer; these reactions were classified as being of insufficiently established differentiation, and they are marked in the Table 1 with 0^+ , 0^{++} , depending upon the intensity of the associated orientation reaction.

In the experiment using the motor defence method, 2 min after an unconditioned stimulation lasting 15 sec, the excess of ammonia which had been formed was eliminated and the normal level restored (Fig. 1, columns 1, 4 and 5).

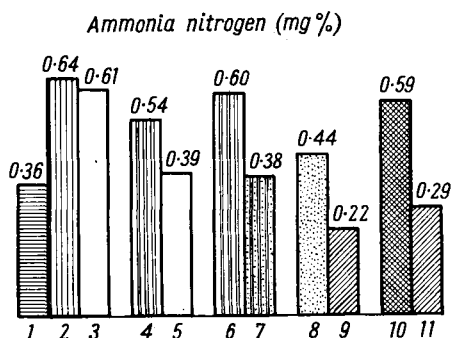


FIG. 1. Changes in the content of free ammonia in the brain of rats in a state of excitation and inhibition (motor-defensive method): 1—normal conditions; 2—conditioned stimulus for 15 sec; 3—after effect of 120 sec; 4—unconditioned stimulus for 15 sec; 5—after effect of 120 sec; 6—unconditioned stimulus for 15 sec; 7—unconditioned stimulus for 600 sec; 8—the fact of the transfer of the animal into the chamber + latency period of 2–3 sec; 9—state of conditioned inhibition lasting 600 sec; 10—orientation reaction of 15 sec; 11—state of inhibition for 120 sec.

The buzzer was sounded every 2 min to secure a constant background. Our photokymogram of rat No. 5 shows a positive motor alimentary reaction to the sound of a bell which was rung 3 sec after the buzzer which had been switched on 3 sec after the bell had been switched off. Another rat, No. 4, showed a positive motor alimentary reaction to the ringing of a bell 2 sec after the buzzer, and an absence of reaction to the bell when the animal was somnolent or asleep. There was no differentiation reaction to the buzzer switched on 0.5 sec after the bell had been stopped. Yet another, No. 3, showed a positive motor alimentary reaction to a bell switched on half a second after a differentiation reaction, and the absence of a differentiation reaction after a buzzer which started half a second after the bell had stopped. One hundred and twenty sec after the end of the positive conditioned reflex,

TABLE 1. THE AMMONIA CONTENT (in mg % nitrogen per fresh substance) IN THE BRAIN OF RATS UNDER NORMAL CONDITIONS DURING CONDITIONED ALIMENTARY STIMULATION AND IN CERTAIN PHASES OF DIFFERENTIATION

Experimental condition	Duration of the isolated action of the conditioned stimulus (sec)	Conditioned motor reaction	No. of experiments	Average and extreme data	Standard deviation	Statistical significance	Increase in % of the normal value	Remark
Normal conditions	—	—	45	0.38 (0.28-0.45)	± 0.0065	—	—	State of relative rest, orientation reaction $\pm$
Conditioned alimentary stimulation (Bell)	15-20	++	24	0.52 (0.40-0.65)	± 0.0158	Increased significance	36.8	The rats rush to the feeding bowl, gnaw the edges of the door and of the surface
Same	30-45	++	25	0.55 (0.42-0.68)	± 0.0152	Same	44.7	The rats rush to the feeding bowl and intensively gnaw the edges of the surface
Interval of 120 sec before the action of the buzzer	—	Orientation reaction	16	0.38 (0.30-0.44)	—	—	—	The rats are quiet, no orientation reaction (—)
Buzzer used for the first time instead of the bell	60	+++	14	0.63 (0.48-0.82)	± 0.0271	Increased significance	66.0	The rats rush rapidly to the feeding bowl, orientation reaction ++++, dyspnoea

Incomplete differentiation — buzzer	60	0 +	18	0.61 (0.46–0.86)	± 0.0258	Same	60.5	The rats do not react to the opening of the feeding bowl but walk about in the chamber, sniffle, listen to the buzzer, orientation reaction +
Complete differentiation — buzzer	60	0	34	0.37 (0.24–0.45)	—	—	—2.6	No reaction to the opening of the feeding bowl some animals carry out intensive washing movements, no orientation reaction (—)
Buzzer for 60 sec, then bell for 2 sec without further reinforcement	15	+ +	18	0.57 (0.40–0.77)	± 0.0247	Increased significance	54.0	The rats rush to the opening of the feeding bowl, intensive motor—alimentary reaction, dyspnoea, orientation reaction + + /

the brain ammonia content was that found normally at rest, an average of 0.38 mg % (Table 1). Under similar experimental conditions, other workers have shown that the heart rate returns to normal within $1\frac{1}{2}$ –3 min (7).

If the differentiating stimulus, the buzzer, was used instead of the positive conditioned stimulus (the bell), an intense motor alimentary reaction accompanied by marked dyspnoea occurred at the beginning of differentiation. Sixty seconds after the beginning of the buzzing the brain ammonia reached a level of 0.63 mg %, corresponding to 66 per cent above normal.

If the so far insufficiently consolidated differentiation was increased, at a time when, following the differentiating stimulus, there was no further positive alimentary reaction but still an orientation reaction, the brain ammonia rose to 0.61 mg %—60.5 per cent higher than normal. In both cases the increase of ammonia compared with normal was statistically significant (Table 1).

If, as in the above experiments, complete differentiation was achieved the animal showed neither a positive motor alimentary reaction to the negative conditioned stimulus, nor an appreciable orientation, the brain ammonia remained normal, at an average of 0.37 mg % of ammonia-N (Table 1).

When one regards the ammonia changes in the cerebral hemispheres as a biochemical index of the functional state of the central nervous system and compares it with the whole complex of physiological reactions which characterize differentiating inhibition, one can distinguish stages in the development of differentiation, with transitions from one stage to the next. This idea is supported in the literature by the demonstration that during negative conditioned stimuli and full differentiation, when the positive conditioned reflex has become completely extinct, the heart and respiratory rates remain within normal limits.

The question then arises whether the functional state of the central nervous system during fully-blown differentiation corresponds to rest in unstimulated animals. To answer this we carried out investigations of the brain ammonia following applications of positive and negative conditioned stimuli at short intervals after full differentiation.

Our photokymograms show that a positive motor alimentary reaction appears within 2–3 sec after the bell, but within only 0.3 sec after the buzzer had been switched off with full differentiation.

Within 15 sec after application of only the positive conditioned stimulus brain ammonia increases by 54 % (to 0.57 mg %) (Table 1): this increase was statistically significant.

This quick appearance of the motor alimentary reaction supports our view that during absolute differentiation the processes of excitation and

inhibition are in a state of balance. Furthermore, later experiments show that the action of the same conditioned stimulus, the bell, against an inhibitory background like somnolence delays or sometimes even completely stops the motor alimentary reaction in our rat No. 4.*

If the buzzer is sounded within 2-3 sec after the bell stops the animals immediately ceases feeding and shows only a weak orientation for a minute; at this time the brain ammonia averages 0.47 mg % (Table 2). On the other hand, with the same negative conditioned stimulus soon after the positive motor alimentary reaction, but sounded $\frac{1}{2}$ -1 sec after the bell is turned off, the animal continues to go towards the feeding dish and is incapable of distinguishing the sound of the buzzer from that of the bell (rats Nos. 3 and 4). Sixty seconds later the brain ammonia was average 0.58 mg % (Table 2). The increases in ammonia in the first and second cases were statistically significant.

It is of interest that within a minute after the positive motor alimentary reaction, the level of ammonia in the brain rose to 0.57 mg %, 9.6 per cent above the original level, and 50 per cent higher than the normal level (Table 2), although no additional excitatory stimuli had been used. The reason for this increase in the ammonia was probably the agitated state of the animal, as this was the first time that the positive conditioning had not been reinforced with food.

It is interesting to compare the ammonia values in these conditions. The action of a differentiating stimulus following a positive motor alimentary reaction without reinforcement not only prevents a further increase in the ammonia (0.57 mg %), but also to a certain extent eliminates the excess of ammonia; the content decreases from 0.52 mg % to 0.47 mg % i.e. becomes 2.6 per cent lower than the original level and 17 per cent lower than the level found in the brain a minute after the motor alimentary reaction. We are inclined to regard the decrease during differentiating inhibition in the period of the after-effect mentioned above as a manifestation of the interrelation between the processes of internal inhibition and excitation.

This is confirmed by experiments with the motor defence method illustrated in Fig. 1. The increased ammonia (0.64 mg %) found within the first minute of reflex excitation remains at the same high level, 0.61 mg % (for 120 sec) (Fig. 1, columns 1, 2 and 3), whereas during inhibition the ammonia during the same period of time decreases from 0.59 mg % to 0.29 mg % of ammonia-N (Fig. 1, columns 10 and 11). A constant decrease occurs during

* All numbers of particular rats refer to an illustration which cannot be reproduced from the original text.

TABLE 2. THE AMMONIA CONTENT (in mg % nitrogen per fresh substance) IN THE BRAIN OF RATS IN A STATE OF RELATIVE REST AND DURING THE PERIOD OF SUDDEN ACTIVITY OF BOTH THE PROCESSES OF EXCITATION AND INHIBITION AND DURING CERTAIN PHASES OF EXTINCTIVE INHIBITION

Experimental conditions	Duration of the isolated action of the conditioned stimulus (sec)	Conditioned motor reaction	No. of experiments	Average and extreme data	Standard deviation	Statistical significance of the changes	Increase in % of the normal values	Increase in % of the original values	Remark
Normal conditions	—	—	45	0.38 (0.28-0.45)	± 0.0065	—	—	—	State of relative rest, orientation reaction $\pm$
Conditioned alimentary —motor stimulation, bell	15-20	++	24	0.52 (0.40-0.65)	± 0.0158	The increase is significant	36.8	36.8	The rats rush to the feeding bowl, gnaw the edge of the door and of the surface dyspnoea
Bell for 15 sec without reinforcement, then an interval of 60 sec	—	Orientation reaction	18	0.57 (0.35-0.86)	± 0.0302	Same	50.0	9.6	The animal continued to rush to the feeding bowl 5-10 sec after the bell stopped ringing, then they go away showing signs of motor unrest; orientation reaction +
Bell for 15 sec without reinforcement, then buzzer within 2-3 sec	60	0 $\pm$	27	0.47 (0.28-0.65)	± 0.0175	Same	23.7	-9.6	The animals move away from the feeding bowl 2-3 sec after the beginning of the action of the buzzer; weak orientation reaction $\pm$

Bell for 15 sec without reinforcement, then buzzer within 0.5-1 sec	60	++ +	12	0.58 (0.47-0.74)	± 0.0264	Same	52.6	11.5	Intensive motor—alimentary reaction throughout the peri- od of action of the buzzer ++ +
	15	+	20	0.55 (0.46-0.71)	+ 0.0116	Same	44.7	-6.0	In response to the action of the bell the animals rush to the opening of the feeding bowl, dyspnoea
	15	0	17	0.38 (0.30-0.44)	—	—	0	-27.0	During the action of the bell the animals respond not only with a positive alimentary— motor reaction but fail to show an appreciable orienta- tion reaction

the development of protective inhibition following prolonged conditioned or unconditioned stimuli (600 sec). The amount of free ammonia in the brain decreases from 0.60 mg % to normal (Fig. 1, columns 1, 6 and 7) and if reflex inhibition is applied for the same period, the ammonia falls considerably below normal to 0.22 mg %, and approaches levels found during natural sleep (Fig. 1, columns 8 and 9; Fig. 2, columns 6 and 7).

This decrease from 0.40 mg % to 0.22 mg % occurs within 5 sec of inhibition (Fig. 2, columns 1, 2 and 3). A preliminary insignificant rise from 0.36 to 0.44 mg % can be seen, which could be caused by moving the animal into the chamber, or the effect of conditioned stimuli immediately preceding the assumption by the animal of the characteristic posture of inhibition.

These and our previous findings with free ammonia, as well as parallel studies of heart and respiratory rate, confirm our hypothesis that a dynamic balance exists between the excitatory and inhibitory processes during complete differentiation and that differentiative and other types of inhibition play an active part in the elimination of excess ammonia, and consequently in the return to normal function of the central nervous system.

We next had a look at changes in cerebral ammonia during extinctive inhibition, which represents one of the simplest types of internal inhibition. Extinction was caused by the positive conditioned stimulus (the bell) being rung without reinforcement, at intervals of 30–60 and 120 sec until the animals failed to give a motor alimentary reaction to a bell rung for 15 sec. The brain ammonia then amounted to 0.38 mg %, that is within the normal range (Table 2). If the animal had not completely accommodated and there was still an appreciable orientation reaction, the level of ammonia was on average 0.55 mg %, 44.7 per cent higher than normal. It thus seems that the formation and elimination of ammonia in the cerebral hemispheres in both differentiative inhibitions as well as accommodation inhibition is closely related to the physiological state of the central nervous system. The new relationship between the excitatory and inhibitory processes is mirrored by biochemical changes including processes which produce an excess of free ammonia in the brain.

The elimination of excess ammonia may take place by reaction with glutamic acid to form free glutamine in the brain (10). Enzymic de-amination of glutamine to free ammonia can be demonstrated in brain slices in borate buffers at various pHs. Under these conditions, maximally at pH 9.2–9.3, a difference appears between the brain and skeletal muscle in ammonia formation. We have repeatedly shown the effects of various drugs on the important glutamine-glutamic acid system (12), and changes induced in it by conditioned and unconditioned reflexes.

In the brain in the first 25 sec of the excitatory state, the increase in ammonia was accompanied by a decrease in the free glutamine followed within 1-2 min by an increase somewhat later. As soon as protective inhibition appeared the amount of newly synthesized glutamine returned to normal. This made it possible that the excess of glutamine might be used for the synthesis of more complicated proteins necessary for the restoration of the normal chemical structure of nerve cells (13).

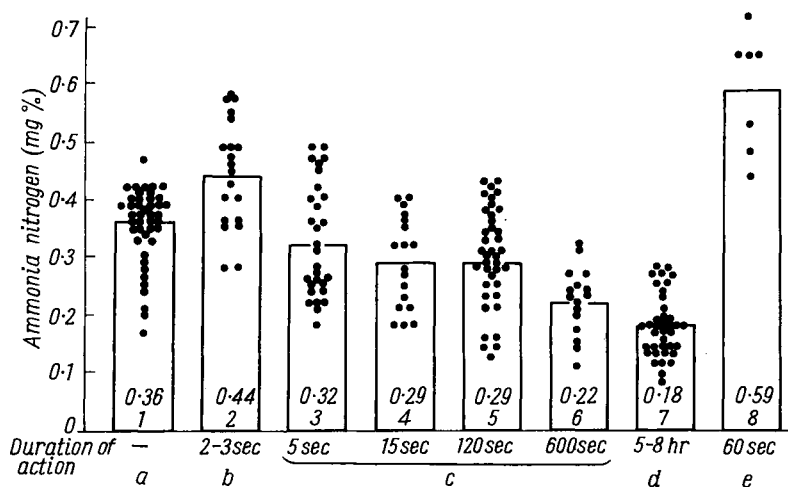


FIG. 2. Decrease in the ammonia content in the brain of rats during the intensification of the state of inhibition and during the period of prolonged natural sleep: a—normal conditions; b—latency period; c—conditioned inhibition; d—sleep; e—the rats are woken up.

Academician Palladin and his co-workers, as well as others, have shown that the cerebral cortex contains more protein than other parts of the brain (14-17): the proteins of the cerebral hemisphere possess the highest turnover rate (18), and therefore the highest capacity of synthesis (19). In well-marked excitatory states, an increase in the specific activity of protein sulphur can be observed after administration of labelled methionine, and also in the activity of protein carbon after injection of ^{14}C -labelled glycine (20, 21). It is interesting that during excitation carbon-labelled glycine is incorporated at a greater rate into the glutamine of the nonprotein fraction, but there are no changes in the activity of glutamic acid, which indicates, in the author's opinion, that there is an increased transformation of glutamic acid into glutamine (20). These observations are consistent with our results of free ammonia in the brain during excitation.

SUMMARY

1. The changes which take place in the ammonia in the brain suggest that the development of differentiative inhibition and extinctive inhibition passes through several phases.

2. The first application of a differentiating stimulus as well as the first extinction of the positive conditioned stimulus without reinforcement are accompanied by well-marked motor alimentary and orientation reactions with a considerable increase in the brain ammonia.

3. When the consolidation of differentiation has been firmly established and when complete extinction of the conditioned positive motor alimentary reflex has occurred, the ammonia in the cerebral hemispheres decreases to the level found in the brains of control rats in a state of "rest", when the excitatory and inhibitory processes are in a state of equilibrium.

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AMMONIA DURING EXCITATION AND INHIBITION

E. E. MARTINSON AND L. YA. TYAKHEPYL'D

Department of Biochemistry, Tartu State University, Tartu,
Estonian S.S.R.

IT HAS been known for a long time that prolonged hypnotics cause a marked disorder of ammonia incorporation into urea in the liver, which can be seen soon after an injection of sodium barbitone (1). As the concentration of ammonia rises in the blood, more ammonia accumulates in the brain (2).

In our experiments the liver was excluded from the circulation by an Eck fistula, a technique first described by Pavlov and Nentsky (3). In the latter experiments, ammonia changes are primary and the changes in the nervous system are only secondary. The ammonia not detoxicated by the liver enters the blood and the brain and causes the marked behavioural disorders described by Pavlov (3) who characterized a sequence of excitation and inhibition developing in dogs with an Eck-Pavlov fistula.

To show its effect experimentally ammonia must be allowed to act on the central nervous system for a prolonged period. We injected white mice subcutaneously with 100–150 mg/kg of sodium barbitone or 60–80 mg/kg amylobarbitone twice a day for 7–15 days. The ammonia in the brain was estimated by rapid freezing of the whole mouse in liquid air, using 4 animals for each estimation. Controls were done on mice not receiving hypnotics.

It appeared that after administration of the hypnotic, in the phase of excitation which sometimes leads to convulsions, the amount of ammonia and glutamine in the brain increased. This excitation was followed by a period of sleep, during which the ammonia decreased, whereas the glutamine continued to increase (Table 1).

Our findings for a single injection of a hypnotic are in agreement with those of Vladimirova (4) who found that the ammonia and glutamine in the brain change during excitation, and during the transition from excitation to inhibition.

If, however, injection of the drugs is continued, ammonia accumulates in the brain (Tables 2 and 3 and Figs. 1, 2 and 3). In 3 series of experiments in which sodium barbitone and amylobarbitone were administered for

TABLE 1. THE AMMONIA-GLUTAMINE CONTENT IN THE BRAIN IN THE PHASES OF EXCITATION AND DURING THE TRANSITION INTO SLEEP AFTER A SINGLE INJECTION OF A HYPNOTIC DRUG (in mg %)

No. of experiments	Sodium barbitone		Amylobarbitone	
	ammonia nitrogen	amino-nitrogen of glutamine	ammonia-nitrogen of glutamine	ammonia-nitrogen
Control				
1	0.49	6.41	control same	control same
2	0.45	7.10		
3	0.52	6.37		
Average	0.49	6.63	—	—
Stage of excitation				
1	1.31	6.19	0.75	7.80
2	1.10	7.80	1.04	8.30
3	1.10	9.60	0.65	10.35
4	0.89	6.27	1.00	10.90
Average	1.10	7.47	0.86	9.34
State of 2 hours sleep				
1	0.45	11.52	0.55	12.46
2	0.40	13.51	0.41	14.59
3	0.50	13.70	—	—
Average	0.45	12.91	0.48	13.53

15–17 and 7 days respectively, the mice were rapid-frozen in their sleep after receiving their morning injections. By the 7th day the amount of ammonia in the brain showed a marked increase compared with the controls, and during the next few days the increased level of ammonia went up much higher than the upper limit of brain ammonia in control animals.

Although there was a marked increase in the glutamine in some cases, this increase was much less than the increase in the amount of ammonia, particularly on the 3rd, 6th and 7th day of amylobarbitone sleep. This could have been because glutamine synthesis in the blood of animals drugged to sleep was depressed (2). After the drugs were stopped, ammonia and glutamine in the brain rapidly decreased and within five days completely returned to normal, as was shown in the sodium barbitone experiments (Table 1). Although repeated injections of hypnotic substances led to a considerable increase in the brain ammonia, this was not accompanied by excitation; in spite of the increased ammonia, the animals continued to sleep and the state of inhibition remained uninterrupted.

TABLE 2. AMMONIA AND GLUTAMINE CONTENT AFTER ADMINISTRATION
OF SODIUM BARBITONE FOR 15 DAYS
(in mg %)

Days of experiment	Control experiments		Experiments with sodium barbitone	
	ammonia- nitrogen	amino-nitrogen of glutamine	ammonia- nitrogen	amino-nitrogen of glutamine
1	0.40	6.10	0.53	14.80
2	0.51	7.60	0.57	13.14
3	0.48	8.00	1.10	12.60
4	0.47	4.30	0.87	10.40
6	0.42	6.90	0.80	12.14
7	0.40	4.90	1.15	15.60
9	0.52	6.68	0.84	15.44
11	0.54	6.92	1.23	11.00
13	0.43	5.40	0.75	8.60
15	0.52	6.80	0.70	15.00
After the sleep				
1	0.50	7.20	0.55	10.40
3	0.60	6.40	0.60	7.60
5	—	—	0.44	7.00
Average	0.48	6.43	—	—

TABLE 3. AMMONIA AND GLUTAMINE CONTENT IN THE BRAIN AFTER ADMINISTRATION
OF SODIUM BARBITONE AND AMYLOBARBITONE FOR 7 DAYS
(in mg %)

Day of experiment	Control experiments		Experiments with sodium barbitone		Experiments with amylobarbitone	
	ammonia nitrogen	amino- nitrogen of glutamine	ammonia- nitrogen	amino- nitrogen of glutamine	ammonia- nitrogen	amino- nitrogen of glutamine
1	0.42	6.17	0.50	13.70	0.41	14.59
2	—	—	0.64	12.47	0.75	13.55
3	—	—	1.16	12.81	1.09	15.93
4	0.50	6.85	0.99	16.25	0.80	12.74
5	—	—	0.77	11.87	1.13	9.25
6	—	—	0.80	15.73	2.05	8.15
7	0.55	6.08	1.10	10.65	1.73	9.84
Average	0.49	6.37	0.85	13.35	1.14	12.01

*Amino-nitrogen of
glutamine in mg%*

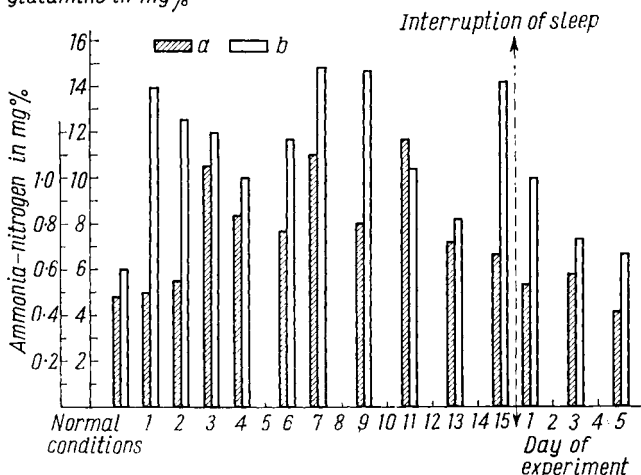


FIG. 1. Ammonia and glutamine content in the brain after prolonged drug-induced sleep (sodium barbitone): a—ammonia-nitrogen; b—amino-nitrogen of glutamine.

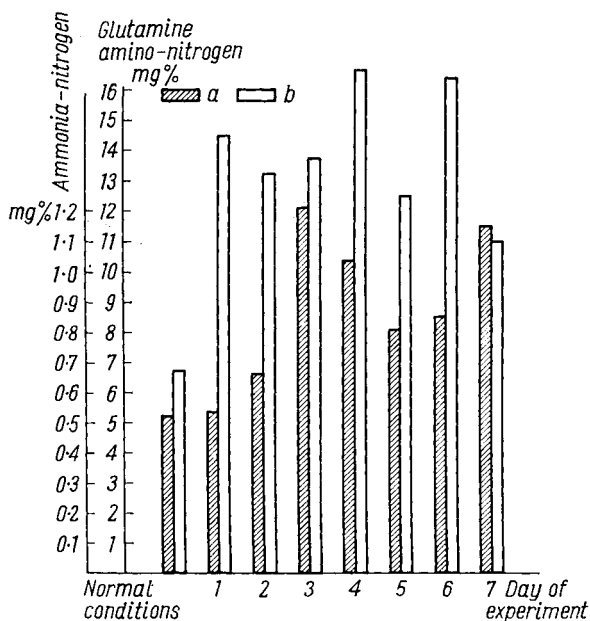


FIG. 2. Ammonia and glutamine content in the brain after prolonged drug-induced sleep (sodium barbitone) 2nd series: a—ammonia-nitrogen; b—glutamine amino-nitrogen.

This apparent contradiction of previous findings about the relation between ammonia and the states of excitation and inhibition could be explained by the fact that during the administration of hypnotics, ammonia itself acts as an endogenous stimulus instead of the usual external stimuli like an electric current, for example.

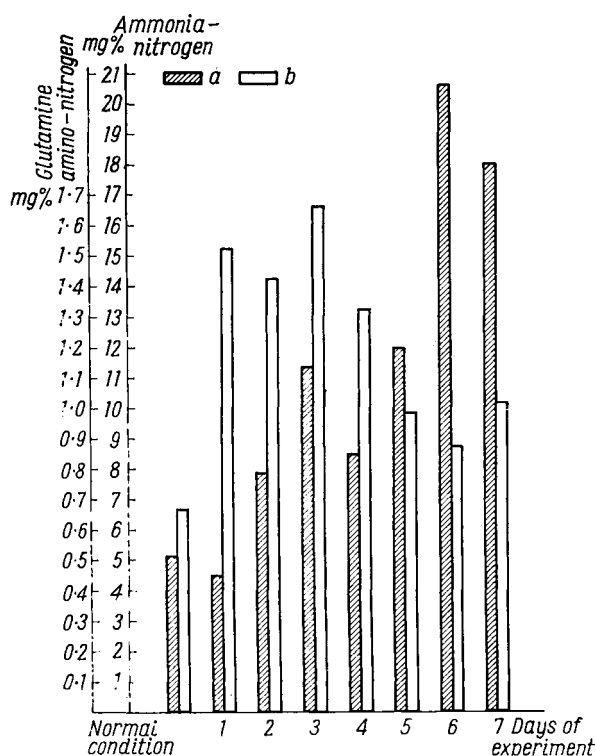


FIG. 3. Ammonia and glutamine content in the brain after prolonged drug-induced sleep (amylobarbitone): a—ammonia-nitrogen; b—glutamine amino-nitrogen.

The ammonia which forms when the drug is injected excites the central nervous system. Due to the continued action of ammonia this excitation becomes an inhibition, which is accompanied by an increased amount of ammonia, due to its fixation into glutamine so characteristic of the inhibitory state. As, however, under our experimental conditions the formation of ammonia does not cease, this substance gradually accumulates in the brain. The nervous tissue, which is now in a state of transliminal inhibition, ceases to react to ammonia or becomes less sensitive to it.

It may well be that at this stage there is competition between excitatory and inhibitory processes. This competition against a background of ammonia in the brain may cause the convulsions which are sometimes observed during the drug induced sleep. The animals, apparently in a deep sleep, react with a sudden jerk to a stimulus like high-frequency sound.

This state resembles the much more intensive reaction of animals which have previously been given parenteral ammonium chloride. They are also immobile but the slightest stimulus causes jerks and convulsions.

In the behaviour of Pavlov's dogs with Eck fistulas, there is competition between excitation and inhibition and, as he stresses, inhibition predominates (3). The marked state of depression in these dogs was also emphasized by Veselkin and Gordon (5). The Eck-Pavlov fistula, however, leads to the formation of that constant endogenous stimulant—ammonia—which enters the brain from the blood. Under the conditions of our experiments with prolonged drug-induced sleep, there is a sort of functional Eck fistula instead of an anatomical one, as urea synthesis in the liver is suppressed. The free ammonia which enters the brain from the blood, together with the ammonia which may form in the brain itself, acts as a stimulus of the central nervous system.

This idea of the biochemical nature of inhibition during drug-induced sleep was supported by the following experiment. If glutamic acid neutralized by carbonate (0.5–1.0 g/kg) is injected subcutaneously at the same time as the hypnotic, the first phase of excitation is less apparent, there is less increase in the ammonia of the brain, and an increase in the glutamine (Table 4).

TABLE 4. THE INFLUENCE OF GLUTAMIC ACID UPON THE AMMONIA CONTENT IN THE BRAIN IN THE PHASES OF EXCITATION AFTER ADMINISTRATION OF SODIUM BARBITONE (in mg %)

No. of experiment	After administration of glutamic acid		Without glutamic acid	
	ammonia nitrogen	glutamine amino-nitrogen	ammonia nitrogen	glutamic amino-nitrogen
1	0.62	8.38	0.82	6.04
2	0.32	10.20	0.98	6.27
3	0.47	8.21	1.10	7.47

If, on the other hand, 1.035 g/kg of ammonium chloride is administered, the control animals die within 10–15 min, whereas the animals in drug-induced sleep survive in many cases (Table 5). The nervous tissue during inhibition becomes insensitive to ammonia and also ceases to react to other

external stimuli. Survival for even longer after other hypnotic and narcotic drugs was shown by Kosyakov (6), who used a smaller dose of ammonium chloride than we did.

TABLE 5. SURVIVAL RATE OF ANIMALS AFTER ADMINISTRATION OF AMMONIUM CHLORIDE AGAINST A BACKGROUND OF DRUG-INDUCED SLEEP

Total number of animals	Among them perished	Survived	
NH ₄ Cl			
20	after 10 min—9	—	
	after 10—14 min—6		
	after 17—20 min—5		
Sodium barbitone with NH ₄ Cl			
13	after 12 min—1	4	
	after 30 min—1		
	after 1 hr—2		
	after 8 hr—1		
	after 11 hr —1		
	after 13 hr—1		
	after 16 hr—1		
30	after 18 hr—1	10	
	Amylobarbitone with NH ₄ Cl		
	after 15 min—1		
	after 25—35 min—10		
	after 40—60 min—5		
	after 1 hr 39 min—2		
	after 7—14 hr—2		

TABLE 6. AMMONIA AND GLUTAMINE CONTENT IN THE BRAIN AFTER ADMINISTRATION OF AMMONIUM CHLORIDE AGAINST A BACKGROUND OF DRUG-INDUCED SLEEP (in mg %)

NH_4Cl			Sodium barbitone with NH_4Cl			Amylobarbitone with NH_4Cl		
time elapsed after the administration (min)	ammonia nitrogen	glutamine amino-nitrogen	time elapsed after the administration (min)	ammonia nitrogen	glutamine amino-nitrogen	time elapsed after the administration (min)	ammonia nitrogen	glutamine amino-nitrogen
5	4.83	6.83	5	3.65	12.35	10	5.33	12.27
8	6.34	14.91	8	4.06	15.84	20	7.53	11.67
beginning of general convulsions			15	6.73	9.24			
			20	6.98	11.02			

We found that administration of ammonium chloride during drug-induced sleep was accompanied by an increase in brain ammonia of the same order of magnitude as the increase in animals which received ammonia when the lethal convulsions began, but, unlike the latter, these animals survived.

Finally, we are left with our main problem of the causative relation between the ammonia content of the brain and its functional state. Does the increase in ammonia precede excitation? Is it the cause of excitation, or is it incidental to it? Is it nothing but a passive, albeit very sensitive, indicator of the functional state of the brain? Rozengart (7) poisoned rabbits with picrotoxin, and watched their electroencephalograms; he found no ammonia increase in the pre-convulsive phase—the increase occurring actually during the convulsions. On the other hand, Vladimirova found an increase in brain ammonia within 15 sec of unconditioned or conditioned stimulation (8).

If the assumption that the increase in the ammonia *precedes* excitation is correct, what is the mechanism of its action? It is possible that there may be a physico-chemical change in brain proteins due to the ammonia, for example, as a result of the release of free carboxyl groups, or carbonic acid in the protein molecule. If this were so, the transition to a state of inhibition could mean that some of these carboxyl groups could have become combined.

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OXIDATIVE PHOSPHORYLATION IN THE BRAIN *IN VIVO*

N. A. VERZHBINSKAYA

Laboratory of Comparative Biochemistry of the Nervous System, Pavlov
Institute of Physiology, Soviet Academy of Sciences, Leningrad

IN PREVIOUS investigation we have shown that the content of high-energy phosphates in the brain increases during development (1). In the present study we have measured the rate of turnover of these phosphates in the brain *in vivo*.

METHOD

The animals were given 0.2 μC of ^{32}P in 10 μg of ^{31}P per g by intraperitoneal injection. Each day the brains of the animals were investigated at fixed times after exposure to the same dose of radioactive phosphate. The brains of cold-blooded animals were analysed 30, 60 and 120 min after the injection, while the warm-blooded ones were estimated at 10, 15, 30 and 60 min. Unlike other authors who have investigated labile phosphates in the brain (2-6), we looked at changes in the specific and relative radioactivity (SA and RSA) of the phosphates of the brain and blood. After exposure, the animal's head was submerged in liquid oxygen, the frozen brain chipped out and finely ground in liquid oxygen. The powder was transferred with a chilled spatula into weighed flasks containing cold 4 per cent trichloroacetic acid; the flasks were weighed again and the brain suspension diluted with chilled acid to 1 in 20. The extract of acid-soluble phosphates was fractionated in the cold. Inorganic phosphate was precipitated by the method of Delory (7). Creatine phosphate in the centrifugate was estimated after 60 min hydrolysis in a weakly acid medium at 40°C, then measuring the liberated inorganic phosphate by precipitation with magnesium salts (8).

ATP and ADP were precipitated as mercury salts. The precipitate was thoroughly washed and dissolved in 1 N HCl and hydrolysed. Part of the sample was used for estimation of inorganic phosphate liberated after

hydrolysis, and the larger part used for radioactive measurements. Inorganic phosphate was precipitated with magnesium salts and addition of 1 mg ^{31}P to obtain more complete precipitation of ^{32}P . To a separate portion of the TCA extract, 1 mg ^{31}P was added, and the inorganic phosphate precipitated by the method of Delory. This procedure purified the creatine phosphate fraction from traces of inorganic phosphate possessing a high radioactivity. In this portion the RSA of the creatine phosphate was estimated.

From the separated trunk of the animals we took blood and estimated its inorganic phosphate IP and ATP, and their RSA. Blood samples were treated in the same way as the brain. The amount of blood in the brain powder used for analysis of the phosphates was estimated by the benzidine method (9, 10). This enabled us to calculate the radioactivity of the blood in the brain, and to subtract it from the radioactivity of the inorganic phosphates of the brain; this was essential, as only short times of exposure were used. Results were expressed as rates of increase of specific activity per unit time ($\text{SA} = \text{counts/min/mg phosphate fraction}$) and the increase in relative specific activity of the fraction (RSA) as a proportion either of the specific radioactivity of the inorganic phosphate of the brain (RSA^{M}), or of the specific radioactivity of the inorganic phosphate of the blood (RSA^{R}).

When estimating the specific radioactivity of brain ATP, we calculated the ^{32}P which had been incorporated into the creatine phosphate due to the reaction with ATP and inorganic phosphate. For this we counted the total number of counts in 1 g of brain due to the ATP and creatine phosphate fraction. The total impulses divided by the micrograms of ATP phosphate present in 1 g of brain gives a value for the specific activity of ATP and for the RSA^{M} of brain ATP, and this gives a fairly precise measure of the radioactivity of the total labile high-energy phosphates in the brain. The early parts of the curves of the RSA^{M} and ATP were used to calculate the "turnover time" of high-energy phosphate ATP in the brain *in vivo*.

Zilversmit and others worked out equations for the rate of metabolic reactions (11). From expressions for the SA of substances "A" and "B", where $\text{A} \rightarrow \text{B}$, they calculated the turnover time of substance B during metabolism. The turnover time, (t_r) is defined as the time required for the breakdown and resynthesis of a substance B, normally present in the tissue. The equation they give is:

$$\frac{r}{p} \left(\frac{d \frac{x}{r}}{dt} \right) = \left[f(t) - \frac{x}{r} \right] \quad (1)$$

where p is a constant for the rate of transformation of A into B

r is a constant for the amount of B present in the tissue

x is the quantity of radioactive B in the tissue

$f(t)$ is the SA of substance A as a function of time

From this general equation the authors derive simple relationships. When the SA of substance A is linear with time, i.e. $f(t) = bt$, equation I becomes:

$$\frac{x}{r} = \frac{br}{p} \left(\frac{p}{r} t - 1 + e^{-\frac{p}{r}t} \right) \quad (\text{II})$$

From these equations it follows that:

$$\frac{x}{r} = \text{SA of B}; \quad \frac{r}{p} = t_t; \quad \frac{bx}{p} = \text{SA of A}$$

at time t_t when a complete cycle of turnover has occurred.

It further follows that at time t^{-1} the relation:

$$\frac{\text{SA of B}}{\text{SA of A}} = \frac{\frac{x}{r}}{b \cdot \frac{r}{p}} = \frac{1}{t_t} t_t - 1 + e^{-\frac{1}{t_t} t_t} = l^{-1} = 0.37$$

Consequently at the moment the cycle for brain ATP is completed, the relation is:

$$\frac{\text{SA of ATP}}{\text{SA of IP}} \text{ or } \text{RSA}^M \text{ of ATP of brain} = l^{-1} = 0.37$$

At any other time $T = \frac{t_t}{n}$

$$\text{RSA}^M \text{ of ATP of brain} = n \left(\frac{1}{n} - 1 + e^{-\frac{1}{n}} \right) \quad (\text{III})$$

This equation was used to calculate the turnover time of ATP in vertebrate brains *in vivo*. From it we find a value of n for the arbitrarily chosen time $T = t_t$. When $T = \frac{t_t}{n}$ we find a value of t_t or the turnover time of P-ATP in the brain. Knowing the content of ATP phosphorus in the brain (r) we find the rate of turnover.

$$\text{P of ATP (p)} = \frac{r}{t_t}$$

Equation III can be solved approximately by Newton's method, as

$$\frac{1}{2n} - \frac{1}{6n^2} = A$$

This is a simple quadratic equation. In practice, one selects the value RSR^M of brain ATP for each species of vertebrate, corrects it by 0.37 and solves the equation.

$$ATP^* = \frac{1}{2n} - \frac{1}{6n^2}$$

In this way values for n , t_t and P can be found.

To make similar calculations for cold-blooded animals, experimental points for shorter periods of exposure than the turnover time of brain ATP had to be obtained. For all terrestrial vertebrates, the first point on the RSR^M -ATP curve unfortunately turned out to be outside the range of the turnover time of the high-energy P-ATP, but in most cases it was very near to it. In these cases the RSA^M -ATP curve was extrapolated to zero.

RESULTS

Table 1 shows values for the SR of inorganic phosphates of the blood, the SR for inorganic phosphates and ATP of the brain and the RSA^M -ATP of the brain. These are the average from a number of experiments. In each experiment we calculated the SA of phosphate fractions in 1 g of brain, the SA of the inorganic phosphates in 1 g of blood and the phosphorus content of the corresponding fractions.

Table 1 shows that in the course of evolution of the vertebrates, the rate of turnover of P-ATP increases, indicating a greater rate of phosphorylation. The development of the process of phosphorylation in the brain parallels the development of oxygen-consuming energy metabolism in vertebrates. The curves of RSA^M -ATP of vertebrate brains can be divided into two groups. One group—fishes and amphibia—show a low rate of turnover of P-ATP, while the other with a higher rate are the reptiles and warm-blooded animals.

It has often been reported that the energy metabolism of the brain of aquatic vertebrates is very different from that of terrestrial vertebrates. It differs by the type of metabolism, the structure of the hydrogen ion and electron transport systems, and the intensity of oxygen-consuming metabolism. These *in vitro* findings are consistent with our results from the whole animals. The rate of turnover of the high-energy phosphate in the brain of aquatic vertebrates is quite different from that of terrestrial vertebrates.

TABLE 1. THE P-ATP TURNOVER RATE IN THE BRAIN OF VERTEBRATES
(a correction was made for the blood present in the brain)

Class and species. Temperature (°C)	Exposure (min)	SR* - ATP* of the brain (counts/min/1 μ g P)	SR - IP** of the brain (counts/min/1 μ g P)	RSR ^B *** - ATP* of the brain (%)	<i>t</i> _f - P-ATP-turnover time (min)	P-ATP content (μ M P in 1 g brain)	P-ATP turnover rate (μ M P in 1 g brain in hr)	SR - IP of the blood (counts/min per/1 μ g P)
Fishes								
Pike, 18.	30	1.2	8.3	14.4				
	60	10.7	32.8	32.5	90	3.5	2.3	300
	120	16.2	34.8	46.6				1520
Carp, 20	30	7.7	44.5	17.3				920
	60	12.7	47.5	26.8	75	2.9	2.3	5200
	120	16.7	42.5	39.4				3040
								2270
Amphibia								
Frog— <i>Rana</i> <i>temporaria</i> , 20	30	6.9	27.8	24.8				
	60	7.2	18.0	39.7	48	3.7	4.6	2340
	120	14.8	25.0	59.0				1520
								1680
Reptiles								
Turtle— <i>Testudo</i> <i>hornsfeldi</i> , 20	30	9.3	15.8	59.0				
	60	9.4	14.0	67.0	16	3.7	14.0	2130
	120	18.3	20.0	91.5				1380
								1530
Birds								
Pigeon, 37.5	15	7.6	19.0	40.0				
	30	16.7	27.2	61.0	12	7.4	37.0	1520
	60	19.7	29.6	67.0				1700
								1130
Mammals								
White rats 37.5	10	14.2	36.1	39.4	8	7.4	56.0	3700
	15	15.2	33.3	45.5				2660
	30	16.3	25.8	63.0				610
	60	22.2	32.0	69.5				1030

* SR = specific radioactivity

** IP = inorganic phosphorus

*** RSR^B = relative specific radioactivity of the brain

Another previous finding that the brain of reptiles possesses an energy-producing system which is identical with the metabolism of homoiotherm animals is confirmed. Figure 1 shows that the rate of turnover of bound phosphate in the brain of reptiles at rest is the same as that of warm-blooded animals. The *in vitro* results agree with the *in vivo* experiments. The content of

high-energy phosphates in turtle brain is known to be about half, and its inorganic phosphates known to be about 50 per cent higher than homoiotherms (12). Hence the fact that the turtle and homoiotherms possess equal RSA^M values for P-ATP does not necessarily prove that this animal has the same rate of oxidative phosphorylation as the homoiotherms.

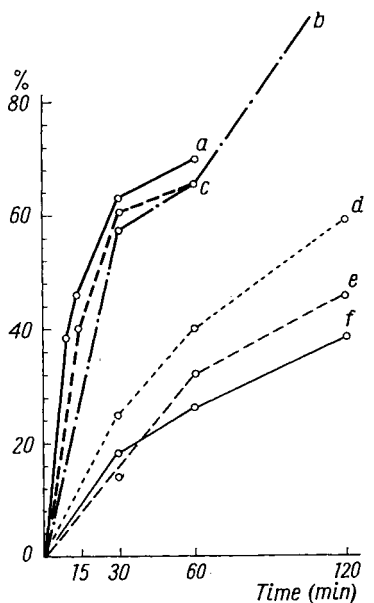


FIG. 1. Curves showing the changes in the RSR^B-ATP^* time in the brains of vertebrates: a—rats; b—turtle; c—pigeons; d—frog; e—pike; f—carp.

The turnover time of high-energy phosphorus ATP of the turtle is very similar to the homoiotherms. At 20°C it is 75–90 min in fish brain, 48 min in the frog, 16 min in the turtle and 8–12 min in warmblooded animals at their body temperature. The *rate of turnover* of high-energy phosphate ATP, however, shows a quite different distribution; at 20°C it is 2.3 μ moles $\sim P$ per g brain tissue/hour in the fish: in the frog 4.6 μ moles $\sim P$: in the turtle 14 μ moles $\sim P$, and in warm-blooded animals 40–60 μ moles $\sim P$ at their body temperature.

These values indicate that in terrestrial animals the increased oxidative metabolism in the brain is accompanied by development of the process of oxidative phosphorylation. This process has already reached a fair degree of development in the turtle. The difference between the rates of oxidative phosphorylation of the brain of the turtle and warm-blooded animals can be accounted for largely by the difference in body temperature.

From our data we can calculate P:O ratios of the brain, using the respiration rate *in vitro* at 20–22°C in poikilotherm animals, and at 37.5°C in homoiotherms. The QO_2 values for the forebrain were decreased by 20 per cent for the whole brain, to give the number of oxygen atoms respired by the brain in unit time (13). The rate of turnover of high-energy phosphate ATP corresponds to the rate of esterification of inorganic phosphate. These values were used to calculate the P:O ratios. These are shown in Table 2.

TABLE 2. COEFFICIENT OF PHOSPHORYLATION P/O IN THE BRAIN OF VERTEBRATES (calculated on the basis of the mean intensity of respiration in the brain *in vitro* and the "P-turnover rate" of the brain *in vivo*)

Class and species Temperature °C	Respiration of the brain QO_2	Consumed per 1 g moist brain substance in 1 hr		P/O
		μ -atoms of oxygen	μ meles P	
Fishes				
Carp, 20	3.6	46	2.3	0.05
Amphibia				
Frog, 20	2.2	26	4.6	0.2
Reptiles				
Turtle, 20	2.4	36	14.0	0.4
Birds				
Pigeon, 37.5	8.0	119	37.0	0.3
Mammals				
White rat, 37.5	9.0	134	56.0	0.4

These calculations show that the P:O ratio does not exceed 1.0 in the brain *in vivo* as in warm-blooded animals during rest. Our values for P:O ratios in the brains of homoiotherms were 0.5–0.6, that is 5 or 6 times lower than *in vitro* (14, 15).

We suggest that under normal conditions in homoiotherms at rest, the brain respiration is not parallel with phosphorylation. Otherwise one would have to explain the P:O ratios of 3 found *in vitro* in dialysed homogenates of mitochondrial suspensions as being due to the exercise of a maximum capacity which is not being used *in vivo*. There are many reports in the literature of oxidation of various substrates by mitochondrial suspensions, which occur without phosphorylation.

The other interesting point is that the P:O ratio of the brains of vertebrates gradually decreases as one descends in the evolutionary scale. The brains of higher bony fishes which show a high rate of respiration *in vitro* show a negligible rate of phosphorylation, with a P:O ratio of only 0.05. Their respiration is probably glycolytic, and not mainly phosphorylative. This is probably a primitive form of biological oxidation, which is seen in

the brain of aquatic vertebrates. Amphibia have a low rate of brain respiration compared with higher fish, and their P:O ratio is 0.2, which is more phosphorylative. In the turtle the P:O ratio of 0.4 is of the same order as that found in warm-blooded animals. In this feature, too, biological oxidation in reptile brain is similar to the terrestrial type. The low P:O ratio in terrestrial vertebrates is probably explained by the fact that the two oxidative systems coexist in their brains. The phylogenetically older system of the "aquatic" type is a glycolytic one, and the more recently evolved "terrestrial" type is a phosphorylative one. It is probable that these two systems are at different sites within the cell, as has been suggested by Lehninger (15).

The oxidation of hydrogen in the substrate by either system can be visualised as a mechanism to regulate the efficiency of energy metabolism of the brain. Brain respiration during excitation is to a greater extent phosphorylative than at rest, as is suggested in the literature.

It thus appears that oxidative phosphorylation in the brain gradually develops in the course of evolution of terrestrial vertebrates.

THE PERMEABILITY OF THE BLOOD-BRAIN BARRIER TO PHOSPHORUS

Along with the development of energy metabolism during the evolution of vertebrates, there develops a blood-brain barrier which regulates the rate of phosphorus entry from the blood. The development of this permeability barrier parallels the development of energy metabolism.

In aquatic vertebrates, the rate at which labelled ^{32}P enters from the blood is appreciably higher than in terrestrial cold-blooded vertebrates, as estimated from the rate of increase of RSA^{K} of inorganic phosphate of the brain in unit time (Fig. 2 and Table 1). Furthermore, it is several times higher than the rate of phosphorus *metabolism* of the brain (Fig. 2). The RSA^{K} of the ATP of the brain is low compared with the RSA^{K} of the inorganic phosphate of the brain.

$$\text{RSA}^{\text{K}} \text{ of ATP of brain} = \frac{\text{SA of ATP of brain}}{\text{SA of IP of blood}}$$

In terrestrial cold-blooded animals, the RSA^{K} of IP is low, in that the rate at which ^{32}P crosses the blood-brain barrier is low. Gradually the values for RSA^{K} of IP and RSA^{K} of ATP of the brain approach each other. This is an indication of the development in terrestrial vertebrates of a barrier which separates the brain from the blood in terrestrial vertebrates and regulates substances entering the brain in relation to the requirements of metabolism. Judged by the close correspondence between the values of

RSA^{K} of IP and RSA^{K} of ATP of the brain, the blood-brain barrier is fully formed in representatives of this class of reptile. In warm-blooded animals the value of the RSA^{K} of ATP of the brain increases at the same time as the value for RSA^{K} of IP, due to the high rate of brain metabolism. The barrier function is well marked.

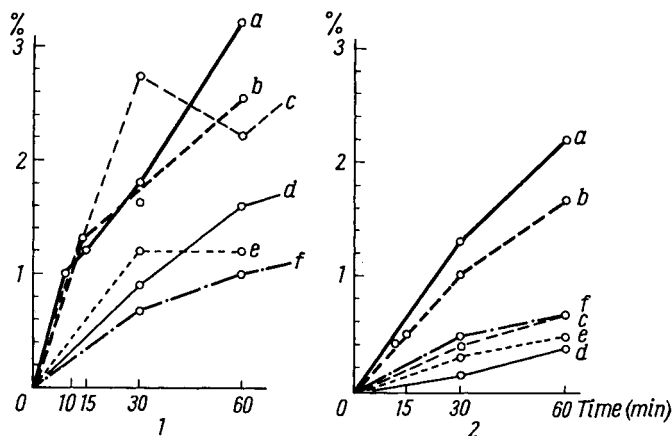


FIG. 2. Curves showing the changes in the $\text{RSR}^{\text{B1}*}$ of IP time of the brain and the RSR^{B1} of ATP time of the brain of vertebrates: 1— RSR^{B1} of IP of the brain of vertebrates in experiments lasting one hour; 2— RSR^{B1} of ATP* of the brain taking into account the radioactivity passing over to IP in one hour; a—rat; b—pigeon; c—pike; d—carp; e—frog; f—turtle.

If one regards the RSA^{K} of IP value as a measure of the permeability to phosphorus of the blood-brain barrier, it gives one an incorrect idea of the extremely slow rate at which ^{32}P enters the brain through the barrier. In vertebrates, the RSA^{K} of IP of the brain does not exceed 2–3 per cent in 2 hr of the experiment. All authors agree that even after 60 hr there is no equilibrium between blood and brain, that is the SA of IP of the brain and blood do not become equal.

If one looks at curves of the changes of SA of IP of the brain in unit time, one gets a different idea of the permeability of the barrier to phosphorus. The SA of IP of the brain of all vertebrates investigated by us increases during the first 30 min in cold-blooded animals, and 10–15 min in warm-blooded animals, and then settles down to a constant level. The maximum level of SA of IP of the brain which is rapidly reached is characteristic for

* RSR^{B1} the relative specific radioactivity of the blood.

each class of vertebrates and changes only if marked shifts occur in the SA of IP of the blood (Fig. 3, Table 1). In fish, this level is higher than in terrestrial cold-blooded animals, but in warm-blooded animals it approaches the level found in fish. The SA of IP of the blood is always many times, as much as 100, higher than the SA of IP of the brain.

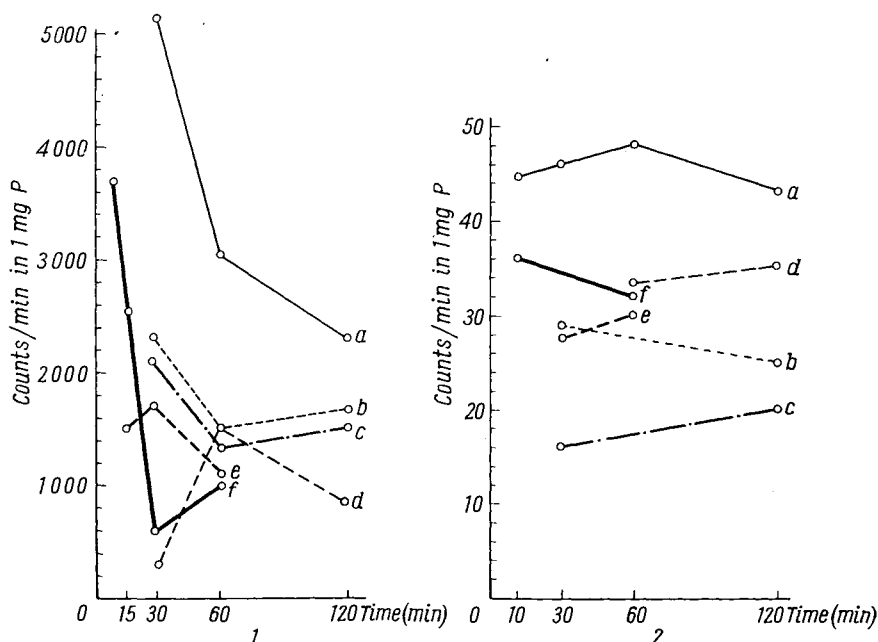


FIG. 3. Curves showing the changes in the SR of IP time of the brain of vertebrates and the SR of IP of the blood: 1—SR of IP of the blood at all periods of the experiment; 2—SR of IP of the brain in the beginning and at the end of the experiment; a—carp; b—frog; c—turtle; d—pike; e—pigeon; f—rat.

We cannot explain these findings as a blood-brain barrier with a membrane structure, but they can easily be understood within the framework of the adsorption theory of cellular permeability.

Our tentative view of the barrier between brain and blood localises it either in the glial membrane of Hess, the capillary endothelium or the so-called "basal substance" of the brain (18). All these structures have been stated to be selectively permeable, and some have secretory characteristics. This role is suggested by the fact that these structures are found all over the brain round small blood vessels, i.e. in those places where exchange between blood and brain take place. Hess has adduced considerable physio-

logical evidence for a connection between the barrier function and a natural membrane (16). Brain tissue cannot be stained with acid vital stains injected intravenously; this feature develops in mammals at the same time as the development of the glial membrane and the basal substance. If the brain is damaged mechanically or by heat, the barrier function is damaged at the same time, according to Hess (18); after trauma the barrier function to vital stains is regained at the same time as return of basal substance. However, such a common time course does not necessarily mean that they are causally related.

The adsorption theory of cellular permeability has been confirmed by the demonstration that cells generally do not possess selective permeability (Troshin, 19). Recent experiments with radioactive isotopes have shown rapid movements of ions in both directions between the medium and the cells. The accumulation of so-called intra-cellular ions (K^+ , HPO_3^-) in the cells and of extra-cellular ions (Na^+ , and Br^-) in the medium are explained not by a selective permeability, but by different affinity of the cellular protoplasm to these ions. Intra-cellular ions are present within the cells, mainly in a bound or adsorbed state. Only an insignificant proportion of them are capable of being exchanged with plasma by diffusion.

The maximum SA of IP of the brain reached within 10–30 min after injection of ^{32}P in vertebrates, can be regarded as a measure of the equilibrium with the plasma. This equilibrium is reached in warm-blooded animals in 10–15 min and in cold-blooded animals in 30 min.

The low values of SA of IP of the brain in equilibrium with the plasma can be explained by the low solubility of the phosphorus in protoplasm compared with its solubility in water. The higher level of inorganic phosphates in the brain compared with the plasma is due to greater adsorption or loose chemical binding to the colloids of the protoplasm.

Our data indicate that phosphate ions diffuse into the brain from plasma at the same rate as into other tissues. It is incorrect to think that they enter at a very low rate. The low values of RSA^k of IP in the brain are due to the low phosphate solubility in the protoplasm, and the fact that a large proportion of the IP being adsorbed on to protoplasmic colloids and not being directly exchanged with the plasma does not become radioactive during short experiments, but “dilutes” the radioactivity of the dissolved phosphate of the cells. The increase in the RSA^k of IP of the brain is due to a decrease of the SA of IP of the blood without changes in brain SA of IP.

In the course of evolution of terrestrial vertebrates, the protoplasm of brain cells acquires an increased capacity for adsorption and the phosphate present in brain tissue becomes less soluble, which leads to a fall in the maxi-

mum SA of IP of the brain. The brains of fishes and amphibia, which have a higher water content, have a higher maximum SA of IP than reptiles.

The rate at which ^{32}P enters the brain from the blood depends not on the permeability of the barrier to phosphate, which equilibrates within a short time, but on the rate of oxidative phosphorylation, i.e. the rate at which free phosphate is "extracted" from the solution by metabolism. From this "extract" the process of oxidative phosphorylation represents an auto-regulatory system in which the necessary rate of reinforcement by substrate is automatically ensured.

This concept of the blood-brain barrier does not require the assumption of a discrete structure possessing selective permeability. The blood-brain barrier to phosphate ions ought to be localized in those cellular elements of the brain which require phosphate for their metabolism.

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THE INFLUENCE OF TEMPERATURE ON THE METABOLISM OF LABILE PHOSPHORUS COMPOUNDS IN THE BRAINS OF COLD-BLOODED ANIMALS

R. I. VOLKOVA

Laboratory of Comparative Biochemistry of the Nervous System, Pavlov
Institute of Physiology, Leningrad

IT HAS BEEN established that severe hypoxia, interruption of the blood supply, electrical stimulation of the exposed brain and convulsions caused by drugs, cause a rapid fall in the creatine phosphate (CP) and adenosine triphosphate (ATP) levels of the brain and a rise of inorganic phosphate (IP).

Powerful electrical stimulation of the skin (unconditioned and conditioned) and brief stimulation of the central nervous system by drugs, however, do not cause appreciable changes in the levels of these phosphorus compounds (1-5). This is apparently due to the rapid resynthesis of CP and ATP under these conditions in the brain of warm-blooded animals. The high rate of turnover of CP and ATP has been confirmed by data from cerebral slices (6, 7).

Measurement of the rate of incorporation of ^{32}P into CP and ATP of the brain cannot be derived from the relative specific activity (RSA) values, because of the very high turnover rate of these compounds (8). Vladimirov and Rubel showed in rat brain that the RSA of ATP phosphorus reaches 100 per cent in one hour allowing for contamination by blood; it seems that CP and ATP are completely and perhaps repeatedly renewed within this time (8, 9).

Kerr (10) had noticed that CP of the brain broke down much more slowly after death in the frog and turtle than in warm-blooded animals and Verzhbinskaya (11) showed that CP and ATP in fish, amphibia and reptiles broke down after stopping the blood supply more slowly than in birds and mammals. We have, therefore, studied CP and ATP turnover in cold-blooded animals.

Experiments were carried out on the land turtle (*P. concinna*) and the swamp turtle (*E. orbicularis*) at 3, 5, 10, 17, 20 and 30°C, measured rectally

with a small mercury thermometer. 0.3 mc per kg body weight of radioactive sodium phosphate was injected intraperitoneally after the animal had been brought to the desired temperature. The head was frozen in liquid oxygen to preserve labile phosphorus compounds and extracted with 4 per cent TCA. IP was precipitated with magnesium and calcium salts by the method of Delory, then reprecipitated with magnesium. The CP was estimated by hydrolysing the supernatant at 37°C for 40 min under mildly acid conditions, then measuring the IP released as described. ATP was precipitated as the mercury salt and hydrolysed for 10 min at 100°C in N HCl to release the "labile ATP phosphorus". In our experiments we estimated the contamination of the tissue with blood by the method of Bing and Backer making a corresponding allowance for the specific radioactivity (SA) of the brain IP (12).

Contents of CP, ATP and IP are shown in Table 1.

TABLE 1. CP, ATP AND IP IN THE BRAIN OF TURTLES
(in mg % per 100 g moist tissue)

	M	σ	m	n
Terrestrial turtles				
CP	10.8	1.82	0.27	45
ATP	10.5	1.10	0.16	45
IP	20.3	4.34	0.65	45
Aquatic turtles				
CP	11.1	1.85	0.28	45
ATP	10.8	1.48	0.22	45
IP	16.0	4.40	0.66	45

Note. M —arithmetical means,

σ —mean square deviation, m —standard deviation, n —No. of experiments.

According to Verzhbinskaya, the CP content of vertebrate brains hardly changes during phylogenesis whereas the content of ATP gradually increases with the complexity of the central nervous system (11).

In our experiments the CP content of the brains of turtles was similar to that of rats and rabbits but the ATP content was less. All the results for CP, ATP and IP contents in turtle brain are given in one table because no differences related to body temperature could be found. The rate at which ^{32}P -labelled IP entered brain tissue from the blood was expressed thus:

$$\text{RSA of tissue IP} = \frac{\text{SA of tissue IP}}{\text{SA of blood IP}}$$

Figure 1 shows these RSA values at various body temperatures. As the body temperature rises, the rate of ^{32}P incorporation increases due to increased cardiac activity, increased blood flow, etc.

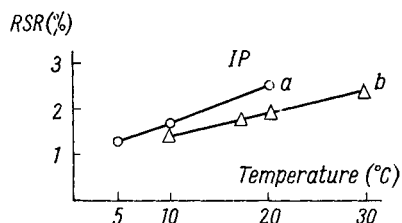


FIG. 1. The influence of the temperature upon the rate of uptake of ^{32}P from the blood into the brain tissue (time of experiment 1 hr): a—aquatic turtle; b—terrestrial turtle.

A temperature increase of 10°C produces a threefold or higher increase in the reaction rate of chemical processes, whereas purely physical processes, such as osmosis and diffusion increase by one and a third. In our experiments, a temperature increase of 10°C produced less than a twofold increase in the rate of penetration of brain tissue by ^{32}P . In *P. concinna*, the RSA increased 1.4 times for a temperature rise from 10 to 20°C and 1.3 times for a rise from 20 to 30°C . In *E. orbicularis*, the value was 1.8 for a rise from 10 to 20°C . These figures indicate that the limiting process for phosphate entry into the brain is of a physical rather than a chemical nature.

CP and ATP turnover was expressed in the following way:

$$\text{RSA of CP or ATP} = \frac{\text{SA of CP or ATP}}{\text{SA of IP in the tissue}}$$

These RSA values at various body temperatures are given in Fig. 2.

In *P. concinna* a rise of body temperature from 10 to 20°C caused a sharp increase in the CP-RSA value but a rise from 20 to 30°C produced little further change. In *E. orbicularis*, CP turnover was more rapid and changes of body temperature from 5 to 20°C caused an increase in the CP/RSA value from 4.2 to 21.1 per cent within an hour.

The labile ATP phosphorus showed a much higher rate of turnover than CP, a finding similar to that of Ernster and Lindberg (13) who injected ^{32}P intracranially into rats.

ATP turnover responded even more sharply to changes in body temperature. In *P. concinna* the ATP turnover rate was increased 2.7 times for a rise from 10 to 20°C and a further 12 times for a rise from 20 to 30°C .

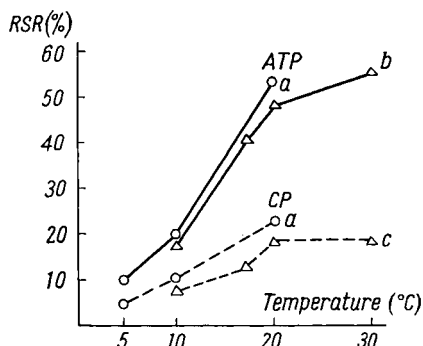


FIG. 2. The influence of the temperature upon the turnover rate of CP and labile ATP phosphorus in the brain of turtles (time of experiments 1 hr): a—aquatic turtles; b—terrestrial turtles.

E. orbicularis had a slightly higher ATP turnover rate. From these values it seemed that the limiting process for ^{32}P incorporation into these compounds was chemical.

It is interesting that an increase of body temperature from 20 to 30°C did not increase the CP turnover rate and only slightly increased labile ATP phosphorus turnover. It is likely that the temperature optima for the enzymes involved in CP and ATP turnover was about 20°C and activity decreased above this value.

Knowledge of cerebral metabolism of cold-blooded animals exposed to cold or during hibernation is very scant. While studying the relationships between body temperature and rate of CP and ATP turnover, we also examined the effect of wakefulness and hibernation. We compared animals in a state of hibernation for the previous 3–4 months to awakened animals at the same temperature. Artificial hibernation was induced by maintaining the turtles at 2–5°C for long periods during which they did not feed and remained virtually immobile; awakened turtles were kept in a warm aquarium where they fed and moved freely.

Hibernating and awake turtles were studied at body temperatures of 3 and 18°C. The awake turtles which had a normal body temperature of 18–20°C were artificially cooled to 3°C in about 1½ to 2 hr. Hibernating turtles were warmed up to 18°C. To assess the clinical state of the animals, respiration and pulse rates were measured. The CP, labile ATP phosphorus and IP contents are given in Fig. 3.

In hibernating turtles at 3 or 18°C there was a small drop in the CP and IP and a 30 per cent drop in ATP, compared with awake animals. Our data showed that during hibernation, when metabolism and nervous

activity in the brain were minimal, energetic requirements could be satisfied by a very low level of ATP. Decreases in the phosphorus compounds in hibernating animals, both warm and cold-blooded, have been observed by other authors (14, 15).

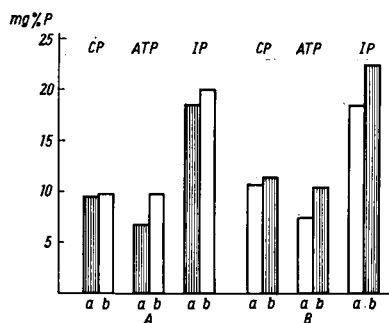


FIG. 3. Content of CP, labile ATP phosphorus and IP in the brain of sleeping and awake turtles at various body temperatures: a—sleeping turtles; b—awake turtles; A—body temperature 3°C; B—body temperature 18°C.

TABLE 2. OXYGEN CONSUMPTION BY SLEEPING AND AWAKE TURTLES AT VARIOUS BODY TEMPERATURES (in ml O₂ per 1 kg weight in 1 hr)

	3°C		18°C	
	Sleeping	Awake	Sleeping	Awake
Arithmetical mean	6.17	3.29	25.55	16.88
Mean square deviation	2.68	2.38	7.13	9.30
Standard deviation	0.65	0.58	1.68	2.25
No. of experiments	17	17	18	17

If the body temperature of hibernating animals was raised from 3 to 18°C, the ATP content remained low, although oxygen consumption and physiological activity increased greatly (Table 2). CP- and ATP-RSA values are given for awake and hibernating animals in Fig. 4. Experiments at 18°C lasted 1 hr. At 3°C metabolic reactions in the turtle brain were too slow to measure the CP- and ATP-RSA values accurately in 1 hr, so the experiments were prolonged to 3 hr.

At 3°C in either hibernating or artificially cooled awake animals, we could detect no differences in the CP- and ATP-RSA values, hence rapid cooling of awake animals decreased the metabolic rate to the same level as that found in prolonged hypothermia.

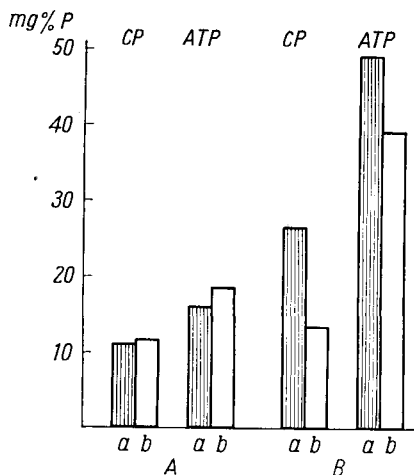


FIG. 4. Turnover rate of CP and labile ATP phosphorus in the brain of sleeping and awake turtles at various body temperatures: a—sleeping turtles; b—awake turtles; A—body temperature 3°C; duration of experiment 3 hr; B—body temperature 18°C; duration of experiment 1 hr.

The raising of body temperature of hibernating turtles in $1\frac{1}{2}$ to 2 hr to 18°C produced a marked increase in the CP- and ATP-RSA. Values obtained reached and even exceeded those in awake animals at 18°C especially for CP. This suggests that arousal from hibernation by warming produces an exceptionally high CP and ATP turnover. It is generally believed that the marked increase in metabolic rate of mammals suddenly roused from hibernation is due to the large production of body heat necessary to raise the body temperature to normal (16) and our data support this view.

Respiration of hibernating and awake turtles was measured in special chambers in which the gases could be analysed by a Haldane apparatus. Experiments were carried out at 3°C for 5 hr and at 18°C for 2 hr. Results are given in Table 2. These show that gas exchange at 3°C is one-fifth to one-quarter of the rate at 18°C. Similar relationships between body temperature and rates of gas exchange have been described elsewhere (17–19).

Another interesting fact is that in hibernating turtles the O_2 consumption at 3°C and at 8°C is $1\frac{1}{2}$ times as great as in awake turtles. Similar findings have been recorded elsewhere (17, 20, 21). Gelineo, using black Dalmatian lizards, showed that O_2 consumption at 13–30°C was higher in animals maintained at 11–15°C than those maintained at 18–22°C, or 24–28°C (21). We found that turtles rapidly cooled to 3°C, showed a lower gas exchange rate than those adapted over a long period to this temperature.

An important relationship exists between our values for CP and ATP turnover and gas exchange rates. Both these processes were more active in animals adapted to low temperatures. We visualize tissue adaptation at low temperatures which attains the maximum use of the enzymes' potential activity. This would allow higher metabolic rates in the brains of such adapted animals transferred to warm conditions than in unadapted ones.

The connection between the changes in CP and ATP turnover rate and rate of oxygen consumption of turtles at various body temperatures is probably of a causal nature since in reptilian brain, energy is supplied mostly by oxidative phosphorylation (11). When the body temperature of turtles is raised to 18°C, as the CP and ATP turnover increases so does the oxygen consumption.

These measurements have enabled us to differentiate between true hibernation of cold-blooded animals and the rigidity produced by temporary hypothermia since certain processes during hibernation seem to be more active than in rapidly cooled animals. We believe that this adaptation during prolonged hypothermia is due to a neurohumoral mechanism—a theory based also on the evidence of Rodbard *et al.* (22).

Terrestrial cold-blooded animals tolerate variations in body temperature of up to 25°C in less than 2 hr (23) and the resultant metabolic changes one would expect are reflected in our results for CP and ATP in the turtle brain.

SUMMARY

1. Increasing the body temperature of turtles from 3–30°C produces little increase in the rate at which ^{32}P enters the brain from the blood (temperature coefficient $Q_{10-22} = 1.3-1.8$), but a considerable increase in the rate of incorporation into CP and labile ATP phosphorus ($Q_{10-20} = 2.4$ for CP and 2.7–2.8 for ATP).

2. The rate of turnover of CP and ATP has been studied in the brains of awake and hibernating turtles. In the latter group slightly less CP and IP and 30 per cent less ATP has been found than in awake animals.

3. O_2 consumption was higher in hibernating than awake turtles at 3 or 18°C.

4. The rate of incorporation of ^{32}P into CP and ATP was higher in hibernating turtles raised to 18°C than awake animals at this temperature.

5. From our data we conclude that in hibernating cold-blooded animals a special form of adaption occurs whereby some metabolic processes in the brain are maintained at a higher rate or potential than in non-adapted animals suddenly cooled down.

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LIPOPROTEIN PHOSPHORUS IN THE BRAIN OF RABBITS DURING ONTOGENESIS

K. G. MANUKYAN

Laboratory of Comparative Biochemistry of the Nervous System, Pavlov
Institute of Physiology, Soviet Academy of Sciences, Leningrad

MOST of the lipids in nervous tissue are believed to be complexed with proteins. These complexes have recently been intensively studied because they play a very important role in the function of the brain.

Of particular interest is a trypsin-resistant lipoprotein detected in cerebral white matter by Folch and Le Baron (1). In this complex the phospholipid is stably bound to protein and does not possess the solubility characteristics of simple lipids. After the bond between the lipid and protein moiety has been broken, however, the lipid fraction can be extracted by chloroform-methanol.

According to Folch (2) and Folch and Le Baron (3), the lipid fraction contains diphospho-inositide bound to peptides (phosphatido-peptide) and probably contains other phosphatides as well. The phosphorus in this lipoprotein constitutes about 40–50 per cent of the protein-bound phosphorus in the brain. Some authors name it “residual protein phosphorus”, others “inositide phosphorus”.

Strickland (4), De Luca, Rossiter and Strickland (5), Vladimirov, Ivanova and Pravdina (6) showed that lipoprotein phosphorus is characterized by a high rate of turnover which greatly exceeds the rate of turnover of nucleic acids or phospholipids, and is second only to the rate for phosphoprotein phosphorus.

Streicher and Gerard (7) conclude from the higher turnover rate of lipoprotein phosphorus in brain homogenates compared with other phospholipids that the most active ones serve as carriers of phosphate from ATP and CP to other phospholipids possessing a lower turnover rate. Another theory considers that protein-lipid complexes concentrated in the external layers of nerve cells and their processes are involved in permeability control and therefore influence excitability and conductivity characteristics. The high concentration of lipoprotein phosphorus in cere-

bral white matter and the extensive depletion of this compound during Wallerian degeneration (Logan, Mannel and Rossiter 8), supports the theory that lipoproteins are components of the myelin sheath. According to Schmitt (9), Elkes and Finean (10), the proteolipid molecules in the myelin sheath are distinctly orientated.

In this report we present studies on the content and metabolism of lipoprotein phosphorus of the cerebral cortex and spinal cord of rabbits during ontogenesis, particularly in the early post-natal period when myelination commences.

METHODS

Rabbits were injected subcutaneously with 7–8 mg of sodium orthophosphate 1.0–1.5 ml per kg body weight, containing 0.5 mc ^{32}P . Three hours later the animals were decapitated under narcosis. For investigating the cerebral cortex, the grey matter was removed from the entire surface of the hemisphere; the spinal cord was studied whole. Tissue samples were quickly freed from blood and cerebral membranes, and frozen in solid CO_2 . Tissue suspensions were extracted with 7 per cent TCA and inorganic phosphate in the extract was precipitated by the method of Delory (12). The residue was washed repeatedly with ice-cold 5 per cent TCA and twice with ice-cold ethanol. For estimating lipoprotein phosphorus by the method of Folch (2), the washed residue was extracted with mixtures of hot ethanol-ether, ethanol-chloroform, chloroform-methanol and methanol-ether at 75–80°C for 50 min to remove lipids. After extraction with the second solvent, the residue was washed with ether and vacuum dried; the chloroform-methanol was added and it was left to stand overnight. The protein-containing residue was next washed with ether and vacuum dried, then 10 ml of acidified chloroform-methanol were added (200 parts chloroform: 100 parts methanol: 1 part concentrated HCl, see Folch and Le Baron (3)), left overnight in the cold and the residue extracted twice more for one hour with the same mixture. All acid chloroform-methanol extracts were pooled and analysed for inorganic and phospholipid phosphorus content, as well as their specific radioactivity. Phosphorus turnover rates in these fractions were assessed by the Relative Specific Activity (RSA) which is given by the ratio of the Specific Activity (SA) of the fraction to the SA of the total inorganic phosphorus of the brain per cent. Extraction with acid chloroform-methanol for 2 hr followed by washing gives maximal phosphorus content in the lipoprotein fraction. Continued extraction for 24 hr or more as well as raising the extraction temperature from 2° to 24°C did not increase

the yield of lipoprotein phosphorus. The optimal concentration of the HCl, which is the most critical factor, proved to be 0.03–0.04 N. Lower concentrations led to considerable losses (see (2)) and increasing the concentration to greater than 1 N caused an increase in the yield of phosphorus due to the extraction of nucleic acids (see Table I).

TABLE 1. RELATION BETWEEN THE CONTENT OF LIPOPROTEIN PHOSPHORUS EXTRACTED FROM THE BRAIN OF RABBITS AND THE CONCENTRATION OF ACID IN THE EXTRACTING CHLOROFORM–METHANOL MIXTURE

Concentration of HCl in the extracting chloroform–methanol mixture	Phosphorus content (mg %)		
	Lipoproteins	Nucleic acids (as judged by the absorption in ultraviolet light. Extracted by the method of Schneider (13) after extraction of the lipoproteins)	Nucleic acids + lipoproteins
0.04 N HCl	20.4	—	—
1 N HCl	21.7	—	—
0.04 N HCl	19.4	14.7	34.1
1 N HCl	27.4	7.6	35.0
Lipoproteins not extracted	—	15.2	—

Since lipoprotein is extracted with ribonucleic acid in Schmidt and Thannhauser's method, we felt it necessary to determine how completely lipoprotein was extracted by acidified chloroform-methanol. Lipoproteins were extracted as described, and nucleic acids extracted with two lots of hot 10 per cent NaCl. The residue was then treated by Schmidt and Thannhauser's method and the phosphoproteins were precipitated from the hydrolysate. This solution still contained 3–4 mg % of phosphorus whose RSA was much higher than the RNA fraction but lower than the lipoprotein phosphorus. Hence part of this quantity of phosphorus might have been derived from unextracted lipoprotein.

The method for measuring lipoprotein phosphorus described above was compared with another method which consisted of measuring the total phosphorus associated with RNA isolated by Schmidt and Thannhauser's method (11) and subtracting from it the theoretical quantity of phosphorus associated with the true RNA determined spectrophotometrically at 265 m μ . Logan, Mannel and Rossiter, (8) have shown that lipoprotein phosphorus is the main contaminating constituent of the RNA fraction isolated by Schmidt and Thannhauser's method.

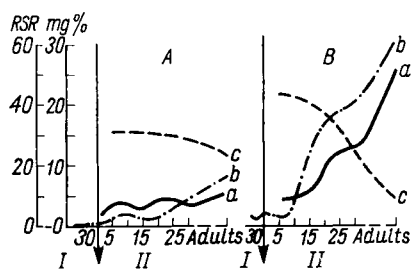


FIG. 1. Content and metabolism of lipoprotein phosphorus in the brain of rabbits in the course of the ontogenesis: A—cerebral cortex; B—spinal cord; a—phosphorus content in mg per 100 g tissue (moist weight), estimated by the difference (1st method); b—phosphorus content in the acid chloroform-methanol extract (in mg per 100 g tissue) (2nd method); c—RSR (%) phosphorus in the chloroform-methanol extract (2nd method); I—embryonal period, II—post-embryonal period.

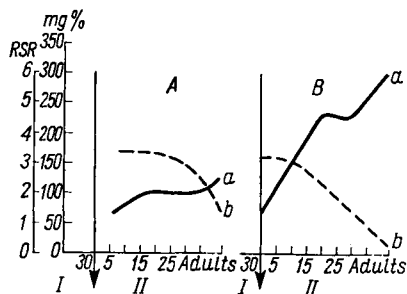


FIG. 2. Content and metabolism of phospholipid phosphorus of the brain of rabbits in the course of the ontogenesis: a—phosphorus content in mg per 100 g weight (moist weight); b—RSR of phosphorus in %; c—RSR (in %) phosphorus in the chloroform-methanol extract (2nd method); I—embryonal period; II—post-embryonal period.

RESULTS

Both methods gave similar results. The curves indicating changes in lipoprotein content during ontogenesis were similar whether expressed per unit dry or wet weight. Lipoprotein could scarcely be detected in rabbit brain either during the pre-natal period or the first day after birth. It began to increase after the second week, attaining a maximal rate at 1 month. These time-course changes were more pronounced in the spinal cord than the cerebral cortex. Figure 3 gives the lipoprotein values in mg % of 6-day-old rabbits. Note the higher levels in spinal cord compared with grey matter.

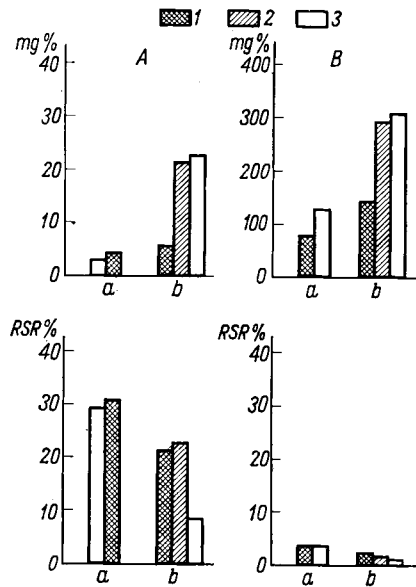


FIG. 3. Content and metabolism of lipoprotein phosphorus and phospholipid phosphorus in various parts of the rabbit brain: a—6-day-old rabbit; b—adult rabbit; A—lipoprotein phosphorus; B—phospholipid phosphorus; 1—cerebral cortex; 2—white matter of the cerebral hemispheres; 3—spinal cord.

Lipoprotein phosphorus turnover: This is very high during the first two weeks after birth in both spinal cord and cerebral cortex; thereafter the RSA of the spinal cord lipoprotein phosphorus falls markedly but remains virtually unchanged in the cerebral cortex. (Note that the turnover rate of cerebral lipoprotein phosphorus in the adult rabbit is three times that of the spinal cord.) The rate of incorporation of phosphorus into the lipoprotein of cerebral white matter in adult rabbits is about the same as

in grey matter and considerably higher than in the spinal cord, whereas the lipoprotein phosphorus content is the same in the cerebral white matter as the spinal cord (Fig. 3). This can be explained by differences in composition as well as a higher metabolic activity of the lipids constituting the cerebral white matter.

The decrease in turnover rate 2 weeks after birth is simultaneous with the accumulation of lipoproteins in the brain. This decrease is probably due to the onset of myelination when more metabolically inert material is synthesized.

Lipoprotein phosphorus accumulates at the same time ontogenetically as phosphoprotein—a fact which links it closely with the process of myelination but by no means excludes it from being a constituent of nerve cell structures other than the myelin sheath or from participating in cellular metabolism.

The turnover rate of lipoprotein begins to decrease at the same time ontogenetically as the phospholipids but this decline is not so rapid. For instance, the phospholipid content of an adult rabbit is 2.5–3.0 times that of a 6-day-old animal and the lipoprotein content 5.5 times, but in the adult the phospholipid turnover rate has decreased to one-twelfth, whereas the lipoprotein rate has decreased to one quarter of that of the 6-day-old animal.

After 3 hr exposure to ^{32}P in adult rabbits the lipoprotein RSA of the cerebral grey matter is 14 times, of the cerebral white matter 29 times, and of the spinal cord 30 times the corresponding phospholipid fraction. It is apparent then, that metabolically inert phospholipids accumulate along with more active lipoproteins during myelination.

CONCLUSIONS

1. The content of lipoprotein phosphorus is low during the pre-natal and immediate post-natal periods but increases rapidly at 2 weeks.
2. The lipoprotein phosphorus increases to much higher levels in the spinal cord than in the cerebral grey matter. In an adult rabbit the lipoprotein phosphorus content is almost the same in the spinal cord as cerebral white matter (24.0 mg %) but much lower in grey matter (5.3 mg %).
3. The RSA of lipoprotein phosphorus remains at a high level which is similar in both the cerebral cortex and spinal cord for the first two weeks after birth and then decreases, being more pronounced in the cerebral cortex than the spinal cord.

4. The rate of incorporation of phosphorus into the lipoprotein of cerebral white matter of the adult rabbit is similar to that of the cerebral grey matter and much higher than that of spinal cord.

5. Accumulation of lipoprotein phosphorus occurs simultaneously with the accumulation of phospholipids due to myelination. It thus appears that during myelination not only do the metabolically inert phospholipids accumulate in the cortex, but also lipoprotein phospholipids with a high rate of phosphorus turnover.

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ENZYME ACTIVITY IN DIFFERENT AREAS OF THE CEREBRAL CORTEX OF RABBITS AND DOGS DURING GROWTH

Z. D. FIGAREVA

Biochemistry Laboratory, Institute of Normal and Pathological Physiology,
Academy of Medical Sciences, Moscow

COMPARISON of the rate of development of various parts of the brain shows that biochemically the cerebral cortex is slower to mature than other parts of the brain. The respiratory rate and activity of oxidative enzymes in the cortex is, as a rule, higher than in the older parts of the brain, which one might expect from its more complicated structure and function (1). On the other hand, unlike the oxidative enzymes, the activity of cholinesterase is lower in the cortex than in the subcortical parts of the brain. It has also been shown that in the development of mammals all components of the acetylcholine system decrease (2). The authors attribute this decrease to a decrease in the density of neurones, and to an increase in weight of the brain. The literature is, however, very poor in reports of localization of enzyme activity (3-8).

This paper is concerned with the development of the activity of the cytochrome system and of cholinesterase in the motor, visual and auditory areas of rabbit and dog. These animals were chosen because they are born in an immature state, and are similar in their type of maturation, but are very different in their cortical histology and the development of their cortical activity. Oxidative enzymes and cholinesterases were studied together to correlate the development of general metabolic enzymes with the specific acetylcholine metabolism of nervous tissue (9-10).

Enzyme activity in the different areas was assayed by conventional manometric techniques, using a Warburg apparatus (11-12). The brains of 14 adult dogs, 15 rabbits, 54 puppies, 60 newly born rabbits, 16 rabbit foetuses and 3 dog foetuses were used. The concentration of cytochrome oxidase and carbonic anhydrase in the cortex of adult animals is shown in Fig. 1. The activity of the former enzyme is $1\frac{1}{2}$ -2 times higher in the dog cortex than in the rabbit, while cholinesterase, on the other hand, is 2-3

times higher in the rabbit. The activity of the cytochrome system and of carbonic anhydrase in both species is almost equal. Although the activities of the enzymes we investigated are subject to considerable variation, the distribution of activity between zones is fairly constant for a particular species.

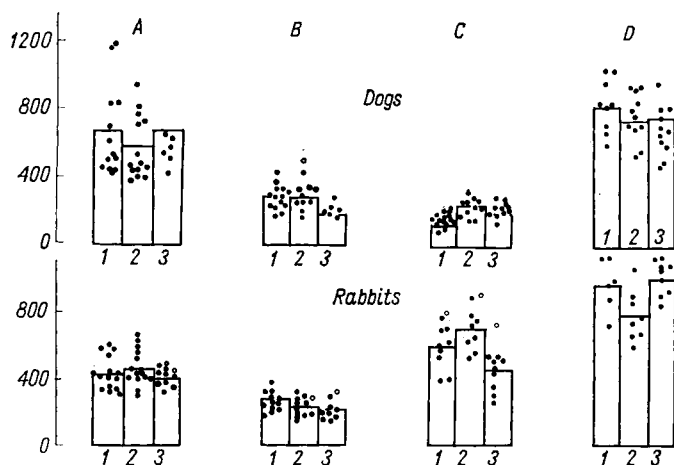


FIG. 1. Variations in the activity of enzymes in different zones of the cerebral cortex of adult animals: 1—optic zone; 2—auditory zone; 3—motor zone; A—cytochrome oxidase; B—cytochrome system; C—cholinesterase; D—carbonic anhydrase.

Table 1 shows that the enzyme activity of the auditory area and of the motor area expressed as a percent of the activity in the optic area, arbitrarily taken as 100, displays marked differences between zones. This difference is most marked in the case of cholinesterase, and least with cytochrome oxidase.

TABLE 1. ACTIVITY OF ENZYMES IN THE ACOUSTIC AND MOTOR ZONE OF THE CEREBRAL CORTEX OF DOGS AND RABBITS EXPRESSED IN PER CENT OF THE ACTIVITY IN THE OPTIC ZONE ARBITRARILY DEFINED AS 100

Enzyme	Dogs			Rabbits		
	optic zone	auditory zone	motor zone	optic zone	auditory zone	motor zone
Cytochrome oxidase	100	87	99	100	109	98
Cytochrome system	100	99	68	100	85	79
Cholinesterase	100	173	144	100	118	75
Carbonic anhydrase	100	91	91	100	80	103

TABLE 2. RELATION OF THE ENZYME ACTIVITY IN VARIOUS ZONES OF THE CEREBRAL CORTEX OF DOGS AND RABBITS

Zones in the cerebral cortex	Dogs			Rabbits		
	cyto-chrome oxidase	cyto-chrome system	cyto-chrome oxidase	cyto-chrome oxidase	cyto-chrome system	cyto-chrome oxidase
	cholin-esterase	cholin-esterase	cyto-chrome system	cholin-esterase	cholin-esterase	cyto-chrome system
Optic zone	44	1.9	23	7.0	0.45	15
Auditory zone	22	1.1	20	6.5	0.33	20
Motor zone	30	0.9	33	9.0	0.48	19

Table 2 shows that the dog cortex differs from the rabbit by the greater activity of oxidative enzymes relative to cholinesterase. In dogs, the differences in respect of all enzymes between the motor, auditory and visual areas are more marked than rabbits. It appears that individual areas of the cortex of adult dogs and rabbits are characterized by particular levels of activity of oxidative enzymes and cholinesterases. These activities in the different areas of the brain during growth are illustrated in Figs. 2-5. The activity of the enzymes is expressed as microlitres O_2 (CO_2) per 10 mg moist tissue per hour, and also as a percentage of the activity in the corresponding area of the adult animal.

The curves in Fig. 2 show that the activity of the cytochrome system in the cerebral cortex of dogs develops in a similar way to that of rabbits. In the last 10 pre-natal days the activity of the cytochrome system in the cortex of rabbits is higher than in dogs, but the latter shows a steeper rate of rise during this time. During foetal development and in the first 10 days of post-natal life, the difference between the enzyme activity of the different areas is hardly significant. Later on, however, particularly after 30 days, the characteristic curves of activity of the individual areas becomes apparent. In dogs younger than 60 days, the activity of the cytochrome system is higher in the motor area than in the auditory and visual areas; this relationship is reversed after 60 days.

In rabbits the activity of the cytochrome system is higher at all stages of development in the motor and visual areas than in the auditory area. After 2 months, however, its activity in the visual area becomes higher than the auditory and motor areas. The curves of the development of activity

in the visual and auditory areas in the dog are similar, but are quite different from the curve of the motor area.

In rabbits the differences between these curves are much less marked, but within this general trend the curves of the development of the motor and auditory areas are more similar to each other than the auditory and visual areas, respectively.

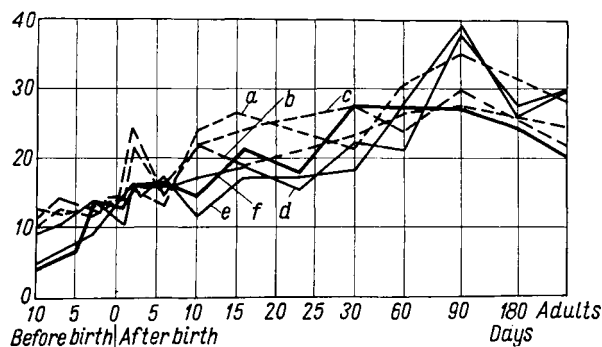


FIG. 2. Changes in the activity of the cytochrome system in various zones of the cerebral cortex in the course of the ontogenesis in rabbits and dogs (in $\mu\text{l O}_2$ per 10 mg moist tissue). Rabbits: a—optic zone; b—auditory zone; c—motor zone. Dogs: d—optic zone; e—auditory zone; f—motor zone.

During early development the activity of the cytochrome system in dogs and rabbits runs parallel, but between 60 and 90 days its activity in puppies becomes considerably higher than in rabbits of the same age. Figure 3 illustrates changes in levels of activity of the cytochrome system. In the last third of embryonic life, the activity of the cytochrome system in the cortex of rabbits is higher than in dogs; in newly born rabbits it is 54–66 per cent of adult activity compared with 34–61 per cent in puppies.

In rabbits the activity of the cytochrome system increases in all areas to a greater extent than in dogs. The order of “maturation” of various areas in both species is the same. Adult levels are achieved first in the motor area then in the auditory and visual areas. The times at which the adult levels are reached in the different areas are closer together in rabbits than in dogs. For example, the adult level in the motor area of rabbits is reached between the 2nd and 10th day, in the auditory area around the 10th day and in the visual area between the 12th and 15th days. In puppies the adult level is reached in the motor area between the 10th and 15th day, but in the auditory and visual areas between the 60th and 70th day. Between the 10th and 90th days the cytochrome activity in the motor area

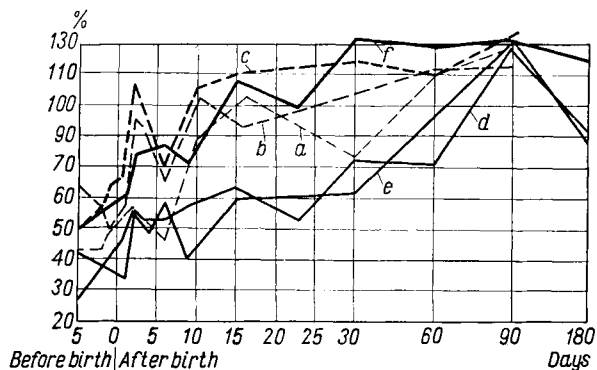


FIG. 3. Changes in the activity of the cytochrome system in various zones of the cerebral cortex in the course of the ontogenesis in rabbits and dogs (expressed in per cent of the activity found in adult animals). Rabbits: a—optic zone; b—auditory zone; c—motor zone. Dogs: d—optic zone; e—auditory zone; f—motor zone.

in both species is greater than the “adult” level. In the visual and auditory areas a greater-than-adult level is seen only by the 30th day in rabbits and the 60th day in dogs.

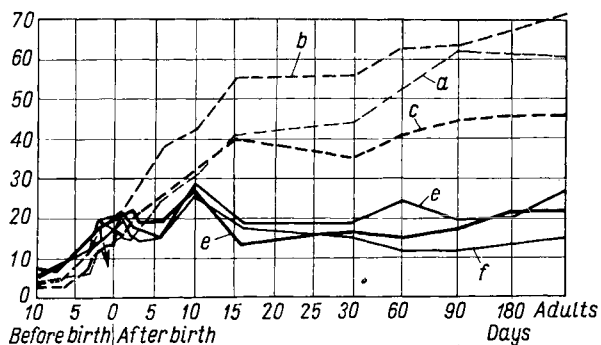


FIG. 4. Changes in the cholinesterase activity in various zones of the cerebral cortex in the course of the ontogenesis of rabbits and dogs (in $\mu\text{l CO}_2$ per 10 mg moist tissue within one hour). Rabbits: a—optic zone; b—auditory zone; c—motor. Dogs: d—optic zone; e—auditory zone; f—motor zone.

Development of cholinesterase activity is shown in Figs. 4 and 5. In dog embryos, between 5 and 6 days pre-natal, the cholinesterase activity is higher than the corresponding zones of rabbits. Cholinesterase is first found in dog embryos in the 10th to 12th pre-natal days long before there is any trace in the rabbit. In newly born rabbits the cholinesterase level

is 24–34 per cent of the adult rabbit, while in newly born dogs it is as high as 82–129 per cent of the adult level, although their absolute levels of activity are almost equal.

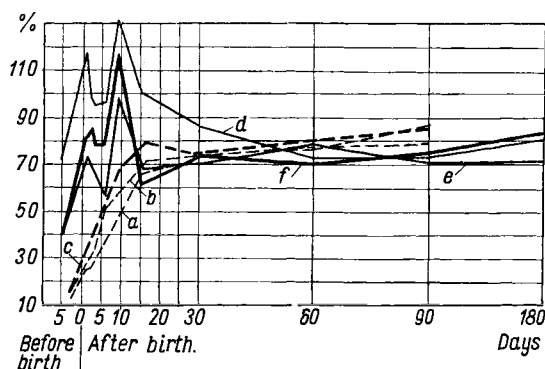


FIG. 5. Changes in the cholinesterase activity in various zones of the cerebral cortex in the course of ontogenesis in rabbits and dogs (expressed in per cent of the activity found in adult animals). Rabbits: a—optic zone; b—auditory zone; c—motor zone. Dogs: d—optic zone; e—auditory zone; f—motor zone.

Immediately after birth a rapid increase in cholinesterase begins in the brain of young rabbits. In the first 15 days the auditory activity increases quicker than the visual and motor areas, but after this there is a slowing down, and increase is relatively more rapid in the visual area. In young puppies the cholinesterase increases in the first 15 days of life but by the 15th day it is falling: Subsequently, activity gradually rises to the adult level. The differences in cholinesterase between the zones are less marked in their first fortnight than in their subsequent development; the greatest changes occur in the visual area. Up to 30 days the activity in the visual area is higher than in the motor area but then becomes lower.

It would seem that, unlike oxidative enzymes, the activity of cholinesterase develops in a different way in the cortex of dogs and rabbits. In dogs there is characteristically a higher level in the late embryo and early post-natal stages.

It was of interest to try to correlate changes in enzyme activity with the times at which morphological and functional changes appear. Very early on "maturation" of the motor area can be correlated with its motor function. Then the olfactory, skin, auditory and visual areas become functional. The order in which the analysing systems become functional depends upon the coordination of their peripheral and central parts. The capacity to form

protective conditioned reflexes, for example, depends upon the degree of structural maturity of the cortical elements and appears later in dogs than in rabbits. Such reflexes conditioned by auditory stimuli appear in puppies from the 17th to 20th days, and conditioning by visual stimuli can be achieved from about the 30th to 36th days; in rabbits auditory reflexes can be developed from the 10th to 14th days and visual ones from the 17th to 20th days. Subsequently conditioned reflex activity becomes complete by the development of cortical processes of excitation and inhibition. At the same time the structure of individual cells and their bio-electric activity becomes established. The highest level of cortical activity is observed in rabbits and dogs 2-3 months old (13-20).

In agreement with the data just quoted, our results show a parallel achievement of adult levels of cytochrome activity. It reaches these levels earlier in the motor area than in the auditory and visual areas which is the same order in which the development of conditioned reflexes using the corresponding sensory organs can be induced. The development of the cytochrome system ends earlier in rabbits than in dogs, which is consistent with the earlier functional and histological maturation of the brain of rabbits compared with dogs. Finally, the more marked localization of function in the cerebral cortex of the dog compared with the rabbit is mirrored in the greater difference of cytochrome activity of dogs between the motor area, on the one hand, and the auditory and visual areas on the other hand, than of rabbits.

The significance of the correlation is that the energy supplied by the oxidative processes is being used for nervous tissue activity, including specific cortical functions. During the first 2 or 3 post-natal weeks it appears that a certain level of enzymes—one of which is cytochrome—is reached in the cortical "analyser", and this mediates the conditioned reflex.

The correlation of maturation of the cortical areas with development of cholinesterase activity is much less obvious. After 15 days all areas investigated except the visual area of dogs show an equal activity of cholinesterase, which is then 70-90 per cent of the adult level, and is increasing slowly (Fig. 5). Maturity of cortical activity in the rabbit is accompanied by an increase in its cholinesterase, but this is not the case in dog.

Finally, the differences between the curves of this enzyme activity in the different areas in the dog is less marked than in the rabbit. It seems then that the cholinesterase activity of the dog cortex reflects the development of cortical activity less precisely than do the oxidative enzymes. This conclusion seems to be at variance with other views, particularly Nachmansson (10) who showed a close correlation in chick embryo muscle and pig em-

bryo nervous tissue, between increased cholinesterase activity and the development of conduction (26). The difference may be due to the difficulty of differentiating in the cerebral cortex between that part of the cholinesterase which is free and that part associated with the neurones.

The relation between cholinesterase and neurone and neuroglia in the cortex of animals is still unknown (22, 23, 24). There is a low density of neurones in the cortex (2) and in the dog the concentration of enzyme is relatively low; the importance of other cortical components which mature at the same time as the neurones could have a marked effect on the total cholinesterase activity. This could explain the considerable differences in the cholinesterase curves in the cortex of dogs in the first 10 days after birth, as well as the small differences in the curves of the various areas later on. The high level of cholinesterase in dogs during the first 10 post-natal days may reflect the differentiation of glia as well as neurones (21). At the same time, the development of many neurones containing large quantities of enzyme in the cortex of rabbits causes normal development of the activity of cholinesterase, which is in this case correlated with the development of cortical processes.

TABLE 3. RELATIVE ACTIVITY OF THE CYTOCHROME SYSTEM AND CHOLINESTERASE IN CORTICAL ZONE OF RABBITS AND DOGS

Age before and after birth (days)	Rabbits			Dogs		
	optic zone	auditory zone	motor zone	optic zone	auditory zone	motor zone
Embryonal period						
10	—	—	—	1.48	0.65	1.33
7	—	—	—	—	0.90	1.26
4	1.63	1.11	1.73	0.75	0.96	0.87
2	0.87	0.67	0.70	—	—	—
1	0.96	1.38	1.22	—	—	—
Post-embryonal period						
1	0.96	0.63	0.92	0.52	0.63	0.59
2	1.01	0.99	1.25	0.97	0.90	0.72
3	—	—	—	1.00	0.83	0.85
6	0.56	0.41	0.59	1.01	1.09	0.88
9	0.77	0.52	0.70	0.64	0.41	0.54
15	0.66	0.33	0.60	1.08	0.98	1.58
23	—	—	—	0.93	0.92	1.16
30	0.46	0.40	0.74	1.40	0.98	1.67
60	0.59	0.42	0.60	1.81	1.21	1.70
90	0.57	0.42	0.66	3.14	2.57	1.55
180	—	—	—	1.91	1.26	1.33
adult animals	0.45	0.33	0.48	1.90	1.1	0.91

Table 3 indicates the oxidative enzyme and cholinesterase activity of the cortex of dogs and rabbits at different ages. It shows that the differences between areas appear very early. The relation between the activities of oxidative enzymes and cholinesterases in the cortex of dogs and rabbits changes to a different degree. During development, their ratio in rabbits decreases, whereas in dogs it increases; in rabbits the cytochrome system increases less than the cholinesterase, whereas in dogs it is the other way round.

Inasmuch as the potential activity of enzymes reflects the level of tissue metabolism (2, 7, 25) it may be that the development of cortical activity is correlated with changes in relative proportion of oxidation processes and acetylcholine metabolism (see, 27). It thus appears that we have established a correlation between enzyme activity and functional activity of the different areas of the brain.

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THE HISTOCHEMISTRY OF THE NUCLEOPROTEINS OF THE NEURONES IN RELATION TO THEIR FUNCTIONAL ACTIVITY

A. L. SHABADASH

Institute of Biological Physics, Soviet Academy of Sciences, Moscow

THE presence of nucleoproteins in the cytoplasm of neurones has been known for the last 65 years, since Nissl, by use of a staining method he developed, described specific granules in the cytoplasm—the “tigroid” bodies (1). This observation has been frequently confirmed in the last ten years, and the presence of 2–4 per cent of the total proteins in the “tigroid” substance has been demonstrated (2–9). In view of the extremely important role of nucleoproteins in biological processes (10–12) and of their presence in pre-natal and adult neurones, these substances have attracted the interest of many workers.

Ever since the “tigroid” substance was detected, attempts have been made to correlate the amount of nucleoprotein with the biological state of the neurone. Although undoubted changes have been shown under pathological conditions (13–15), there is very little data on changes in nucleoproteins in the physiological conditions of stimulation and inhibition. Changes in nucleoproteins of spinal motoneurones during exhausting muscular work (3, 8, 16) or in the sensory cells of spinal ganglia (17–19) may be secondary to other physiological reactions, and not a direct consequence of the experimental exhaustion.

Two factors have led us to the study of nucleoproteins in neurones: (a) the results of protein analysis of the nervous system by Academician Palladin and his co-workers: and (b) the discrepancies between estimation of nucleoproteins by spectrophotometry and single cell chemical analysis.

Palladin and Goryukhina extracted the brain with water, 4.5 per cent potassium chloride and, finally, 0.1 N sodium hydroxide, and detected protein fractions with 3 iso-electric points at pHs 4.6, 5.2 and 5.6 (21). The cytoplasmic nucleoprotein was characterized by a powerful bond between the nucleic acid and the protein with a ratio of N to P of 8 : 1: the

protein component was a lipoprotein and contained considerable amounts of tryptophane and tyrosine (22). In the nucleoprotein of the nucleolus other N to P ratios were found, ranging from 3.7 to 4.2 (23). These findings have not yet been followed up with histochemical studies.

Nucleoprotein in motoneurons of the spinal cord estimated by absorption spectrophotometry in the ultra-violet range shows a concentration of 1.9 per cent while the same authors chemically estimating it on single teased cells, found 2.4–2.6 per cent (18–20). It seems unlikely that this discrepancy is due to a difference in the method or to neuroglial contamination; it is probably due to an undetected source of nucleoprotein in the neurone itself.

Using histochemical techniques previously described (24–27), we re-examined the nucleoproteins in various neurones. To detect the whole nucleoprotein complex, including the desoxynucleoproteins, histochemically, we used the basic stain methylene blue at various pHs. The range of isoelectric points of the nucleoprotein complex makes the stain highly selective, and this is a very good physico-chemical method.

Before discussing our results the term “nucleic acid” should be defined more precisely (28, 29). Absorption spectrophotometry estimates only the purine and pyrimidine bases, while staining with pyronine and methylene green—which is not very specific—stains the free acid groups incompletely. Neither of these methods gives clear evidence that the cell contains free nucleic “acids”. It has been previously shown that nucleic acids are very reactive in the body, and bind closely to proteins and glycogen (26). Excluding particular conditions, such as chromosomes during mitosis (28) or bacteria (30), desoxyribonucleic acids (DNA) and ribonucleic acids (RNA) are present in the cells as unstable, “saltlike” and “true” stably complexed nucleoproteins. In undamaged tissues one is not dealing with free nucleic acids but with nucleoproteins or their complexes (26). Realization of this fact enables us to understand the wide variety of tissue and particle specificity of nucleoproteins (31–34), and to develop methods to detect them histochemically. From the findings of Palladin and his co-workers already quoted (22, 23), we know that cytoplasmic and nucleolar nucleoproteins have different N : P ratios and different iso-electric points. The spread of isoelectric points of the complexes of nucleic acids with proteins, lipids, polysaccharides and mucopolysaccharides will be even greater and this pattern will enable us to understand the stages of biological changes in the cells.

We used the technique of rapid fixation of the whole nervous system in 60–90 sec by injection into the circulation of excess alcohol-formalin solution of copper nitrate under 2–2.5 atm pressure (26); this mixture inac-

tivates intracellular ribonuclease and other enzymes and precipitates the copper salt of the nucleoproteins in the alcoholic medium. Serial sections of the spinal cord and brain embedded in paraffin are stained with a mixture of M/300–M/500 methylene blue in McIlwain's citrate buffer, or with equivalent solutions of citric acid, phthalic acid and other organic acids at pHs between 3.2–5.6. With such techniques one can detect the pH for optimal staining of each nucleoprotein site under different experimental conditions. In 1953 we emphasized that our differential staining of nucleoproteins was different from that widely used for staining with pyronine and methylene green, and its wide pH range has added to its usefulness (27). The control for the presence of nucleoprotein was carried out by Kunitz's method with McDonald's modification; crystalline ribonuclease (1 mg/ml) was incubated for 1 hr at 37°C and DNA detected by the Feulgen reaction and removal of DNA in control experiments.

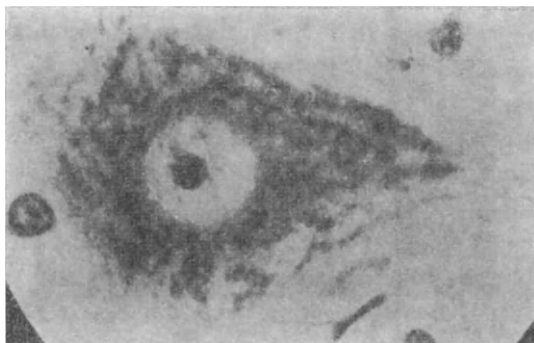


FIG. 1. Ganglion cell from the anterior horns of the spinal cord of a cat (cervical region) stained by the method of Shabadash at a pH of 4.5. In the cytoplasm large "tigroid" bodies and in the nucleolus a coherent staining caused by the presence of ribonucleoproteins can be seen. The nucleus of the neuron and the nuclei of the neuroglia contain desoxyribonucleoproteins. Microphotograph ($\times 1200$).

In the brain and spinal cord of normal animals we found 3 groups of proteins with different iso-electric points localized in different structures; (1) in the "tigroid" substance of the neurones a group of proteins appeared at a pH of 3.8–3.9; (2) in the axoplasm they appeared at a pH of 4.9; and (3) in the neuroglial cytoplasm at a pH of 5.4. In cats, rabbits, rats, mice, cows and horses, the pHs varied somewhat but their pattern and distribution were similar.

These findings should be interpreted cautiously, since it is well known that the pH value for a protein often depends upon whether it is measured

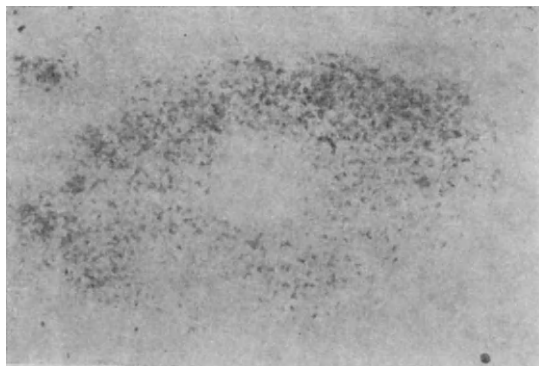


FIG. 2. A ganglion cell from the anterior horn of the spinal cord of a cat (cervical region) stained at a pH of 3.7. The cytoplasm contains numerous small staff-shaped mitochondria, which can be well discerned from the "tigroid" bodies shown in Fig. 1. A motor ganglion cell in the state of rest after drug-induced sleep lasting $1\frac{1}{2}$ hours. The nuclear structures are unstained, the nucleolus falls outside the plane of dissection. Microphotograph ($\times 1200$).

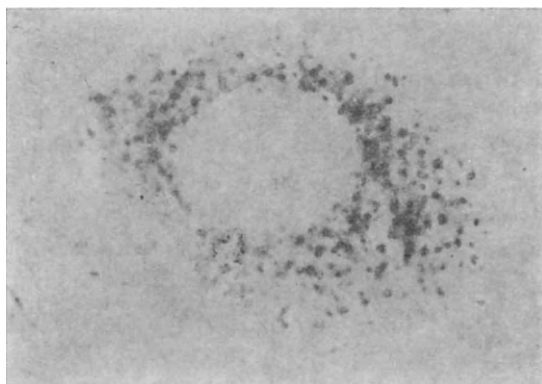


FIG. 3. Motor ganglion cells from the nucleus of the nervus phrenicus (5th cervical segment in the spinal cord of a cat). Staining by the method of Shabadash at a pH of 3.7. The mitochondria are of spheroid shape; the upper left quadrant shows annular mitochondria with a more dense marginal zone and a more transparent centre; they are much larger than staff-shaped mitochondria found in the nerve cell at rest. The nucleolus is hardly stained. Microphotograph ($\times 1700$).

by electrophoresis or by its staining reaction. The physical constants of brain proteins extracted in the cold are not identical with those for tissue proteins in stained, fixed, paraffin-embedded histological preparations, as is seen in gelatine and fibrin. The shift of iso-electric point of the brain

proteins in our sections towards the acid side by 0.5–0.6 pH units is probably explained by this phenomenon.

Ultra violet spectrophotometry (2–4, 6–9), gallocyanine and chrome-alum staining at pH 1.75 (37), and methylene blue staining at pH 4.3–4.5 (27) all demonstrate the presence of nucleoproteins in the “tigroid” substance and in the nucleolus. The mitochondrial fraction from nervous tissue homogenates contains 9–20 per cent nucleoproteins (38) which is more than that in the “tigroid” bodies. The important role played by mitochondria in intra-cellular metabolism is well known (39, 40).

The shape, number and intracellular distribution of mitochondria in neurones can be seen on methylene blue staining at a pH of 3.7–3.8. In the motor cells of the spinal cord and brain stem, in afferent ganglion cells, in cortical neurones and in the basal ganglia, the mitochondria have a characteristic shape and size; they are oblong, 1.5–3.5 μ long and 0.5–1.0 μ across. In the cells of the spinocerebral ganglia of animals, mitochondria became longer, or formed complexes, up to 5–7 μ in length, after narcotic sleep lasting 1½–2 hr. At a pH of 4.0–4.1, mitochondria are seen layered in the cytoplasm free of the “tigroid” bodies. It would seem that mitochondria are more widely distributed than “tigroid” bodies.

Our method of detecting nucleoproteins enables us to examine mitochondria in different functional states. Their number is very considerable; in 10 μ^3 there are 50–70 mitochondria. If we take the volume of the anterior horn motoneurone to be 20,000–24,000 μ^3 , (41), each of these cells contains 120,000 to 170,000 mitochondria; a similar figure was calculated by Harmann (42). This enormous number of mitochondria presents an extremely large biologically-active surface to the cytoplasm.

There are two important features of the nucleoprotein complex in the mitochondria which are different from that of the “tigroid” bodies; firstly, its iso-electric point is 0.4–0.5 pH units lower: secondly, with phthalate buffer and methylene blue, it stains red-violet, while the “tigroid” body stains blue. These features suggest that the nucleoprotein of mitochondria contains a greater number of free phosphate groups.

Electron microscopy of mitochondria enables one to discern a core consisting of protein and a double lipid layer which gives out septa into the interior of the mitochondrion: in nervous elements these septa are mainly orientated in the long axis (43, 44). The composition of the protein “stalk” cannot be elucidated by electron microscopy, but our observations indicate that nucleoproteins are present in the external part of that stalk.

The nucleoproteins of the microsomes are still a matter of dispute.

The high concentration of nucleoprotein within mitochondria may require us to reconsider the nucleoprotein content of the neurones. First of all, changes of complex nucleoproteins cannot be used as indices of biological change, as the nucleoproteins extracted are not homogeneous. Secondly, if the nucleoproteins of mitochondria and "tigroid" bodies are not the same, the cytochemical differences we detected may correspond to "active" and "reserve" nucleoproteins. Thirdly, redistribution of nucleoproteins may occur while their total amount does not change.

There are two diametrically opposed views about the activity of the mitochondria. According to one view (45) mitochondria are extremely stable, whereas the other view (46) insists on their continuous biological activity. We were able to show a series of changes which can be detected as the cell becomes active. In motoneurones, ganglion cells and cerebral cortical cells, activity is accompanied by marked changes in the shape of the mitochondria; they change from an oblong to a round shape, and their density increases; then their sizes become equal but some appear to swell further and become 1.5–3 times larger than their neighbours. The next stage is the formation of a dense, ring-shaped girdle with a paler centre in the shape of a disc. Finally, with physiological stimulation, spheroid formation can be seen as the nucleoprotein is concentrated in one hemisphere in the shape of a half moon or sickle and the other part of the mitochondrion remains unstained by our method. We are at present trying to find out the chemical composition of this unstained part, and preliminary work suggests that following irradiation it contains a high quantity of lipids.

This sequence of changes in the mitochondria was well marked in different experimental conditions. In kittens exposed to thiopentone anaesthesia for short periods, round active mitochondria could be seen in the majority of cells in the optic area, whereas in the parietal area, red-shaped mitochondria predominated. A similar, well-marked contrast can be seen in anaesthetised animals between motoneurones of the phrenic nerve in C3–5, and the neighbouring motoneurones in the same segments innervating the neck muscles and shoulder girdle; in the former, the mitochondria are round or ring-shaped, and in the latter they are rod-shaped. Well-marked changes in the mitochondria are characteristic of ganglion cells in which, during prolonged narcosis, rod-shaped mitochondria appear and increase to 2–3½ times their normal length. These findings have been repeatedly confirmed in our laboratories.

Reports of physiological or biochemical correlations of these findings are scant in the literature, probably because of the variety of mitochondrial fractions obtainable from homogenates. However, analysis of mitochondria

isolated from different muscles is very instructive; their number, size and enzymatic activity are proportional to the activity of the muscles in question (47-49). The studies of Harmann are of particular interest (50-53): he has shown a close correlation between the shape of the mitochondria in the liver and muscles and their biochemical activity, using differential centrifugation. In hypertonic sucrose, mitochondria are normally rod-shaped. If they are incubated in artificial media, the rod swells and gradually becomes spherical, discoid and then target-shaped; next, a vesiculous outgrowth arises from the denser ring on the outside and pushes towards one of the poles, to form a sickle-shaped segment of an enlarged sphere (50). He showed that each shape corresponded with particular chemical characteristics. The change of shape from rods to spheres and then discs is accompanied by an increase in respiration and oxidative phosphorylation. The intactness of the mitochondria is very important in the preservation of this order and the coordination of the enzymatic activity of the cyclophorase. The preservation of the shape of the mitochondria depends upon the resynthesis of ATP, and under good experimental conditions, can be preserved for many hours (50-53).

It can be seen that there is considerable similarity between the observations of Harmann and ourselves although we used different systems. It is also interesting that after exposure of animals to X-rays, their mitochondria become spherical with a narrow sickle-shaped waistband of nucleoproteins.

The physico-chemical characteristics of the nucleoproteins in the "tigroid" substance and in the mitochondria are very different, and this probably reflects biological differences in these particles. Gale has recently shown that RNA is a highly specific catalyst for the synthesis and metabolism of glutamic acid and proteins, and an activating factor for a number of enzymes (54, 55). These properties are more characteristic of the nucleoproteins in mitochondria than the "tigroid" substance. Changes of the nucleoproteins take place more slowly in the "tigroid" substance than in the mitochondria, and would appear to be secondary to them.

SUMMARY AND CONCLUSIONS

1. A histochemical method for the detection of nucleoproteins in nerve cells based on their iso-electric points is described. Enzymatic degradation of small preparations has been used to study them.

2. The activity of the neurones is reflected in qualitative and quantitative changes in the nucleoproteins of the mitochondria, nucleolus and "tigroid" substance, which take place in a particular sequence.

3. Various degrees of activity up to maximum stress are reflected in physico-chemical and morphological changes in the mitochondria of nerve cells. These changes are related to respiration, and oxidative phosphorylation.

4. The presence of 3 groups of proteins in the nervous system of mammals with 3 iso-electric points, which was first shown by Palladin and his colleagues, is confirmed, and it is shown that each belongs to particular structures, the "tigroid" substance, the axons and the cytoplasm of the neuroglia.

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THE EFFECT OF INTERRUPTION OF THE CIRCULATION OF THE BRAIN DURING HYPOTHERMIA ON CEREBRAL METABOLISM

G. YE. VLADIMIROV

Kirov Military Medical Academy, Leningrad and the Pavlov Institute
of Physiology, Soviet Academy of Sciences, Leningrad

STUDIES of biochemical changes in the brain during hypothermia throw light on the relation between the metabolism and function of the brain, and may help to characterize hibernation and "suspended animation". Artificially induced hypothermia has been known to be an effective way of changing the activity of the body. As long ago as a hundred years, Wal'ter (1) wrote that, "cooling anaesthetizes an animal better than chloroform". It can suppress allergic reactions in the body (2, 3), prevent the development of shock after transfusion of incompatible blood (4), and inhibit the development of traumatic shock (5, 6). Surgical operations involving interruption of the oxygen supply to the brain and heart which could not be done formerly have now become possible by the use of hypothermia (7, 8, 9). This has stimulated the present study of hypothermia with interruption of cerebral blood flow.

THE EFFECT OF HYPOTHERMIA ON THE ENERGY-RICH PHOSPHATES OF THE BRAIN

The energy for brain metabolism is derived from the oxidation of glucose and glycogen. The energy liberated by glucose oxidation is used for the synthesis of energy-rich substances like adenosinetriphosphate (ATP) and phosphocreatine. When oxidative processes are inadequate, anaerobic glycolysis increases, with a rise in the lactic acid of the brain. Due to the low energy yield of that process, the synthesis of ATP and phosphocreatine lags behind their breakdown, and the content of these substances decreases, with a concomitant increase of inorganic phosphate.

Lowering the body temperature of warm-blooded animals to 25°C decreases the oxygen uptake and glucose consumption of the brain consider-

ably (10–12). The levels of ATP, phosphocreatine, inorganic phosphates, glycogen and lactic acid can be used to assess whether oxygen insufficiency is present in the brain, and also the level of phosphate energy.

METHODS

Rabbits were cooled under ether anaesthesia by immersion in cold water; their body temperatures fell to 20–25°C within 40–80 min. After some time at this temperature, the head was frozen from the occiput with liquid oxygen by the method of Rozengart *et al.* (13). The frozen brain was taken out, homogenized, and its phosphates, lactic acid and glycogen analysed. The inorganic phosphate was precipitated by Delory's method, and estimated colorimetrically by the method of Fiske and Subbarow. ATP was estimated following barium precipitation by the phosphate liberated after 7 min hydrolysis in 1N HCl; the phosphocreatine was estimated by the phosphate liberated in the presence of molybdate in the filtrate from which inorganic phosphate had been precipitated by Delory's method. The hexose-phosphates were separated by Umbreit's method (14). Glycogen was measured by Kerr's method, and lactic acid by the method of Freidemann, Katonino and Schaffer.

RESULTS

Table 1 shows that neither ether anaesthesia nor short or long periods of hypothermia had any effect on the level of labile phosphates or glycogen. The inorganic phosphate and lactic acid were slightly raised after brief hypothermia, but if this state was continued for more than 2 hr, they returned to normal. It thus appears that by these most sensitive indices there is no change in the energy status of the brain during hypothermia at 23–24°C.

Profound changes, however, do occur when hypothermia is combined with interruption of the blood supply, that is when the oxygen and glucose supplies to the brain are stopped. We did this by exclusion of the heart from the circulation. A surgical approach was made in the right chest in the region of the 3rd–6th rib and sternum, the superior and inferior venae cavae and vena azygos were closed, and then the aorta and pulmonary artery clamped. The blood supply both to and from the heart was thus interrupted. 10 min later the rabbits were immersed in liquid oxygen. Other experiments, lasting a shorter time, were carried out with animals at normal body temperature. In further controls operative pneumothorax was induced on the right side. In a final series we examined the brain 5 and 10 min

TABLE 1. CONTENT OF COMPONENTS OF THE CARBOHYDRATE-PHOSPHORUS METABOLISM IN THE BRAIN OF RABBITS UNDER NORMAL CONDITIONS IN ETHER NARCOSIS AND IN THE STATE OF HYPOTHERMIA

(Average data from the experiments of O. N. Savchenko)

Experimental conditions	Phosphorus (mg %)				Lactic acid (mg %)	Glycogen (mg)*
	inorganic	labile ATP	phospho-creatine	total sum of hexose-phosphates		
Normal conditions	12.0	14.0	11.2	—	23	87
Ether narcosis	6.7	14.1	11.8	10.6	29	89
Short lasting hypothermia**	14.1	13.2	10.2	11.5	35	75
Hypothermia lasting 2 hr***	8.5	13.0	10.5	11.2	29	82

* Experiments by T. P. SEREBRENIKOVA.

** The experiment was interrupted after a temperature of 20–25°C had been reached.

*** After a temperature of 20°C–25°C had been reached the animal was kept in a state of hypothermia for 2 hr.

after restoration of the blood supply, to investigate the extent and time course of the changes engendered by interruption of the blood supply. The results are illustrated in Table 2.

From Tables 1 and 2 it can be seen that operative trauma and surgical right pneumothorax had no effect themselves on the brain inorganic phosphate, ATP, phosphocreatine, or glycogen, but the lactic acid was slightly raised. As opening the chest was necessary to exclude the heart from the circulation, this condition must be regarded as the relevant control.

Exclusion of the heart without cooling the animal was carried out for 5–10 min depending on the clinical state of the animal. Interruption of the blood supply caused marked changes: inorganic phosphates increased up to almost 4 times the normal level to 39 mg %; labile adenine phosphates decreased from 12 to 3.3 mg %, and phosphocreatine disappeared altogether. Hexosephosphate levels doubled (20 mg %), and analysis of the glucose-6-phosphate, fructose-6-phosphate, and fructose-1,5-diphosphate showed that this increase was mainly due to raised glucose-6-phosphate. Lactic acid increased up to an average of 1–7 mg %, and there was a marked decrease in glycogen content. The accumulation of lactic acid was probably limited by the fact that interruption of the blood supply decreased the oxygen and glucose available to the brain.

TABLE 2. CONTENT OF THE COMPONENTS OF THE CARBOHYDRATE-PHOSPHORUS METABOLISM IN THE BRAIN OF RABBITS AFTER THE PERFORMANCE OF A PNEUMOTHORAX, AFTER INTERRUPTION OF THE BLOOD SUPPLY TO THE BRAIN AND AFTER SUBSEQUENT RESTORATION OF THE BLOOD SUPPLY

(Average data from the experiments by O. N. Savchenko)

Experimental conditions	Phosphorus (mg %)				Lactic acid (mg %)	Glycogen* (mg %)
	inorganic	labile ATP	phospho-creatine	total sum of hexose-phosphates		
Opening of the chest	11.0	12.0	10.5	10.4	33	81
Interruption of cardiac activity for 5-10 min at normal body temperature	39.1	3.3	0	20.1	127	28
Interruption of cardiac activity for 10 min in a state of hypothermia (23°C-25°C)	29.9	6.6	0	16.8	121	51
Five min after restoration of the circulation which had been interrupted for 10 min under conditions of hypothermia	13.0	11.6	6.7	15.6	69	46
Same after 10 min	12.1	12.2	10.3	17.7	90	65

* Experiments by T. P. SEREBRENKOVA

If the heart was excluded from the circulation during hypothermia, the same changes took place in all the substances discussed, but they were less well marked; only phosphocreatine disappeared completely, as in the previous experiments. In evaluating these results, it should be noted that all animals were capable of tolerating a 10-minute interruption of blood supply. It appears that during hypothermia oxygen insufficiency was accompanied by a marked increase in glycolysis which was slightly smaller than the increase observed at normal temperatures. The consumption of high energy phosphates was less marked, and after 10 min without blood supply, the level of ATP still remained high—6.6 mg %. Thus ATP was better preserved during hypothermia than at normal body temperature. That ATP is maintained better in hypothermia during anoxia has been reported

elsewhere (15, 16, 17). We confirm the finding of Raiko (18) that restoration of glycogen and elimination of excess lactic acid is a very slow process. After the slight decrease in lactic acid during the first 5 min, its rise was apparently connected with the aerobic glycolysis that takes place when the glucose supply is stopped (16).

The intensive glycolysis which takes place after the oxygen supply has been interrupted and the absence of accumulation of lactic acid on restoration of its supply, the disappearance of creatinephosphate and ATP in oxygen deficiency, and the absence of these changes in hypothermia without anoxia, indicate the following facts. Firstly, that the glycolytic mechanism is of high capacity even in hypothermia; secondly, that under these conditions, the Pasteur effect is well-marked, and thirdly, that the synthesis of energy-rich phosphates keeps up with their breakdown during hypothermia, provided that there is no interruption in the oxygen supply. The absence of profound changes in the glycolytic system connected with the synthesis of these energy-rich substances is confirmed by the experimental finding that re-connection of the blood supply for as short a time as 6–10 min restored the ATP and phosphocreatine levels to normal (Tables 2, 4).

At a body temperature of 20–23°C, the oxidative and phosphorylative processes decreased to about a third, whereas the glycolytic processes were

TABLE 3. THE CONTENT OF COMPONENTS OF CARBOHYDRATE METABOLISM IN THE BRAIN OF RABBITS AFTER ADMINISTRATION OF CHLORPROMAZINE
(Average data from the experiments of O. N. Savchenko)

Experimental conditions	Phosphorus (mg %)				Lactic acid (mg %)
	inorganic	labile ATP	phospho-creatine	total hexose-phosphates	
Normal conditions	12.0	14.0	11.2	—	23
After administration of chlorpromazine at normal body temperature	12.2	13.0	10.4	13.6	34
Interruption of cardiac activity under conditions of hypothermia without chlorpromazine	29.9	6.6	0	15.3	121
Interruption of cardiac activity under conditions of hypothermia after administration of chlorpromazine	31.5	7.0	0	15.9	103

inhibited much less. However, some aspects of brain function are inhibited before the temperature has been lowered as much as this. Cooling dogs to 27–30°C for example, causes inhibition of respiratory conditioned reflexes (19). Between 31 and 33°C there is a considerable slowing of electro-encephalogram rhythms in the cortex, and at 20°C electrical activity ceases altogether (20, 21). Maistrakh found that at 26°C the rabbits are unable to balance, and pupillary reflexes disappear. At 20°C the function of the mid-brain and mesencephalon is almost completely inhibited (19). Finally, Savchenko injected a ganglion blocking substance, chlorpromazine, (10 mg per 1 kg body weight) into a cool animal, and this had no effect (Table 3). Although chlorpromazine has an effect on some enzyme systems, it thus seems to have no effect on energy metabolism (23).

It would then appear that one can have serious disorders of nervous function without any effect on the energy metabolism of the brain. The nervous functions are more sensitive to hypothermia than the energy metabolism.

THE EFFECT OF HYPOTHERMIA ON THE METABOLISM OF LIPIDS AND PROTEINS IN THE BRAIN

Both proteins and lipids are very important in brain and cellular metabolism (24, 25, 26). Lipids occur in brain tissue both in a free form and complexed with proteins. The influence of hypothermia on the metabolism of these compounds can be followed by the use of radioactive tracers.

TABLE 4. THE INFLUENCE OF INTERRUPTION OF THE CARDIAC ACTIVITY UPON THE TOTAL CONTENT OF PHOSPHORUS, INORGANIC PHOSPHATES, ATP, PHOSPHOCREATINS, AND HEXOSE-PHOSPHATES

(Average data from the experiments of O. N. Savchenko)

Experimental conditions	Total phosphorus (mg %)
Short-lasting hypothermia without interruption of cardiac activity	49.0
Hypothermia lasting 2 hr without interruption of cardiac activity	43.2
Opening of the chest	43.9
Interruption of cardiac activity for 5–10 min at normal temperature	62.5
Interruption of cardiac activity for 10 min in a state of hypothermia (23°C–25°C)	53.3
Five min after restoration of the circulation which had been interrupted for 10 min under conditions of hypothermia	46.9
The same after 10 min	52.3

From Table 4 it can be seen that ATP, phosphocreatine, hexosephosphates, and other phosphorus-containing compounds are sources of inorganic phosphates during anoxia. Their total amount of phosphorus shows a slight increase from 43–49 to 53 mg % after stopping the blood supply during hypothermia, but a much greater increase, up to 62.5 mg %, if the blood supply is cut off at normal body temperature. This suggests that during hypoxia a certain amount of inorganic phosphorus comes from proteins or phospholipids. To examine this possibility the incorporation of radioactive phosphorus into the lipids and proteins was studied.

Sodium amylobarbitone was injected into white rats at a dose of 7 mg per 100 g weight. As soon as the rat was anaesthetized it was placed in a container with ice and its body temperature observed. When it had fallen to 25°C the animal was taken out and a dose of 0.3–0.5 μ c per g radioactive di-sodium hydrogen phosphate was injected subcutaneously. Controls were carried out on animals which were anaesthetized at normal body temperature; the experiments lasted from 80–120 min. The rate of incorporation of radioactive phosphate into proteins and phospholipids is expressed as the relative specific activity (RSA) which is defined as the percentage of activity of the phosphorus in the compound under study compared with the inorganic phosphate in the brain tissue. This gives a measure of phosphorus turnover which is independent of the dose of radioactive phosphorus.

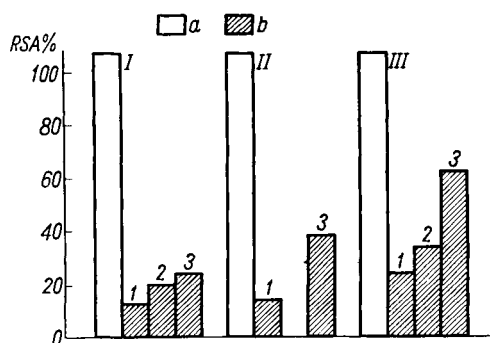


FIG. 1. The relative specific activity of phospholipid phosphorus, lipoprotein and phosphoprotein phosphorus in the brain of a rat in a state of hypothermia after various intervals of time. The relative specific activity for rats with a normal body temperature was defined as 100 white columns: 1, 2, 3—hypothermia lasting 80, 100 and 120 min respectively; I—phospholipid; II—lipoprotein; III—phosphoprotein; a—normal conditions (amylobarbitone); b—hypothermia (26°C–16°C), (G. E. Vladimirov, T. N. Ivanova and N. I. Pravdina).

The mean figures for the turnover of phosphorus in phospholipids, lipoproteins and phosphoproteins are shown in Fig. 1. It can be seen that phospholipid metabolism decreases as much as 4–6 times during hypothermia, depending upon the duration of the experiment. In fact we were using here as normal levels the levels during anaesthesia when the metabolism of the above compounds is already considerably decreased (27). If comparison had been made with the turnover in conscious animals, this difference would have probably been even greater. Not so well-marked, but still considerable, is the difference in rate of phosphorus turnover of lipoproteins and phosphoproteins. In contrast to these are the hexosephosphates whose incorporation of phosphorus is decreased to a lesser extent in hypothermia (Fig. 2). This fact fits in with the observation that glycolytic processes are not inhibited so much during hypothermia, and the same is true for oxygen consumption (Fig. 3).

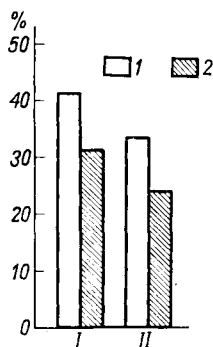


FIG. 2. Relative specific activity (in % of the specific activity of ATP phosphorus) of hexose-phosphates in rats *in vivo* and in tissue sections: I—*in vivo*; II—*in vitro*; 1—normal conditions (33°C–37°C); 2—hypothermia (16°C–24°C). The experiment lasted for one hour (G. E. Vladimirov, and L. N. Rubel').

Amino acid metabolism of protein shows a particularly marked decrease in hypothermia. Nechayeva (28) has shown that the bulk of labelled amino acids of brain proteins are involved in protein synthesis and are not purely structural substances. The use of labelled amino acids gives some indication of the intensity of biosynthesis of proteins. In our investigations, carried out by Vladimirov and Urinson, ^{14}C - and ^{35}S -labelled glycine, methionine and tyrosine were used. In all cases we found a marked decrease in the intensity of amino acid metabolism during hypothermia. For example, Fig. 4 shows the results of experiments with labelled methionine.

Tyrosine, labelled with radioactive carbon, is particularly convenient for studies of amino acid metabolism. This isotope can be estimated quantitatively in brain protein and in the protein-free filtrate (29). Furthermore, tyrosine is not converted into other amino acids. This enables us to estimate it in the protein and protein-free fractions, and the relative activities give us some guide to the turnover of tyrosine-containing proteins.

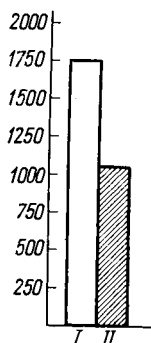


FIG. 3. Oxygen consumption by brain sections at 37°C (I) and at 24°C (II) in mm³ per 1 g tissue per hour (G. E. Vladimirov and L. N. Rubel¹).

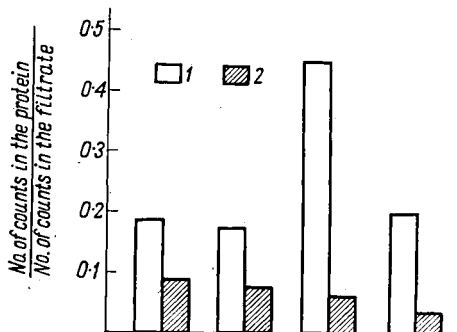


FIG. 4. The relative specific activity (relation between the activity of proteins to the non-protein filtrate per 1 g brain). Of brain proteins under normal conditions and in a state of hypothermia 30–50 min after administration of methionine: 1—normal conditions; 2—hypothermia (20°C–23°C) (G. E. Vladimirov and A. P. Urinson).

The protein fraction of the brain contains on an average 0.48 per cent tyrosine and the soluble fraction contains 0.0022 per cent or 220 times less (Table 5). The ratio of ¹⁴C in the protein fraction to the ¹⁴C of the non-protein tyrosine in experiments lasting 1½ hr was 1.06. As there is 220 times more tyrosine in the protein fraction this ratio will be $1.06/220 = 0.48$ per cent. From this it can be calculated that the average turnover time for brain protein is $206 \times 1.5 = 310$ hr. This value is more reliable than the value of 100 hr calculated from glycine (28), but it is nevertheless tentative.

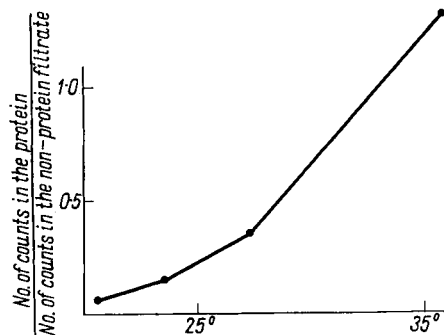


FIG. 5. The relative specific activity of the protein to the activity of the non-protein filtrate of the brain of rats $1\frac{1}{2}$ hr after administration of labelled tyrosine in a state of hypothermia (G. E. Vladimirov and A. P. Urinson).

The results of experiments on cooled white rats are shown in Fig. 5. It can be seen that hypothermia considerably decreases the rate of incorporation of tyrosine into proteins. At 20°C this incorporation is only 1/16th to 1/20th of the normal rate. It thus appears that hypothermia has a much more marked effect on the rate of lipid and protein metabolism than would have been predicted from van't Hoff's factor for a purely chemical reaction. Plastic metabolism is very weak at 20°C , although energy metabolism is well maintained.

TABLE 5. THE SPECIFIC ACTIVITY OF TYROSINE IN PROTEINS AND NON-PROTEIN FILTRATES IN THE BRAIN OF RATS $1\frac{1}{2}$ HOURS AFTER ADMINISTRATION OF LABELLED TYROSINE (G. E. Vladimirov and A. P. Urinson)

Weight of rat (g)	Dose of labelled tyrosine in counts/min per g body weight	Counts/min per weight unit of protein from 1g brain	Counts/min per weight unit of non-protein tyrosine from 1g brain	^{14}C of proteins
				^{14}C of nonprotein tyrosin
160	8800	630	595	1.06
135	8800	540	620	0.87
145	9100	725	553	1.30
150	8800	640	640	1.00
165	9000	825	655	1.26
155	8700	410	575	0.71
140	8700	560	455	1.23
			Average	1.06

We conclude that the functional activity of the nerve cell ceases as the result of inhibition of the biochemical processes connected with the metabolism of protein and lipids during hypothermia.

Results presented are the work of T. N. Ivanova, N. I. Pravdina, L. N. Rubel, O. N. Savchenko, T. P. Srebrnikova and A. P. Urinson. We would like to acknowledge the assistance of Soviet Academician P. A. Kupryanov and his colleagues, V. I. Burakovsky and V. S. Uvarov.

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THE EFFECTS OF COLD, CHLORPROMAZINE AND SODIUM BARBITONE ON THE METABOLISM OF NERVOUS TISSUE

R. V. CHAGOVETS, YE. V. LAKHNO, A. A. RYBINA, R. S. FRIDMAN
AND Ts. M. SHTUTMAN

Institute of Biochemistry, Ukrainian Academy of Sciences, Kiev

INVESTIGATIONS on the effect of hypothermia on the nervous system have been of two kinds: (1) the metabolism of hibernating animals, and (2) changes induced by artificial lowering of the external temperature. In recent years, the study of hypothermia has been stimulated by its use with drugs in surgery. Particular interest has been shown in the phenothiazine derivatives used during anaesthesia. These compounds are the main constituents of the "cocktail" used in surgical hypothermia (Laborit (1)). Chlorpromazine and its derivatives have been used in psychiatry and other branches of medicine. We were interested in the possible relationship between surgical hypothermia and sleep therapy which is widely used in Soviet clinics. The possible metabolic effects of different periods of sedation are also very interesting.

As a result of our investigations, we are now in a position to characterize the biochemical action of certain hypothermic drugs on the central nervous system and the endocrine glands. In this way we can assess the most useful and safest combinations of the different hypothermic drugs in clinical practice. Despite the known pharmacological effects of the phenothiazine derivatives, it is difficult to classify them as a separate group of hypothermic agents, or to draw a strict borderline between them and the hypnotics which also decrease the body temperature.

It is important in studying the effects of hypothermia to realize that physical methods of cooling, such as cold air, immersion in cold water, or the application of cold compresses, produce the effects on the body distinct from the drugs administered for surgical purposes. We, therefore, had to assess the effect of cold itself.

There is considerable doubt in the literature about the effect of cooling upon metabolism in the body (2), but Van't Hoff's assertion that at low

body temperature the biochemical processes begin to be governed by simple physical and chemical laws seems questionable. These metabolic changes in simple systems are radically different from the reaction of the whole warm-blooded animal to cold. Such literature as there is, is mostly concerned with biochemical investigations of the pharmacodynamics of the hypothermic drugs.

The mechanism by which the phenothiazine derivatives act is of particular interest. According to the important work of Finkelstein, Spencer and Ridgeway (3) 10 mg % of chlorpromazine decreases the respiration of cat's heart muscle and cerebral cortex slices *in vitro* by 45 per cent, and 20 mg % decreases it by 90 per cent. These results were confirmed by Broglie, Jörgensen and Voss (4). Also Abood (5) showed inhibition of phosphorylation, decrease of cytochrome oxidase activity and increase in respiration of brain mitochondria by a concentration of 10^{-5} M chlorpromazine. Higher concentrations inhibit respiration and decrease adenosinetriphosphatase activity.

Laborit (6) believes that the effects of chlorpromazine on the body are due to its suppression of carbohydrate metabolism. Decourt (7) regards its effect as being a general suppression of cellular metabolism, particularly in the central nervous system, due to its suppression of the activity of oxidative and reducing enzymes. Chlorpromazine has also been shown to inhibit the action of other enzymes (8).

Comparison of the effect of hypothermic drugs on the metabolism of the brain with their effect on other tissues indicates a probable specific action of chlorpromazine on nervous tissue.

We studied the reducing properties of the tissue and the activity of dehydrogenases (E. V. Lakhno), labile phosphates (T. S. Shtutman), incorporation of ^{35}S -labelled thiamine and endogenous thiamine (A. A. Rybina), and the level and chemical state of ascorbic acid (R. S. Fridman). To give some idea of the clinical state of the animals, their respiration and rectal temperatures were measured (A. A. Rybina). In all experiments on adult rabbits cooling at 3–5°C was carried out for 1–2 hr; the animals were exposed to chlorpromazine while under sodium barbitone anaesthesia for 2–3 hr, and the combined influence of chlorpromazine *and* cooling was studied for $1\frac{1}{2}$ – $2\frac{1}{2}$ hr.

It is known that both phenothiazine drugs and anaesthesia decrease the basal metabolic rate. Cooling the whole animal increases the intensity of energy metabolism and consequently oxygen consumption, due to the "protective" increase in heat production. The degree to which this latter effect is related to changes in the reducing properties of the nervous tissue

is shown in Fig. 1. Chlorpromazine causes a marked decrease in reducing power, particularly in the cerebral cortex, less in the cerebellum, then the suprarenal glands, and least in the muscles. This distribution of reducing power is altered by the administration of sodium barbitone, cooling, or cooling combined with chlorpromazine. Cooling does not change the

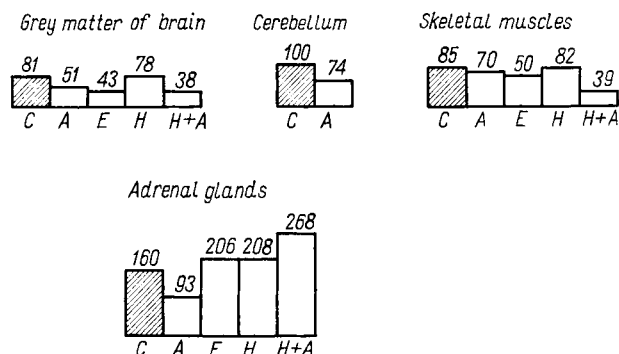


FIG. 1. The influence of chlorpromazine (A), sodium barbitone (E), hypothermia (H) and hypothermia+chlorpromazine (H+A) upon the reducing properties of cortex tissue, cerebellar tissue, skeletal muscles and the adrenal glands of rabbits. C—control. The numbers indicate the quantity of methylene blue (in μg) decolorized per 1 g fresh tissue in 1 min.

reducing power of the cerebral cortex or skeletal muscle appreciably, but increases it in the suprarenals. Reduction by these glands is increased by the sodium barbitone, as well as chlorpromazine with cooling.

Interesting effects of these hypothermic drugs are seen on various dehydrogenases (Fig. 2). Chlorpromazine causes a general suppression of the dehydrogenases of all tissues, with the exception of the isocitric dehydrogenase of the suprarenal glands. The effect of sodium barbitone narcosis, on the other hand, is exactly the opposite—dehydrogenase activity is increased in all tissues. Physical cooling causes slight activation of the brain and muscle dehydrogenases, and a considerable increase in the dehydrogenase of the suprarenal glands, particularly isocitric dehydrogenase. From the latter experiments, which showed a decrease of dehydrogenase activity by chlorpromazine, it might have been expected that cold would have a similar effect 1 hr after chlorpromazine injection. In fact, however, the suppressing effect on the dehydrogenase system was even *more marked* in the cerebral cortex and muscle. Chlorpromazine did not, however, prevent the increase in suprarenal activity as a result of cooling.

One would have expected that decreased energy requirements during cooling would be reflected in increased levels of thiamine-containing compounds

in the tissues. This was in fact the case when one looked at the effect of chlorpromazine, and chlorpromazine with cooling, on the total thiamine and thiamine phosphates (Fig. 3).

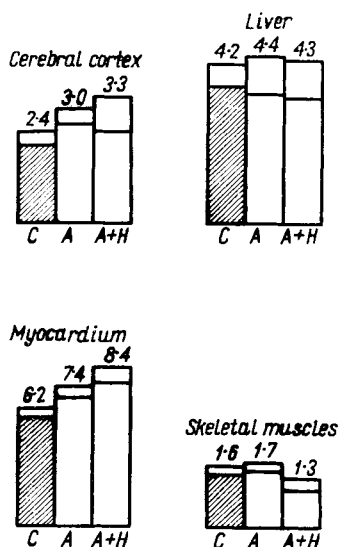


FIG. 3. The influence of chlorpromazine (A) and hypothermia+chlorpromazine (H+A) upon the content and state of thiamine (in $\mu\text{g per g}$) in the cerebral cortex, the liver, the myocardium and the skeletal muscles of rabbits. C—control.

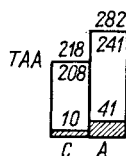


FIG. 4. The influence of chlorpromazine (A) upon the content (in mg %) and the state of ascorbic acid in the adrenal glands of rabbits. C—control. Hatched part of column bound ascorbic acid.

The total ascorbic acid in the tissues is not affected by chlorpromazine except in the suprarenal, where the free and bound vitamin C increases (Fig. 4).

The exchange of compounds between blood and cells is closely related to metabolic processes, so that suppression of metabolism ought to inhibit their incorporation. The radioactivity of ^{32}P -labelled creatine phosphate was used as an index of metabolic incorporation. An hour after dosage with chlorpromazine, a 20 per cent decrease in the rate of radioactive phospho-

rus incorporation was found in rabbits (Fig. 5). It seems likely that shorter exposure times would have demonstrated this effect more clearly, and in fact if the animals were tested 10 min after the chlorpromazine, a 40 per cent decrease was detected.

The excretion of ^{35}S -labelled radioactive thiamine in the urine was increased $1\frac{1}{2}$ times during sleep. At the same time there was an increase in the radioactivity of the blood and a decrease by 9–20 per cent of the amount of thiamine entering the brain, heart and liver. Chlorpromazine does not change the excretion of radioactive thiamine in the urine, although its level in blood is slightly increased. Incorporation of thiamine into the cerebral cortex was decreased by 40 per cent and heart muscle by 60 per cent. The absence of increased excretion in the urine is probably explained by an increased amount entering the liver.

It thus appears that chlorpromazine and barbiturates inhibit thiamine incorporation into most organs. The amount entering the liver, however, seems to be increased by chlorpromazine and decreased by sodium barbitone.

According to Engelhardt (9), Belitser (10, 11), Copenhaver and Lardy (12), and Maley and Lardy (13), the increase in rate of breakdown of high energy compounds or the formation of phosphate acceptors is probably the main factor in the increase of the metabolism of energetic substances. The phosphate acceptors include creatine and adenosine mono- and di-phosphates. By the same token, inhibition of energy metabolism is caused by factors limiting the breakdown of high energy compounds and the formation of phosphate acceptors. This explanation does not, however, exclude the possibility that energy metabolism can be suppressed in other ways, for example, interference with the dynamics of the enzyme systems connected with glycolysis and respiration. If this was so, a disproportion would develop between the rate of formation of phosphate acceptors and metabolism, and there would be an accumulation of phosphate acceptors in the tissues. If this were *not* the case, there would have to be a simultaneous inhibition of enzymes responsible for energy metabolism and high energy compound breakdown.

A decrease in the external temperature causes an increase in energy metabolism in warm-blooded animals, as a means of compensatory heat production. Activation of adenosinetriphosphatase by thyroid hormones is thought to be the biochemical basis for the increased breakdown of high energy compounds (13). Another way of increasing heat production in the muscles is breakdown of the ATP-myosin complex due to stimulation from nerves, of which tremor is the clinical manifestation.

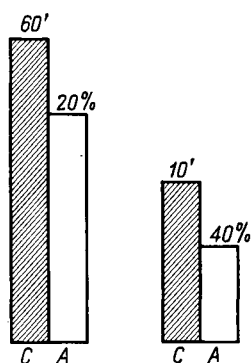


FIG. 5. The influence of chlorpromazine (A) upon the uptake of ^{32}P into the creatine-phosphate of the brain of rabbits after 60 min and 10 min exposure respectively. C—control. Hatched columns: control animals.

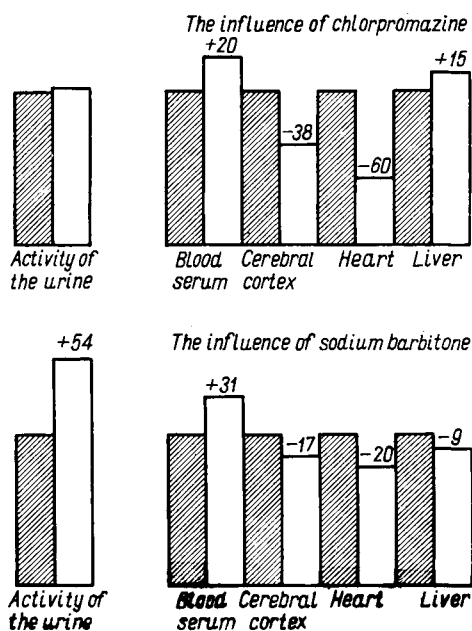


FIG. 6. The uptake and discharge of thiamine labelled with ^{35}S in the tissue of rabbits under the influence of chlorpromazine and sodium barbitone. Hatched columns: control animals.

Fall of temperature, due to environmental change, develops when increased heat production is unable to keep up with increased heat loss. This exhausts the energy sources of the body. Presumably the lower the external temperature, the lower the state of activity achieved, and the more the possibility of returning to normal will be preserved.

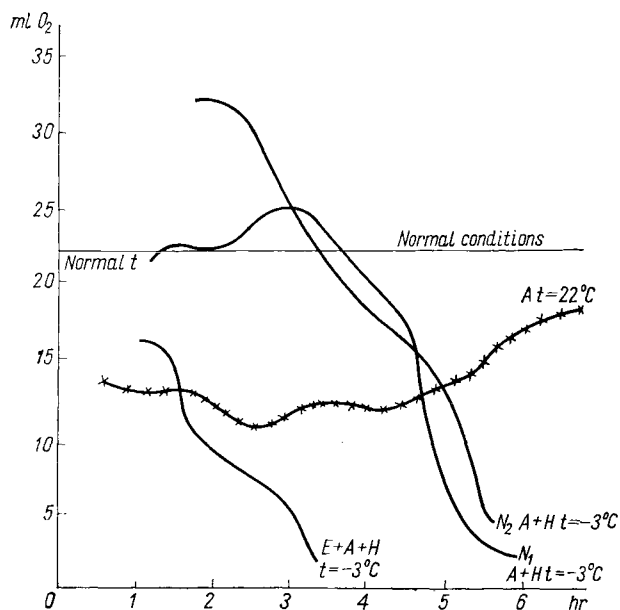


FIG. 7. The influence of chlorpromazine (A), chlorpromazine + hypothermia (A+H) and sodium barbitone in combination with chlorpromazine and hypothermia (E+A+H) upon the oxygen consumption of rabbits, the abscissa shows the duration of the experiment in hours, the ordinate the adsorption of the consumption of oxygen in ml/min.

Chlorpromazine and sodium barbitone are not themselves active inhibitors. They cause only a slight fall in temperature. Chlorpromazine does not prevent the body response to cooling. This can be seen from the curves of respiration of an animal in a cooling chamber after chlorpromazine injection (Fig. 7). Whereas at room temperature chlorpromazine causes decreased respiration, if the animal is cooled, the fall of oxygen uptake does not occur and even sometimes reverses (Figs. 7, A, H). The capacity of the body to resist cold, however, decreases considerably.

Chlorpromazine decreases the activity of all dehydrogenases investigated by us. This would account for the fact that the body's defence reaction to cold of animals treated with chlorpromazine is very short. In view of

reports of its ability to inhibit adenosinetriphosphatase activity in brain sections (5), it is obvious that chlorpromazine has more than one action on enzyme systems. It is not, however, a non-specific enzyme inhibitor. Rybina has shown that the rate of breakdown of thiamine phosphates is not affected by chlorpromazine, while, according to Ostroukhova, prolonged cooling considerably affects thiamine phosphate breakdown.

Probably chlorpromazine decreases "compensatory" heat production by tissues, but does not abolish the function of cold receptors, which ceases only after hypothermia has supervened. The decrease in body temperature caused by sodium barbitone is probably due to another mechanism. The tissue dehydrogenases are activated as we showed that the tissues of anaesthetized animals are incapable of utilizing glycogen. This suggests that barbiturates have a specific action on the enzymes which catalyze the initial stages of carbohydrate utilization. This is in general agreement with Quastel (14), who explained the mechanism of barbiturates as due to dysfunction in the aerobic production of high energy compounds. However, we do not know whether barbiturates have an effect on the *breakdown* of high energy compounds.

The pharmacology of the barbiturates is different from that of the phenothiazines. A combination of chlorpromazine and sodium barbitone suppresses the body's response to cooling. Animals treated with these drugs do not increase their respiration when placed in a cooling chamber (Fig. 7, E, A, H). This is the experimental basis for the administration of chlorpromazine and sedatives when hypothermia is used surgically.

The "potentiating" effect of chlorpromazine on anaesthesia requires further experimental study.

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THE CARBOHYDRATE-PHOSPHORUS METABOLISM OF THE BRAIN DURING SUSPENDED ANIMATION UNDER HYPOTHERMIA AND AFTER SUBSEQUENT RESTORATION OF BODY FUNCTION

M. S. GAYEVSKAYA

Laboratory of Experimental Physiology of Resuscitation, Soviet Academy
of Medical Sciences, Moscow

SEVERAL authors (1-5) have shown that under artificially induced hypothermia, the central nervous system possesses an increased resistance to a temporary drop in the blood supply. In particular, Negovskii and Soboleva (6) established that a state of "clinical death"—apnoea, cardiac arrest and absence of eye reflexes can be maintained for an hour under hypothermia, after which the activity of the nervous system can be fully restored. Other authors (7-11) have shown that in anaesthetized animals under hypothermia, the oxygen consumption of the brain and whole body can fall by 60-80 per cent.

Nikulin (12) investigated the rate of labelled methionine incorporation into tissue proteins of animals under hypothermia, and found that it was only slightly depressed in heart muscle, but considerably so in the cerebellum.

We previously showed that the 5-6 min period of clinical "death" which the brain can survive at normal body temperature is closely related to the time for the energy reserves to be exhausted. Similarly, under hypothermia, the rate at which energy reserves are exhausted seems to determine the maximum period permitting subsequent revival. Under hypothermia exhaustion of energy reserves takes much longer than at normal body temperatures. Negovskii and Soboleva also suggest that revival from clinical death at normal body temperatures is more rapid than revival after hypothermia.

METHODS

Dogs of 7-12 kg, were injected with 0.4 ml of 2 per cent "Pantopan" and 0.1 ml of 1 per cent atropine/kg and 40 min later the femoral artery and vein were exposed under local anaesthetic. A cannula was inserted to record

arterial blood pressure and 0.2 per cent thiopentane was drip-fed into the vein to maintain superficial narcosis as assessed by loss of pain perception, a pupil width of 2–4 mm, and maintenance of eye reflexes. The animals were then placed on ice and covered with ice-filled bags which lowered the rectal temperature by 1°C every 10–12 min. When the temperature had fallen to 25°C they were bled from the artery until clinical death occurred. Note that in dogs dying from loss of blood at normal body temperature, eye reflexes cease before other activities, whereas under hypothermia they are the last activity to cease. Revival by placing on hot water bottles was performed by the method of Negovskii *et al.* (13). Cardiac fibrillation which often occurred during revival was prevented by the method of Gurvich (14) using condenser pulses. In most cases cooling had finished before the dogs were bled but in some cases not until after 20 min of clinical death. The skull was trepanned just before sampling the brain tissue. Where the tissue was sampled outside the period of clinical death it was frozen *in situ* with liquid nitrogen. Freezing of the tissue when sampled during hypothermia was subsequently found to be unnecessary, since enzymatic changes are very slow under these conditions. The tissue was scooped out with a sharp spoon and plunged into liquid nitrogen. This meant one could take successive samples from the same animal during the period of clinical death as well as one during revival. As far as possible, only cerebral grey matter was removed. The tissue was then analysed for glucose, glycogen and lactic acid by the method of Kerr (16); inorganic phosphate by precipitation with magnesium from the TCA filtrate; the labile phosphorus of ATP and ADP by precipitation from the TCA filtrate with mercuric acetate followed by “7 minute hydrolysis”, and phosphocreatine (CP) by the method of Alekseyeva (15).

RESULTS AND DISCUSSION

Before studying the effects of “clinical death”, we had to ensure that the processes of cooling and revival in the absence of bleeding did not affect the equilibrium of synthesis and breakdown of carbohydrate and phosphorus compounds. Four groups of experiments were therefore carried out and the results are shown in Table 1, which gives the amount of variation and mean values for the substances measured.

In the first group of experiments initial contents were measured and found to be similar to those reported by Kerr (16), Stone (17), Palladin (18), Richter (19), Alekseyeva (15) and Ahers, using similar techniques for measurement.

TABLE 1. THE INFLUENCE OF HYPOTHERMIA AND SUBSEQUENT WARMING UP OF NARCOTIZED DOGS UPON THE CONTENT OF COMPONENTS OF THE CARBOHYDRATE-PHOSPHORUS METABOLISM IN THE CEREBRAL CORTEX

(Calculated in mg % moist tissue)

Experimental conditions	ATP + ADP	Phospho-creatines	Inorganic phosphate	Sugar	Lactic acid
Original background narcosis					
average results of six experiments	14.3	8.4	4.8	119	21
Limit of variations	11.3-16.2	7.3-9.6	1.5-9.6	72-197	1.5-36
Cooled to 25°C					
average results of six experiments	14.2	8.1	3.0	91	28
Limit of variations	12.0-15.4	6.7-10.5	1.5-6.4	70-127	5.0-50
Cooled to 25°C					
average results from three experiments	12.5	9.3	5.5	115	29
Limit of variations	11.1-13.3	8.0-10.9	4.2-6.3	85-137	16-49
Cooling and subsequent warming up: average results from three experiments	15.3	7.7	1.5	68	21
Limit of variations	14.0-16.2	5.6-9.9	1.5-1.5	58-78	16-26

In the second group of experiments, the brain was frozen when the body temperature had dropped to 25°C. Table 1 shows that the levels remained similar to those in group I. One animal showed a slight increase in lactic acid (50 mg %) but since the glucose was also high (127 mg %) and CP 10.5 mg %, this increase was not due to hypoxia.

In the third group the brain was frozen after maintaining the body temperature at 25°C for 1 hr. This did not lead to appreciable changes in the initial contents of the compounds under study. It must be noted that when the body temperature was lowered to 25°C, and especially when maintained there, blood pressure fell to 60-70 mm Hg, breathing almost ceased and the colour of the arterial blood deepened. The fact that few changes occurred in the content of the substances measured indicates a lower requirement for oxygen by the brain during hypothermia.

In the last group of experiments, 3 dogs were cooled down to 25°C, maintained for 1 hr, then 2 of them warmed up to 37°C and the other one to 32°C, after which the brains were frozen. During revival cardiac activity improved, the blood pressure increased and breathing became deeper and

more frequent. Again no significant differences in the content of the substances measured could be noted. Hence hypothermia and subsequent revival alone do not appear to affect the levels of these compounds.

The results of experiments where a period of clinical "death" under hypothermia lasted for 8–15 min is given in Table 2. The ATP and ADP levels fell progressively up to 15 min and small quantities of CP were still present. (At normal body temperature, all the CP disappears if the blood supply is interrupted for a few seconds.) Elevated IP levels were higher than could be accounted for by breakdown of CP, ADP and ATP, suggesting release from other sources, such as phosphoproteins.

TABLE 2. CONTENT OF COMPONENTS OF THE CARBOHYDRATE-PHOSPHORUS METABOLISM IN THE CEREBRAL CORTEX AFTER CLINICAL DEATH LASTING 8–15 MIN AGAINST A BACKGROUND OF HYPOTHERMIA

(Calculated in mg % moist tissue)

No. of experiment	ATP+ADP	Phospho-creatine	Inorganic phosph-ate	Sugar	Glyco-gen	Lactic acid	Dura-tion of clin-ical death	Body temperature at the time the brain sample was collected (°C)
1	11.3	1.9	19.4	84	—	45	8 min	23
2	—	—	—	48	58	58	8 min 45 sec	23.8
3	9.2	2.2	25.0	92	84	86	15 min	24
4	4.2	Traces	30.8	110	—	110	15 min	24.3
5	6.3	1.5	30.0	31	51	42	15 min	25.1
10	4.8	0	24.8	32	33	58	15 min	24.5
20	5.0	0.4	—	67	74	37	15 min	24.3

Glucose and glycogen levels fell during the 8–15th min of clinical death in 3 out of 7 dogs but the lactic acid did not increase significantly. This fact could be explained in two ways. Either the substrate was oxidized aerobically to completion, or intermediates prior to lactic acid accumulated. Lactic acid did increase in 2 cases where the glucose and glycogen levels remained normal after 15 min of clinical death. The levels of these compounds during the 8–15th min of clinical death under hypothermia resemble the levels found in dogs dying at normal body temperature at the "terminal" or "agonal" stage (Schuster, (20) Gayevskaya, (21)).

Periods of "clinical death" lasting 30 min were studied in 15 animals (see Table 3). In 7 of them the brain was frozen *in situ*, in the rest brain

samples were scooped out with a sharp spoon and frozen. Table 3 shows that after 30 min of "clinical death" no more CP could be detected and the ADP and ATP levels had fallen by 60–80 per cent. The IP level was similar to that after 15 min of "clinical death".

TABLE 3. CONTENT OF COMPONENTS OF THE CARBOHYDRATE-PHOSPHORUS METABOLISM IN THE CEREBRAL CORTEX AFTER CLINICAL DEATH LASTING 30 MIN AGAINST A BACKGROUND OF HYPOTHERMIA
(Calculated in mg% moist tissue)

No. of experiment	ATP+ADP	Phospho-creatine	Inorganic phosphate	Sugar	Glycogen	Lactic acid	Duration of the process of dying	Body temperature at the time the brain sample was collected (°C)
6	2.7	0	27.0	30.5	—	80	14 min 43 sec	23
7	2.5	0	25.6	45.4	—	155	50 min 40 sec	19.5
8	3.1	0	27.0	133	59	218	13 min 12 sec	21.9
9	2.9	—	28.3	—	36	204	13 min 23 sec	23
10	2.7	0	24.4	18	18	85	9 min 05 sec	24
11	4.8	—	22.2	46	76	107	19 min 53 sec	22.8
12	3.0	—	15.0	67	47	159	16 min 37 sec	21.1
13	—	—	—	49	19	156	5 min 30 sec	23.3
14	—	—	—	83	95	195	10 min 20 sec	25
15	—	—	—	94	39	87	15 min 40 sec	23.8
3	—	—	—	53	30	92	12 min 55 sec	23.8
16	—	—	—	136	35	208	20 min 00 sec	23.2
17	—	—	—	83	—	123	13 min 10 sec	25.9
4	3.0	—	32.6	74	—	101	19 min 15 sec	24.1
5	4.9	—	32.6	35	16	98	10 min 55 sec	24.5

Carbohydrate levels showed very wide fluctuations. In half the animals they were little changed after 30 min of "clinical death", but in the rest they were considerably reduced. The glycogen level remained unchanged in 2 cases and fell in the rest. The high ratio of glucose to glycogen found in most animals suggested that glycogen was utilized more rapidly than glucose. Lactic acid rose 5–10 times the initial value, indicating that glycolysis was the major respiratory pathway for carbohydrates during "clinical death" under hypothermia, as well as during death at normal body temperature.

Neither the duration of hypothermia prior to the commencement of "clinical death", nor cooling the body below 25°C had any further effect on the levels of carbohydrate and phosphates found previously.

Summarizing our results, we can only say that in about 50 per cent of the animals, energy-rich substances were greatly reduced after 30 min "clinical death" under hypothermia; similar to the levels found after 5-6 min of "clinical death" at normal body temperature.

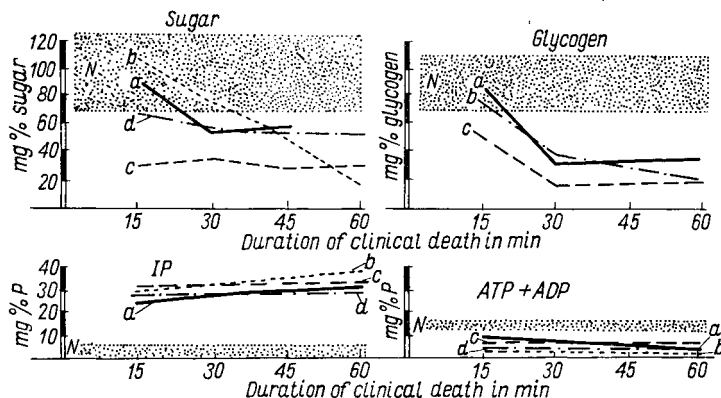


FIG. 1. Changes in the content of components of the carbohydrate-phosphorus metabolism during clinical death: N—limit of variation of the component in question in the original state; a—experiment No. 1; b—experiment No. 2; c—experiment No. 3; d—experiment No. 4.

Further studies were made on tissue samples from animals after the 15th, 30th, 45th and 60th min of "clinical death". Successive sampling from the same brain led to small errors, the range of which is given in Fig. 1. This Figure shows that the major changes in levels of substances measured occurred within 30 min of "clinical death". Glycogen and glucose levels, normal between the 15th and 30th min of "clinical death", almost disappeared between the 30th and 60th min. In this latter period, ADP and ATP decreased only slightly.

Hence prolongation of "clinical death" beyond one hour would lead to irreversible changes in the nervous system and prevent restoration of its activity, so revival was always started after 30 min of "clinical death".

In these revival experiments (see Table 4), one animal (No. 14) took $2\frac{1}{2}$ min to restore regular cardiac activity, due to repeated fibrillation. During the next 7 min, blood pressure was unsteady, varying between 70-120 mm Hg and remained at 50-60 mm after 10 min of revival, when the brain was frozen and sampled. Within 8 min the levels of ATP, ADP, CP and IP had returned to within the normal initial range.

Changes in the levels of carbon compounds during revival were as follows: glycogen, which at the 30th min of "clinical death" was still within

TABLE 4. CHANGES IN THE CONTENT OF COMPONENTS OF THE CARBOHYDRATE PHOSPHORUS METABOLISM IN THE CEREBRAL CORTEX DURING THE RESUSCITATION OF DOGS AFTER CLINICAL DEATH LASTING 30 MIN UNDER CONDITIONS OF HYPOTHERMIA
(Calculated in mg% for moist tissue)

No. of experiment	ATP+ADP	Creatin phosphate	Inorganic phosphate	Sugar	Glycogen	Lactic acid	Blood sugar	Time elapsed since the beginning of resuscitation (min)	Body temperature at the time the brain sample was collected (°C)
2	10.5	4.3	7.1	76	27	61	250	9	25
14	12.0	2.5	11.3	116	55	317	234	10	25.8
17	9.0	5.6	5.9	83	41	130	247	16	26
15	11.9	5.1	7.3	155	42	84	235	45	27
13	10.5	10.5	4.8	110	23	50	105	80	29
18	12.7	6.7	7.1	139	12	20	147	76	30

the initial range (95 mg%) almost doubled: glucose rose from 83 to 116 mg%, and the lactate which had been abnormally high during "clinical death" (195 mg%) rose even higher. The blood sugar rose from 140 mg% to 274 mg% (see similar values found by Gayevskaya (22)). This hyperglycaemia may well be responsible for the aerobic glycolysis and lactic acid accumulation.

In 2 animals (Nos. 2 and 17), after restoration of cardiac activity, the blood pressure was optimally high and stable at 150–200 mm Hg. Eight and 14 min respectively after heart beat was resumed the brain was frozen and sampled. As in animal No. 14, the ATP, ADP, CP and Pi levels reached the initial values during the revival period. However, the CP levels were higher and lactic acid lower than No. 14, indicating better oxidative metabolism resulting from a higher blood pressure. Again tissue glucose levels were normal in spite of hyperglycaemia.

These data show that within 8–14 min of restored blood supply the brain resynthesizes its energy-rich substrates in the presence of intensive aerobic glycolysis.

In 3 subsequent animals the body temperature was raised slowly during revival under artificial respiration. When natural breathing started, the brain was frozen for analysis, at which point the body temperature was 27–30°C. Responses were as follows:

Dog No. 13: natural breathing commenced 41 min 10 sec after revival began and the brain frozen after 80 min: blood sugar normal.

Dog No. 15: Natural breathing began after 45 min and freezing of the brain after 50 min 45 sec: hyperglycaemic.

Dog No. 18: Natural breathing began after 23 min, brain frozen after 76 min: blood sugar normal.

Artificial respiration had to be continued in dogs Nos. 13 and 15, to maintain adequate blood pressure.

By the time the brain was frozen, in all 3 animals lactic acid levels had reached normal values, as did all the other substances except glycogen. This remained at a low level and its final restoration after "clinical death" has not been investigated.

This pattern of changes was similar to the one obtained during revival of dogs after "clinical death" at normal body temperature. The work of Engelhardt (23), Yel'tsina (24) and Lisovskaya (25) on aerobic glycolysis prompts us to assert that rapid restoration of energy-rich compounds during revival is due to intensive activity of this process.

Since during revival more IP disappeared than ADP, ATP and CP appeared, we conclude that other phosphorus compounds (e.g. phosphoproteins) were being synthesized.

Our general conclusion is that hypothermia slows the rate of breakdown of energy-rich compounds during "clinical death" (to about one-tenth) but does not retard their resynthesis during revival.

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CHANGES IN THE CONTENT OF ENERGY-RICH PHOSPHATES OF THE BRAIN DURING HYPOTHERMIA

YA. I. VEKSLER

Department of Biochemistry, Rostov University, and Experimental Laboratory,
S.K.V.O., Rostov-on-Don

RECENTLY hypothermia has been employed in major surgery and treatment of some illnesses. Most authors believe that increased survival due to hypothermia results from the body's greater resistance to injury, and lowered oxygen requirement. Little is known about the metabolic changes in general hypothermia, particularly in the brain, and work on the changes in energy-rich phosphate content has produced contradictory results (1-13). And so we decided to re-investigate the following phosphates: inorganic phosphate (IP), adenosine-triphosphate (ATP) and creatine-phosphate (CP).

METHOD

Two series of white rats were used; in one hypothermia with narcosis was used, and in the other hypothermia alone. For controls narcotized animals without hypothermia were used, and animals clamped into the stands. Hypothermia was produced by circulating cold water through poultices at 4.5°C using a special apparatus. The body temperature was thus evenly lowered to 20-21°C within 60-65 min. Measurements were made at 35°, 30°, 25° and 20°C, corresponding to 15-20, 30-35, 45-50 and 60-65 min after commencement of cooling. Temperatures were measured with small mercury thermometers inserted 4.5-5 cm rectally. 15-20 min before cooling, the rats were fixed in a stand with their heads usually remaining free. The animals were acclimatized to the experimental conditions for several days so that they behaved more quietly during handling. By means of a knife mounted on the stand, they could be quickly decapitated, the head falling into a vessel of liquid air. Freezing the whole rat has also been tried but this method gave low values for the ATP and CP content which was apparently due to brief but powerful excitation on contact with

liquid air (Palladin 14-15). Also, in experiments involving hypothermia, the animals had to be freed from the cooling poultices before they could be immersed in liquid air and this would excite them. After scraping the dura and blood vessels from the frozen brain, it was ground in liquid air and the resultant powder transferred to a chilled vessel of 2.5 per cent TCA, the volume of acid containing about 100 mg/ml of tissue. Further preparation was carried out in the cold room and the following substances measured in the TCA filtrate.

(1) ATP; by precipitation with mercuric acetate or by mild hydrolysis to release IP (no difference between these 2 methods was found).

(2) CP; from the amount of IP released by molybdate reagent.

(3) IP; by precipitation with magnesium salts. (1 per cent ascorbic acid was used to intensify the colour in the phosphate determinations).

The levels of phosphorus compounds in resting controls varied within the following range (18) (values in mg %).

	IP	ATP	CP
Mean values	10.1	162	10.8
(10-15 experiments)			
Variations	± 1.35	± 1.15	± 2.05

In a second series of experiments, the levels of phosphorus compounds during the onset of hypothermia without anaesthesia were measured. Results of these experiments appear at first to be rather scattered, particularly in the initial stages of hypothermia. Different values could be obtained in different individuals at the same stage. For example, the ATP and CP would decrease while the IP rose in one, while in another no changes occurred at all, or else in the reverse relationship. If, for instance, body temperature was decreased to 35°C, the content of labile phosphorus compounds varied within the following ranges:

ATP	CP	IP
16.8-20.6	11.2-18.7	12.2-17.8
10.2-12.7	7.6-14.2	6.4-10.2 mg %

If the temperature was decreased to 30°C, the following values of pairs were found:

ATP	CP	IP
16.4-27.5	13.1-22.3	12.7-15.6
8.8-13.5	8.2-14.8	5.7- 8.4 mg %

At the second stage of hypothermia (20-30°C) variation was much less.

The spread of the results may have been due to the way in which the animals reacted to the cooling. There appeared to be some relation between

phosphate content, stage of hypothermia and the physiological state of the animal. If hypothermia was carried out without anaesthesia, the rats responded to cooling from 30 to 35°C by generalized agitation, convulsive movements, shrieks, a spreading and finally general muscular tremor and a very sensitive response to stimuli. Later on, as the body temperature fell below 30°C, motor activity gradually decreased, the tremor subsided and the animals became unconscious, hardly reacting to external stimuli. However, in another group of animals similarly treated, the general inhibition developed rapidly at the beginning of cooling and became progressively deeper as the temperature fell. The existence of these two types of response to hypothermia was also seen in the phosphorus compounds of the brain. (Table 1 and Fig. 1).

TABLE 1. MEAN CONTENT OF PHOSPHORUS COMPOUNDS IN THE BRAIN OF RATS (in mg%)
EXPERIMENTS WITH HYPOTHERMIA

Degree of hypothermia	State of animal	Inorganic phosphate	ATP	Creatine phosphate
35°	Marked excitation	16.2	11.6	9.8
	Inhibition	8.1	19.7	15.9
30°	Marked excitation	14.1	11.7	10.2
	Inhibition	6.8	20.2	17.7
25°		6.0	22.8	22.2
20°		5.7	24.6	21.9

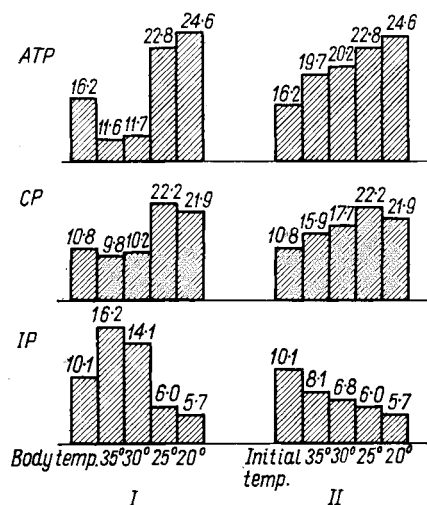


FIG. 1. Pattern of changes in the high-energy phosphorus compounds of the brain in a state of hypothermia (in mg %): I—1st group; II—2nd group.

In animals reacting to being cooled to 30°C by severe excitation, ATP decreased to an average of 11.65 mg%, CP to an average of 10.0 mg% and IP rose to 14.1 mg%. As temperature fell below 30°C, the ATP and CP levels rose and IP decreased. In the group of animals which did not respond at first to hypothermia by motor agitation, there was no initial decrease in ATP or CP level, but they continually increased as hypothermia proceeded, with a corresponding decrease in IP.

Since the clamping procedure necessary to restrain the animals during manipulation might have caused chemical changes in the brain due to excitation, prior to decapitation, control animals were clamped for the same periods as the anaesthetized or cooled animals.

The results of these experiments are given in Table 2 and show that clamping usually causes no decrease in the ATP and CP content, but if occurring, there is a slight decrease during the first few minutes. Either clamping the head as well or leaving it free gives the same results. Thus the changes one finds in ATP, CP and IP content are due to hypothermia alone (see 16-21).

TABLE 2. MEAN QUANTITY OF PHOSPHORUS-CONTAINING COMPOUNDS IN THE BRAIN OF RATS; CONTROL EXPERIMENTS
(in mg %)

Duration of fixation (min)	IP	ATP	CP
15-20	12.3	13.7	9.9
30-35	13.2	13.5	10.2
45-50	11.6	15.8	9.3
60-65	10.6	15.4	10.2

HYPOTHERMIA UNDER NARCOSIS

Twenty to twenty-five minutes before hypothermia was started, 0.1 g per kg sodium amylobarbitone was injected into the rat which fell into a normal depth of sleep for 3-4 hr. The drug alone produced a fall in rectal temperature of 2-3°C in 30 min and a further 2.0°C after 90 min, but artificial cooling proceeded at the same rate as without narcosis. In control, narcotized animals, killed 40-45, 55-60, 70-75 and 85-90 min after amylobarbitone injection, the ATP and CP contents scarcely increased above the normal for anaesthetized animals (Fig. 2).

Hypothermia under narcosis does give a characteristic pattern of changes or ATP, CP and IP levels. ATP and CP gradually increase from the very

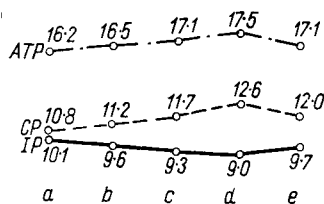


FIG. 2. Content of phosphorus-containing compounds in the brain of rats in a state of narcotic sleep (in mg %). Time elapsed after administration of sodium amylobarbitone: a—before the narcosis; b—40–45 min; c—55–60 min; d—70–75 min; e—85–90 min after injection of sodium amylobarbitone.

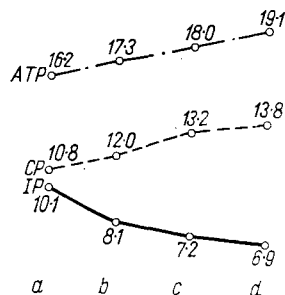


FIG. 3. Pattern of changes in the content of phosphorus-containing compounds in the brain of rats in a state of hypothermia against a background of general narcosis (in mg %). Body temperature: a—initial temperature; b—30°C; c—25°C; d—20°C.

beginning of hypothermia as IP decreases (see Fig. 3). The rate of increase of ATP and CP as the body temperature falls, however, is lower than with narcosis alone, suggesting that it has a depressing effect on the resynthesis of these substances. For example, in animals cooled without narcosis, the ATP and CP contents at 20°C are 24.6 and 21.9 mg% respectively, whereas with narcosis, the values are 19.1 and 13.8 mg%. In narcosis without hypothermia the values are 7.1 and 12.0 mg%. In cases where in spite of amylobarbitone injection, sleep was restless and punctuated by tremor, ATP and CP content was slightly lowered.

SUMMARY AND CONCLUSIONS

In rats subjected to hypothermia, marked changes in the levels of ATP, CP and IP of the brain occur in two stages. At first ATP and CP levels fall, then increase as the body temperature continues to fall. The latter increase is presumably due to well-maintained resynthesis during lowered consumption of these energy-rich phosphates resulting from hypothermia. This conclusion is based on the observation that hypothermia alone leads to the highest ATP and CP contents; hypothermia under narcosis gives slightly lower values; and narcosis alone gives values only slightly above untreated animals. Narcosis did, however, lessen the tendency for ATP and CP to decrease during initial hypothermia in rats that responded at this stage with motor agitation, but in the second stage appeared to inhibit resynthesis of ATP and CP.

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THE EFFECT OF X-RAYS ON THE METABOLISM OF NERVOUS TISSUE

P. F. MINAYEV AND R. I. SKVORTSOVA

Institute of Biological Physics, Soviet Academy of Sciences, Moscow

WE HAVE previously shown that electrical stimulation of the cerebral cortex causes a breakdown of high-energy phosphorus compounds, which are restored when stimulation is stopped (1). We have also established a relationship between the functional state of the nervous system and its metabolism after ionizing radiation. By using local irradiation of the brain—a method we developed—it was possible to show that the central nervous system, particularly its highest part, is extremely sensitive to irradiation, while at the same time showing a considerable resistance to it (2–7).

By the term sensitivity we mean the capacity to react to very small doses of ionizing radiation. By resistance we mean the ability to recover from relatively high doses. The sensitivity and resistance of the nervous system to ionizing radiation are complementary characteristics of its activity under normal and pathological conditions.

What metabolic features are responsible for the sensitivity and resistance of the nervous system? Our studies led us to suspect carbohydrate metabolism. Local irradiation of the brain with small doses of X-rays causes rapid changes in glucose oxidation and higher nervous activity (8). More intense radiation causes more profound changes. The recovery of higher nervous activity after relatively large doses induced us to investigate those metabolic processes which might be responsible for the subsequent development of resistance to radiation. Previous investigations had shown that nucleic acids were relatively stable to the local action of several thousand roentgens on the brain, though there was a slight decrease in the desoxyribonucleic acid (9). Estimates of total nucleic acids did not always reveal these small changes.

In view of the importance of the glutamic acid-glutamine-ammonia system (10–21), it was of interest to establish the effect of local irradiation of various parts of the brain on these substances, and this paper reports

experiments on changes in the amino acids and ammonia in the brain induced by ionizing radiation.

The experiments were carried out on 250–300 g guinea pigs, mostly males, fed on the usual laboratory diet. The animals were irradiated with an RUM-3 apparatus, with a current of $10\mu\text{A}$ at 180 kV, filter 0.5 c, 27 cm from the animal's head. In most experiments the cerebellum had been locally exposed to from 500 to 16,000 r, but in some, whole body irradiation was carried out. The cerebellum and cerebral hemispheres were analysed after decapitation, and freezing in liquid nitrogen. The brain was weighed and homogenized in 5 vol. of distilled water. The proteins were precipitated by 10 vol. of 95 per cent ethanol, and the filtrate was mixed with 3 vol. of chloroform and left overnight to separate. The aqueous residue was separated from the alcohol-chloroform mixture and evaporated to dryness. The sediment was dissolved in a volume of distilled water equal to that of the fresh tissue. A sample corresponding to 10 to 20 mg fresh weight was put on to chromatographic paper. The free amino acids were estimated by ascending chromatography using butyl alcohol, formic acid, and water, in the proportions 75:15:10. For the quantitative estimation of amino acids we used the method of Giri; the error of this method was 2 to 5 per cent. Ammonia was also estimated after local irradiation of the cerebellum with a dose of 16,000 r, and results are shown in Table 1. Estimations were carried out 1 to 2 hr after radiation. By this time the animals showed the classical picture of cerebellar disorder. They did not usually survive this dose for more than 24 hr.

TABLE 1. CONTENT OF GLUTAMIC ACID AND GLUTAMINE IN THE BRAIN OF GUINEA PIGS, THE CEREBELLA OF WHICH WERE EXPOSED TO A RADIATION DOSE OF 16,000 r.
(Average results and extreme values, in mg %)

Part of the brain	Glutamic acid	Glutamine
Cerebellum of the experimental animal	89 (76–95)	62 (50–70)
Cerebellum of a control animal	98 (90–102)	54 (50–57)
Cerebral hemisphere of the experimental animal	98 (75–110)	75 (72–76)
Cerebral hemisphere of a control animal	110 (97–122)	61 (60–61)

Table 1 shows that 16,000 r irradiation lowers the glutamic acid in the cerebellum and the cerebral hemisphere. This decrease is small in relation to the 9 mg % in the cerebellum, and the 12 mg % in the cerebral hemi-

spheres, and it was not constant in all experiments. Chromatographic estimation of the glutamine carried out at the same time as the glutamic acid showed that following this irradiation, glutamine increases in the cerebellum and cerebral hemispheres by 8 to 14 mg % (Table 1). This increase, like the decrease of glutamic acid, is small and also occurs in the adjacent parts of the cerebral hemispheres.

Although the complementary changes in glutamine and glutamic acid did not always occur, whenever they did they bore this relationship. Furthermore, when they occurred in the irradiated cerebellum, they also occurred in the cerebral hemispheres, probably because the initial effect of rays is over the whole nervous system and only later becomes localized at the site of the stimulus. The ammonia content was unchanged.

The effect of X-rays on the cerebellum was also studied with doses of 500, 9000, 10,000, and 12,000 r (Table 2). The cerebral hemispheres and cerebellum were each investigated on the 3rd to 5th day after irradiation. A dose of 500 r had no effect on the content of glutamic acid, glutamine or ammonia. Doses of 9000, 10,000, and 12,000 r caused cerebellar dysfunction, a very slight decrease in glutamic acid, and a corresponding increase in glutamine. Under these conditions ammonia was unchanged.

TABLE 2. THE CONTENT OF GLUTAMIC ACID AND GLUTAMINE IN THE BRAIN OF GUINEA PIGS, THE CEREBELLUM OF WHICH WAS EXPOSED TO RADIATION IN A DOSE OF 9000–12,000 r.
(Average results and the extreme values in mg %)

Part of the brain	Glutamic acid	Glutamine
Cerebellum of the experimental animal	90 (82–97)	60 (60–61)
Cerebellum of a control animal	96 (80–110)	50 (48–51)
Cerebral hemisphere of the experimental animal	109 (95–120)	58 (50–66)
Cerebral hemisphere of a control animal	115 (103–130)	53 (47–66)

Besides studying the effects of local irradiation on the cerebellum, total irradiation of the whole animal was carried out, using doses of 500, 1000 and 2000 r (Table 3). On the 4th to 5th day of this treatment with 500 r, virtually no changes could be found in the content of glutamic acid. On the 3rd to 4th day after irradiation with 1000 and 2000 r—2 and 4 times the lethal doses, respectively—the glutamic acid decreased by 20–38 per cent with a simultaneous decrease in the content of glutamine; in this case the ammonia was increased.

TABLE 3. CONTENT OF GLUTAMIC ACID AND GLUTAMINE IN THE BRAIN OF GUINEA PIGS EXPOSED TO TOTAL BODY RADIATION; AVERAGE RESULTS (in brackets the extreme values in mg %)

Part of the brain	Total body radiation in a dose of 1000 r	Total body radiation in a dose of 2000 r	
	Glutamic acid	Glutamic acid	Glutamine
Cerebellum of the experimental animal	82 (80-84)	72 (71-75)	44
Cerebellum of a control animal	103 (100-105)	92 (81-100)	51
Cerebral hemispheres of the experimental animal	83 (76-90)	80 (75-85)	50
Cerebral hemispheres of a control animal	110 (104-114)	118 (115-120)	65

SUMMARY

1. Local irradiation of the cerebellum by 9000, 12,000 and 16,000 r causes very slight changes in the content of glutamic acid and glutamine in the brains of guinea pigs. The glutamic acid decreases 6-12 mg % and the glutamine increases by 5-14 mg %. These changes are very slight and we conclude that the glutamic acid-glutamine system is rather stable. This may explain the resistance of the nervous system to ionizing radiation.

2. The ammonia is unchanged by local irradiation of the cerebellum.

3. Total irradiation of guinea pigs with a dose 2 to 4 times lethal doses causes irreversible disorders in all systems of the body and decreases the glutamic acid in the brain by 20-38 mg %. There is a simultaneous decrease in the glutamine and a very slight increase in the amount of ammonia.

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THE EFFECT OF β -RADIATION ON THE PHOSPHORUS METABOLISM OF THE BRAIN

G. YA. GORODISSKAYA AND O. N. BARMINA

Department of Biochemistry, Kirov Medical Institute, Gor'kii

THE increasing use of radio isotopes and their effects on human beings led us to study the effects of β -radiation on the central nervous system to which it appears to be particularly sensitive (1-4). The few data available indicate that various forms of radiation have little or no effect on experimental animals.

We have previously shown that general exposure of white rats to X-rays produces within a few hours considerable changes in the rate of incorporation of radioactive phosphorus ^{32}P into various tissues including the brain. The rate of incorporation of ^{32}P into phosphorus-containing compounds of the brain alters with the radiation dose and length of exposure (5, 6).

Here we have studied the effects of internal β -radiation on phosphorus metabolism and on several proteins. Experiments were performed on adult white rats injected subcutaneously with ^{32}P , and decapitated 2 hr later. The ^{32}P not only served as an "internal" source of β -radiation but was also used to trace its uptake into inorganic and organic phosphorus compounds in the brain. The phosphorus was subsequently estimated colorimetrically and its radioactivity measured. The relative specific activity was given as the counts per minute per mg P divided by the total amount of injected ^{32}P expressed as $\mu\text{c}/\text{gram}$ body weight.

These studies showed that within 2 hr of injection of the ^{32}P considerable changes occurred in the rates of incorporation; these were closely related to the dose of internal β -radiation.

Figure 1 shows that the changes in the RSA of the inorganic phosphorus are proportional to the amount of ^{32}P injected. (The Roman numerals in this figure as well as all subsequent figures denote the groups of rats according to their ^{32}P dosage per gram body weight. I = $10 \cdot \mu\text{c}$, II = $1 \cdot 0 \mu\text{c}$, III = $2 \cdot 0 \mu\text{c}$, IV = $4 \cdot 0 \mu\text{c}$, V = $6\text{--}10 \mu\text{c}$.) With a ^{32}P dose of $1 \cdot 0 \mu\text{c}/\text{g}$ or more the RSA of the inorganic phosphorus shows a marked decrease. As the dose increases and the absolute rate of incorporation of the isotope

from the blood into the brain increases, the relative rate of incorporation shows a decrease. This can be explained by a decrease in the permeability of the cerebral vessels to inorganic phosphate, which takes place within the first two hours after the injection of ^{32}P and may possibly be a mechanism which protects the brain to some degree against the effects of radiation.

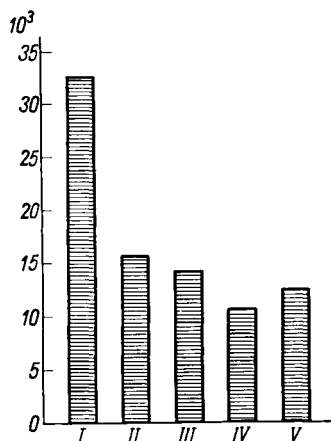


FIG. 1. Relative specific activity of inorganic phosphate in the brain of white rats 2 hr after subcutaneous administration of various doses of a radioactive isotope of phosphorus.

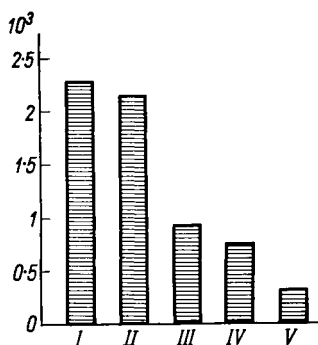


FIG. 2. Relative specific activity of phosphorus in organic compounds in the brain of white rats 2 hr after subcutaneous administration of various doses of radioactive isotopes of phosphorus.

The idea that vascular permeability decreases due to the internal β -radiation seems to be at variance with the findings of other authors (7). It also seems to contradict the finding from our own laboratory by Simonov (8) that hyaluronidase activity of eye tissue increases after generalized exposure to X-rays. However the conditions of our experiments were completely different in that the radiation was not from an external source but emanated from within the body, and the experiment lasting for only 2 hr resulted in a very low total dose of radiation.

In view of our theory of the "protective" effect of decreased vascular permeability following exposure to radiation, it is interesting that Mogilnitskii noted a decrease in the permeability of the tissue barriers after very small doses of radiation.

It is very difficult to say whether the high RSA of inorganic phosphate in the brain following administration of $0.1 \mu\text{C/g}$ of ^{32}P should be interpreted

as an increased or a normal permeability, as one cannot study the relative rate of entry of phosphate into the brain from the blood without a radioactive tracer, and we have shown that even small doses of ^{32}P influence vascular permeability.

As the amount of ^{32}P injected into the animal increases, the RSA of the inorganic and organic phosphorus decreases (see Fig. 2) again probably due to decreased permeability.

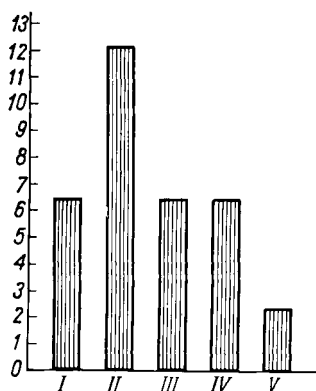


FIG. 3. The specific activity of phosphorus in organic compounds in relation to the specific activity of the inorganic phosphate in the brain 2 hr after administration of various doses of radioactive isotopes of phosphorus.

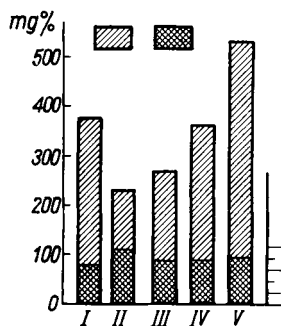


FIG. 4. The influence of various doses of radioactive isotope of phosphorus upon the phosphorus content in the organic compounds in the brain (1) and inorganic phosphate (2).

To achieve greater accuracy the specific activity of the organic phosphorus compound was referred not to the dose of injected ^{32}P but to the specific activity of the inorganic phosphate of the brain, since it is this phosphorus which is used directly in the synthesis of organic phosphorus compounds. These figures are given in Fig. 3, which show the changes in the rates of incorporation of inorganic phosphate from the blood into the brain and may indicate the degree to which inorganic phosphate participates in the synthesis of organic compounds. The large increase in the RSA of organic phosphorus following the injection of $1.0 \mu\text{C/g}$ of ^{32}P compared with the small increase observed after $0.1 \mu\text{C/g}$ suggests an increased phosphorus turnover in the brain. Here again, when assessing the RSA of inorganic phosphate it is hard to say whether the rate of incorporation of radioactive phosphate remains at a normal level after the injection of a minimal dose, or whether this rate is slightly increased. A considerable fall in the RSA of

organic compounds after injecting a dose of 6–10 $\mu\text{c/g}$ would indicate a reduced phosphorus turnover.

Comparison of the values for the actual inorganic and organic phosphorus contents of the brain with the rate of incorporation of phosphorus into these fractions confirms the conclusions mentioned previously (see also Figs. 3 and 4).

If the dose of ^{32}P is increased up to 1.0 $\mu\text{c/g}$ an increased phosphorus turnover is noted, accompanied by a considerable increase in the breakdown rate of organic phosphorus compounds. This is shown by the increase in the RSA of the organic phosphorus compounds a decrease in their content, with an increased inorganic phosphorus content.

If the dose of ^{32}P is increased to 2–4 $\mu\text{c/g}$ the rates of turnover and breakdown of organic phosphorus compounds gradually slow down to the values obtaining in cases where only 0.1 $\mu\text{c/g}$ have been injected. The decrease in these values should not be regarded as a return to normal conditions since on increasing the dose still further to 6–10 $\mu\text{c/g}$ the total phosphorus turnover is greatly reduced and inorganic phosphorus increases.

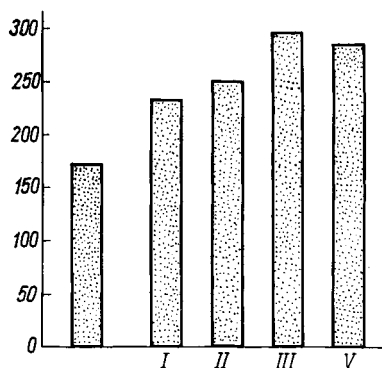


FIG. 5. The influence of various doses of radioactive isotope of phosphorus upon the uptake of ^{35}S -labelled methionine into the proteins of the brain of white rats.

The effects of internal β -radiation on the rate of incorporation of ^{35}S into cerebral proteins were also studied. ^{35}S -labelled methionine was injected subcutaneously at a dose of 0.28 $\mu\text{c/g}$ simultaneously with ^{32}P which acted as a source of β -radiation. After five hours the animals were decapitated and the radioactivity of the cerebral proteins was measured repeatedly at intervals of 12 days on 10 mg samples. This enabled us to differentiate the activity of the ^{35}S and ^{32}P . The results given in Fig. 5 show that after 5 hr exposure to radiation there was an increase in the rate of ^{35}S incorpo-

ration into proteins and this effect was enhanced possibly by increasing the radiation dose.

Our data thus show that exposure to internal β -radiation leads to marked changes in the phosphorus and protein metabolism of the brain within two to five hours. In spite of the small radiation dose employed in these experiments chemical changes in the brain can be sufficiently accurately detected. This can be explained by the particular characteristic of internal as compared with external radiation, and also the fact that we used radioactive phosphorus as our source of β -radiation. Since inorganic phosphorus participates in so many biochemical processes it can be readily understood how rapidly and far reaching its radioactive effects, as an isotope, would manifest themselves.

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THE INFLUENCE OF IONIZING RADIATION ON THE METABOLISM OF BRAIN TISSUE

L. S. CHERKASOVA, B. M. SOSINA, V. G. KARAVAYEV, V. G. REMBERGER
V. A. KUKUSHKINA AND M. I. KOZAK

Biochemistry Laboratory, Institute of Physiology, Belorussian Academy of Sciences; Department of Radiology, Belorussian Post-graduate Medical School; and Department of Radiology, Minsk Medical Institute

DEVELOPMENTS in nuclear physics and the widespread use of radioactive tracers in medicine have made the study of the effects of ionizing radiation of considerable importance. Studies of their biological effects were started soon after the discovery of radium. In 1896 Tarkhanov described the effect of X-rays on the nervous system (9), and in 1903-4 Goldberg (1) and London (6) made neurological observations on mice exposed to radiation. Lebedinskii recently extensively reviewed the reaction of the nervous system to radiation and the symptomatology of radiation sickness, at the Geneva Conference (5).

While there are extensive pathological data on the radiosensitivity of the central nervous system, there are only a few papers on metabolic disorders in the brain in radiation sickness. Sub-lethal and lethal doses of X-rays have been shown to have no effect on the nucleic acids of the brain (7) or the rate of incorporation of labelled methionine into brain protein (3). Gorodisskaya detected changes in the rate of phosphorus metabolism in the brain depending upon the dose and time after irradiation (2). She showed a considerable decrease in the rate of incorporation of ^{32}P into the phosphoproteins and lipids. In the cerebral hemispheres of animals, radiation caused an inhibition of glucose oxidation (8).

At the height of an attack of radiation sickness, changes take place in the nucleic acid metabolism in nervous tissue. In the spinal cord and spinal ganglion cells the ribonucleic acid decreases; in the dorsal horns of the spinal cord and in the cerebral cortex, however, the ribonucleic acid usually increases. After irradiation brain glycolysis becomes more intense, and cerebral hypoxia develops (4).

We were interested in the effects of penetrating ionizing radiation on the carbohydrate and phosphate metabolism of the cerebral hemispheres. We studied the levels and rate of turnover of creatine and adenosine phosphates, and the glycogen content of the brain, and we also examined the glycoproteins and glycolipids.

METHODS

White rats of 175–300 g were exposed to total body irradiation with X-rays or to gamma radiation with radioactive cobalt (^{60}Co). The doses chosen (600–700 r) caused acute radiation sickness. Of these animals, 70 per cent survived, and the natural history of their illness could be studied. When the labile phosphates were studied, the rats were irradiated with an RUM-3 apparatus, with 165 kV, a current of 5 μA , with no filter, at a skin focus distance of 30 cm for 16 min at 38 r per min, giving a total radiation of 600 r. When glycogen was studied, the animals were irradiated with a GUT-Co-400-1 apparatus, under the following conditions: no tubes, skin focus distance 30 cm, 25 r per min for 28 min, giving a total irradiation of 700 r.

Estimations were carried out for 30 days after these treatments, at various times during the radiation sickness. The clinical picture was as follows: weight loss, leucopenia and anaemia, followed by death. For the first 2–3 days after irradiation the rats were quiet, languid, anorexic, their fur became roughened and they had diarrhoea. After 5 or 6 days they became more lively and began to eat. About the 12th–15th days they became aggressive, lost weight, and their stools became semi-liquid. From the 16th–20th day their fur began to fall out and they gradually became hairless. The loss of weight continued up to the 30th–40th day, but then their state improved and they gradually regained weight (Fig. 1).

The rats developed leucopenia 10–12 days after radiation but recovered by the 15th–20th day (Fig. 2). From the 25th–26th days the number of white cells in the blood rose considerably and remained at a high level up to the 40th day. Changes in the labile phosphates of the brain are shown in Table 1.

In the phosphorus turnover experiments, the animals were injected subcutaneously with 0.1 $\mu\text{C/g}$ radioactive phosphorus in phosphate buffer on the day of P-turnover measurement, and 30 min later were killed by immersion in liquid air. Table 1 shows that in the first 7 days after radiation there is a marked fall in brain creatine phosphate. On the 4th day when the fall is most marked, the rate of incorporation of ^{32}P into creatine phosphate is also depressed, its relative activity being only 0.69 compared

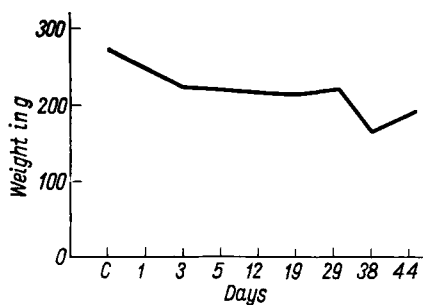


FIG. 1. The influence of radiation upon the animal's weight. C—control.

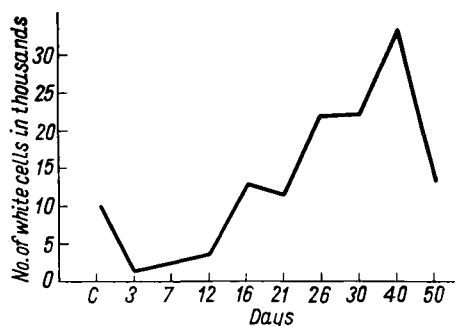


FIG. 2. The influence of radiation upon the white cell count in the blood. C—control.

TABLE 1. INFLUENCE OF EXPOSURE OF THE TOTAL BODY TO X-RAYS UPON THE CONTENT AND TURNOVER RATE OF CREATINE PHOSPHATE AND ADENOSINETRIPHOSPHATE ACID IN THE CEREBRAL HEMISPHERES OF RATS

Time elapsed after radiation (days)	No. of animals	Phosphorus (mg %)		Relative activity of creatine phosphate	Relative activity of ATP
		creatine phosphate	ATP		
Control	14	6.6	9.9	0.83	1.52
2	12	4.7	11.3	0.98	0.62
4	8	4.5	10.3	0.69	0.59
7	6	5.2	6.4	1.80	1.40
10	7	10.8	7.8	0.68	3.11
15	6	7.5	13.7	0.84	1.57
20	6	9.1	6.6	0.86	0.38
24	6	6.7	9.0	0.66	0.44
30	8	6.5	5.6	1.75	0.33
45	6	8.0	20.2	0.73	0.65

with 0.83 in the controls. On the 7th day the ^{32}P incorporation begins to increase, and by the 10th day exceeds the level in control animals. Between the 10th and 20th days the creatine phosphate remains at a high level and returns to normal about the 24th–30th day.

The brain ATP is raised 4 days after irradiation, but the low rate of ^{32}P incorporation into ATP shows that ATP metabolism is depressed. By the 7th day the ATP in the brain decreases to only one-third but the ^{32}P incorporation into ATP becomes normal at about double the rate found on the 10th day of the illness. On the 15th day after radiation the brain ATP is higher than normal while its relative activity is normal. On the 20–30th day the ATP is lower than the controls and the ^{32}P incorporation is decreased by 4–5 times compared with healthy controls. These findings indicate considerable disorders of energy metabolism in the 3rd period of the illness—that period during which the clinical picture is well marked. About the 5th day, in the period of recovery, the creatine phosphate and ATP in the brain show a considerable increase, the low value of relative activity showing that ATP metabolism has not returned to normal.

TABLE 2. THE INFLUENCE OF EXPOSURE TO RADIATION WITH ^{60}Co UPON THE GLYCOGEN FRACTIONS IN THE CEREBRAL HEMISPHERES

Time elapsed after radiation (days)	No. of animals	Glycogen (mg %)			
		Total glycogen	Free glycogen	Glycogen bound to soluble proteins	Glycogen bound to insoluble proteins
Control	32	124.1	22.1	63.4	38.6
1	4	103.7	0	73.1	30.6
2	4	134.1	25.8	73.8	34.5
3	12	130.3	12.8	96.7	20.8
4	4	152.5	16.6	88.1	47.8
5	4	65.6	13.3	38.3	14.0
7	8	108.5	13.1	43.0	52.4
8	5	110.3	20.7	57.1	32.5
10	4	102.5	3.8	89.5	9.2
12	10	121.5	27.5	62.9	31.1
15	4	113.0	5.5	107.5	0
21	8	143.7	17.6	105.6	20.5
30	8	139.4	13.7	93.9	31.8

Table 2 shows the effect of radiation with ^{60}Co on the glycogen fractions. In these experiments also the animals were sacrificed by immersion in liquid

air. The glycogen fractions of the brain were estimated by a method developed at the Biochemistry Institute of the Ukrainian Academy of Sciences by Khaikina (10).

As can be seen, the total glycogen decreases on the 1st day after irradiation. By the 2nd day, however, it recovers and on 3rd and 4th days rises above normal. By the 5th day it again decreases, this time to almost half, and again recovers to some extent on the 7th to 8th days, but only fully at about the 15th day after irradiation. By 3–4 weeks the glycogen again increases above normal.

Disorders in glycogen metabolism can be seen more clearly if one looks at the different proportions of the different glycogen fractions during radiation sickness. The most labile fraction is the free glycogen, which is also physiologically the most active (11). It disappears completely within a day, returns to normal between the 2nd and 12th days, and then falls again to remain low for the month of the illness. One can express the different glycogen fractions as a percentage of the total glycogen (Table 3). The proportion of free glycogen was low throughout the first month of the illness and the fact that on the 2nd, 5th, 8th and 12th days it appeared to approach normal was due to a decrease in the *total* glycogen during that period.

In radiation sickness, the fraction of glycogen associated with proteins and lipids also undergoes changes (Table 2). It shows a gradual decrease in the first day of the illness and except for a slight rise on the 4th–7th days remains at a low level up to the 30th day. Glycoproteins and glycolipids were low 5–10 days after radiation and by the 15th day this fraction disappeared. The *relative* content of glycoproteins and glycolipids also decreased (Table 3).

It was characteristic of the glycogen fraction linked to the soluble proteins that it increased in the first days of the illness, was interrupted by a short decrease on the 5th–8th days, and then continued raised until the 30th day with a maximum between the 15th and 21st days (107.5 and 105.6 mg%, compared with 63.4 mg% in the controls). The increase in total glycogen towards the end of the first month was mainly due to an increase in that fraction.

In short, the illness causes a decrease in the fraction of free glycogen, in the relative proportion of glycogen associated with proteins and lipids, and a decrease in the total brain glycogen in the first weeks and accumulation between the 3rd and 4th weeks. These disorders in the carbohydrate and phosphate metabolism parallel the clinical picture of radiation sickness.

TABLE 3. GLYCOGEN FRACTIONS IN THE BRAIN IN PER CENT OF THE TOTAL GLYCOGEN CONTENT

Time elapsed after radiation (days)	Glycogen			
	Total glycogen	Free glycogen	Glycogen bound to soluble proteins	Glycogen bound to insoluble proteins and lipids
Control	100	17.8	51.0	31.2
1	100	0	70.6	29.4
2	100	19.2	55.0	25.8
3	100	9.9	74.2	15.9
4	100	10.8	57.8	31.4
5	100	20.3	58.4	21.3
7	100	12.0	36.6	48.4
8	100	18.7	52.7	28.6
10	100	3.7	87.4	8.9
12	100	22.6	51.8	25.6
15	100	4.9	95.1	0
21	100	12.3	73.2	14.5
30	100	9.9	67.3	22.8

During the initial reaction there is a considerable decrease in the use of glycogen by the brain resulting in its accumulation and changes in the relative proportions of its fractions; the creatine phosphate content of the brain decreases and the ATP goes up but its metabolism is decreased. In the 2nd period between the 5th and 15th days, when the animal is apparently well, there is a decrease in the content of glycogen and creatine phosphate and an increase in ATP synthesis in the brain. The changes in relative proportion of the glycogen fractions and the instability of ATP and creatine phosphate occur with clinical development of the illness and further reaction of the central nervous system. In the 3rd period of the illness with full clinical radiation sickness, disorders in energy metabolism take place. Creatine phosphate is most resistant to the damaging effects of penetrating radiation. The content in the brain up to the 30th day after radiation is somewhat higher than normal. The ATP at this time is decreased and its rate of ^{32}P incorporation remains low throughout this period. The accumulation of glycogen with a simultaneous disturbance in the relative proportion of its fractions shows that there are serious disorders in the carbohydrate metabolism of the brain and that normal glycogen metabolism is not taking place.

Thus, all the evidence shows that the brain is highly sensitive to penetrating radiation.

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THE AMMONIA-GLUTAMINE-GLUTAMIC ACID SYSTEM AND OXIDATIVE PHOSPHORYLATION IN THE BRAIN DURING OXYGEN INTOXICATION

Z. S. GERSHENOVICH, A. A. KRICHEVSKAYA AND Z. G. BRONOVITSKAYA

University Department of Biochemistry, and Department of Biochemistry
of Biological Research Institute, Rostov-on-Don

STUDIES of small chemical changes during nervous activity often require exaggeration by inhibitors or stimulators to detect them. Narcotics, convulsants, electrical stimulation and drug stimulants have enabled numerous authors (Palladin (1), Vladimirov (2), and Vladimirova (3)), to study chemical changes in the brain during stimulation and inhibition.

We have been studying the effect of high oxygen concentrations on the metabolism of the central nervous system.

The first sign of oxygen poisoning is restlessness, a pre-spastic state which becomes convulsive. The time at which the convulsions occur depends upon the species, the oxygen pressure and the length of exposure. For example, in rats exposed to 6 atm of oxygen, convulsions begin after 7–20 min, while in guinea pigs it takes 20–40 min. Throughout this time the convulsions increase in intensity and the intervals between them become shorter. The period of convulsions alternates with a period of coma; the animal lies down, its respiratory frequency and depth decrease and, very rarely, strong convulsions can be observed. This period is called the “terminal” period. If, at this time, decompression is carried out, the animals usually die. At 4 atm or lower, the picture of oxygen poisoning develops more slowly. In rats slight restlessness is observed in the first 20–30 min, followed by prolonged immobility lasting 2 hr, then 10 to 20 convulsions are followed by the “terminal” state, ending in death.

A group of white rats was exposed to increased oxygen pressure, while another group was used as controls. The pressure was raised in a specially designed chamber with a perspex window for observation. Expired carbon dioxide was absorbed on to an alkali absorbent in the chamber. Both control and experimental animals were quickly immersed in liquid air. The brain was taken out, powdered and transferred into a weighed test tube

containing 5 ml of a 12 per cent solution of trichloroacetic acid at 0°C. The mixture was centrifuged and the centrifugate and supernatant analysed separately. In the supernatant the ammonia, glutamine and glutamic acid were estimated.

Ammonia was estimated by the micro-diffusion method of Conway (4), and the glutamine by the method of Harris (5); in this method the ammonia was liberated by hydrolysis in 12 per cent TCA at 70°C for 75 min. Glutamine gave off ammonia and was converted into pyrrolidone carbonic acid which did not interfere in the measurement of glutamic acid. The ammonia liberated by this method was also estimated by Conway's method.

The glutamic acid was estimated chromatographically. The TCA was extracted with ether from the protein-free filtrate obtained after hydrolysis of the glutamine, and the ether layer was removed. 7 μ l of solution corresponding to 30 mg of brain was spotted on to the paper (Leningrad No. 2). The amino acids were separated by 3 one-dimensional runs with a mixture of butanol acetic acid-water (5:1:4), after which the spots were developed with ninhydrin. The spot which corresponded to glutamic acid was eluted first with water and then with a 10 per cent solution of acetone in water, and the concentration estimated at 570 m μ on a spectrophotometer. Known concentrations of glutamic acid were run as standards.

The protein precipitate was washed twice with 5 per cent trichloroacetic acid and the washings added to the supernatant. To remove the water in the lipids, the precipitate was treated with the following solution: 30 vol. of acetone: a 3:1 alcohol-ether mixture: 2:1 chloroform-methanol. The washed protein was dried at room temperature. A suspension of 10 mg was hydrolysed in 10 ml of 6 N HCl for 20 hr. The hydrolysate was separated from the amines and evaporated in a water bath to remove the hydrochloric acid. The amino acid layer was dissolved in water and again evaporated to dryness; it was then dissolved in 0.2 ml of 10 per cent isopropyl alcohol and 7 μ l were spotted on to paper (Leningrad No. 2). The glutamic acid was estimated as above. Another 50 mg protein suspension was hydrolysed for 5 min in 5 per cent HCl in a water bath, and the amino groups of the protein were estimated by the ammonia liberated.

RESULTS

Figure 1 shows the brain ammonia content during oxygen poisoning. Stimulation of the animal by the increased oxygen pressure was accompanied by a considerable increase in the ammonia concentration in the brain. We were, however, unable to show a clear correlation between the

ammonia concentration of the brain and the onset of convulsions. The ammonia increases some time before the convulsions start and also about 10–23 min after they have been going on.

In one series of experiments the animals were kept immobile for 30 min in the chamber, and they were in a drowsy state; their brain ammonia was $4.24 \mu\text{M}$. If the animals moved round, their ammonia reached $12.54 \mu\text{M}$. Thus it seems that oxygen poisoning causes an increase in the free ammonia of the brain, whether or not the animals were active.

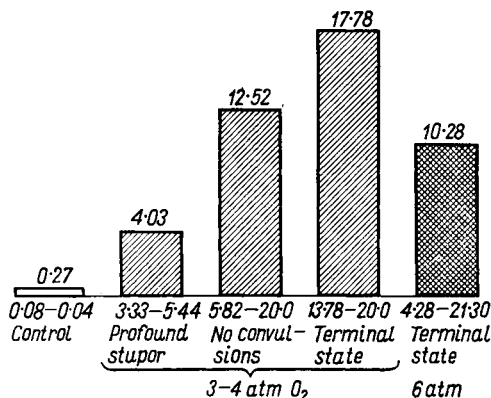


FIG. 1. Ammonia in the brain of rats after exposure to increased partial pressure of oxygen (in μ moles per g).

In 1922, Tashiro (6) reported that peripheral nerves release ammonia on stimulation; since then numerous papers have appeared relating nervous activity to the accumulation of ammonia in the brain (Pravdich-Neminskii (7), Rösch and Te Kamp (8), Vladimirova (9), Krebs (10), Weil-Malherbe (11), Richter and Dawson (12), Rebiling (13), Brühl (14), and Budanova (15)). During stimulation, ammonia formation is increased, but we report here higher values than have been described in other functional states.

We thus have to recognize the presence of rapidly mobilizable sources of ammonia in the brain. Weil-Malherbe showed that brain slices in a glucose-free medium form 5–10 mg of ammonia per g fresh weight per hour, and this ammonia production can go on linearly for 5 hr. This process requires at least 5 enzymes; glutamic acid dehydrogenase, glutaminase, amine oxidase, adenylic acid dehydrogenase and adenosine deaminase.

Ammonia in the brain is usually derived from the deamination of glutamine, adenylic acid and glutamic acid (16). Stoichiometric calculations show that these reactions do not account for all the ammonia which accumulates in some functional states. Furthermore, the physiological activity

of these enzymes has been questioned. Krebs (17) showed that the pH optimum of glutaminase in the brain is about 8.8, and that at a physiological pH its activity is very low. Tsukada and Takagaki (18) and Weil-Malherbe (19) found that de-amination of adenylic compounds hardly occurs in brain slices, only being seen in brain homogenates. Even in the homogenates, however, the de-amination of adenylic acid takes place much more slowly than the ammonia formation. Weil-Malherbe showed that the *total* adenylic compounds in the brain could liberate altogether only about 3 mg % of ammonia.

Deamination of glutamic acid is important physiologically in the brain, as it is the only free amino acid that can support tissue respiration. There is also an active glutamic decarboxylase in the brain.

We are at present studying the possibility of ammonia formation from lipids, neuraminic acid and other compounds.

Investigations show that in various functional states, the rate of ammonia formation exceeds its rate of fixation, and it accumulates in normal as well as in pathological conditions. It is possible that the irreversibility of the reactions connected with its formation or their different localization may be responsible for the disproportion between the rates of these processes.

Amino acids are of great importance in ammonia metabolism, as they interact with it to form amides, the most important one being glutamic acid and its amide, glutamine. The latter was isolated from muscle and brain by Ferdman (20). To assess the main sources of amides we had a look at the free glutamic acid and free glutamine in the protein and protein-free fractions of brain tissue.

Figure 2 shows that increasing the oxygen pressure up to 4–6 atm greatly decreases the free glutamine in the brain. If the animal is kept at 4 atm for 90 min in semi-ccma, the glutamine decreases to half. If, however, the animal showed signs of slight stimulation and walked around, the glutamine showed a less marked fall to $0.31 \mu\text{M}$. After the animal had been at 6 atm in the chamber for 135–140 min, no free glutamine was detectable in the brain; there was, however, wide variation between 0 and $2.43 \mu\text{M}$ due to the quicker development of intoxication of some animals. If we compare the glutamine and ammonia in the brain of the same animal, we see that the glutamine decrease parallels the ammonia increase.

It is well known that glutamine is formed from the reaction between ammonia and glutamic acid using ATP, and catalysed by glutamine-synthetase (Elliott, 21). We have previously shown that during oxygen intoxication the brain contains normal and sometimes excess amounts of glutamic acid. One can calculate that the increase of glutamic acid must have

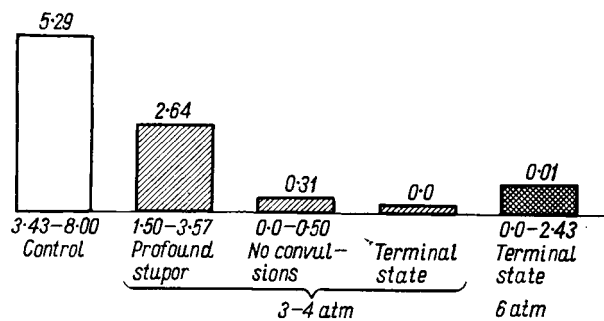


FIG. 2. Free glutamine in the brain after exposure to increased oxygen pressure (in μ moles per g).

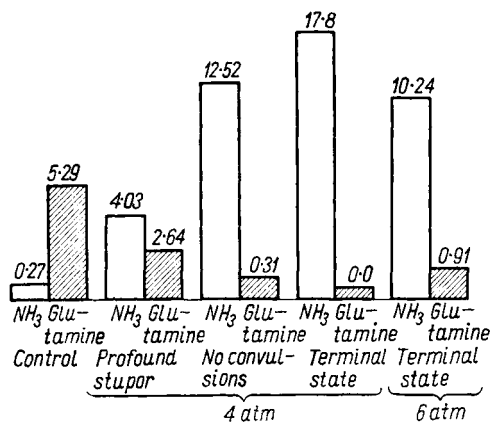


FIG. 3. Ammonia and glutamine in the brain at various periods of oxygen intoxication (in μ moles per g).

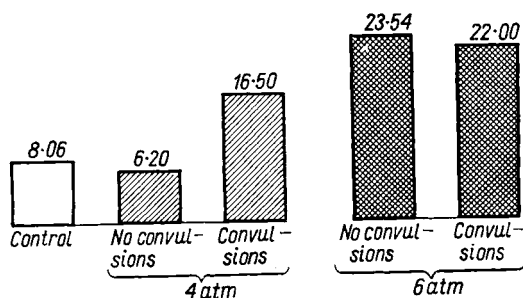
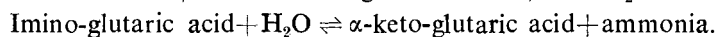
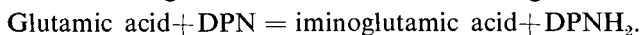


FIG. 4. Free glutamic acid in the brain after exposure to increased pressure of oxygen (in μ moles per g).

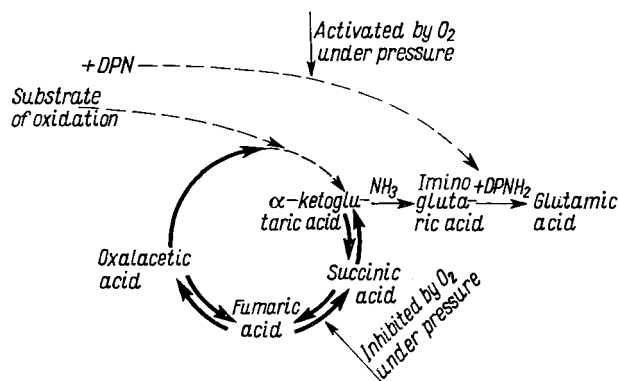
occurred not only by de-amination of glutamine, but also from other sources (Gershenovich and Krichevskaya (22)). Glutamic acid may be derived by (i) mobilization from the blood, (ii) synthesis from non-nitrogen products of intermediate metabolism, (iii) splitting off from proteins and peptides and (iv) de-amination of glutamine.

The permeability of brain tissue to glutamic acid is very low. Klein and Olsen (23), Schwerin, Bessman and Waelch (24), and Dawson (25) have shown that the brain glutamic acid does not increase after intravenous injection. In fact, glutamine passes through the capillary wall more easily than glutamic acid (Handler, Kamin and Harris (26), Tigerman and MacVicar (27)). The glutamine is probably de-aminated in the brain itself. The low permeability of the capillary wall to glutamic acid was also shown by perfusion of cat brains *in vivo* with glutamic acid solution (Waelch). Hence it seems that glutamic acid in the blood cannot serve as a reservoir for the brain.

Formation of glutamic acid from glutamine requires the presence of glutaminase. Probably glutamic acid can be synthesized directly from α -keto-glutaric acid and ammonia (Krebs, Eggleston and Hems (28)). The amination of α -keto-glutaric acid is a reversal of the glutamic acid de-amination.



The first reaction is catalysed by glutamic dehydrogenase. The restoration of the dehydrogenase is connected with the oxidation. The latter causes the equilibrium of the reaction to move to the left. The second reaction takes place spontaneously and its direction is determined by the concentration of ammonia. It is extremely specific, only keto-glutaric acid of the keto-acids is an ammonia acceptor in the presence of dehydrogenase (Euler *et al.* (29)). The whole pathway can be represented by the following scheme:



Evidence that oxygen intoxication interrupts the tricarboxylic acid cycle at the stage of keto-glutarate formation was sought in two ways.

1. Animals in the "terminal" state were exposed to increased oxygen pressure. After decapitation the brain was quickly placed in a McIlwain medium cooled with ice. Sections were prepared and their capacity to bind inorganic phosphate and to form ATP was estimated, the experiment lasting 25 min at 28.5°C.

2. Sections of brain from normal animals in a Warburg vessel with McIlwain's medium were subjected to 8 atm for 1 hr, with controls.

From Figs. 5 and 6 it can be seen that, notwithstanding the poor state of the animals, no serious disorders of oxidative phosphorylation could be detected. In fact, in a second series of experiments, incubated sections exposed to the direct action of oxygen showed stimulation of oxidative phosphorylation.

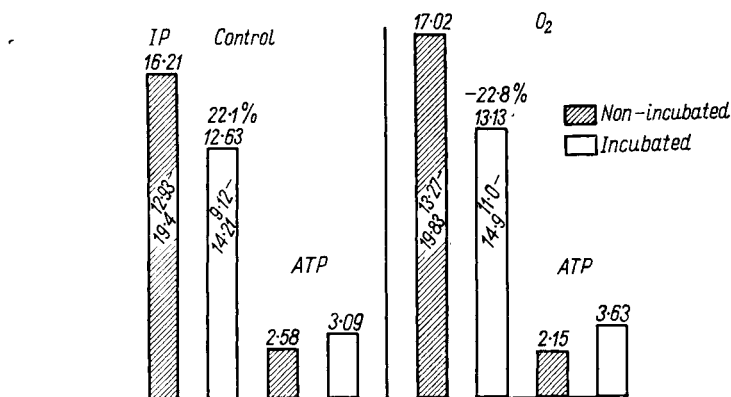


FIG. 5. Oxidative phosphorylation of the brain under the influence of increased oxygen pressure (phosphorus in µg per 100 mg tissue).

Krebs (30), in discussing oxidative phosphorylation, emphasized that only in the decarboxylation of keto-acids is there a mechanism for the incorporation of phosphate into that process to form ATP. The increased oxidative phosphorylation in the brain at increased oxygen pressure might represent an increased rate of passage round the tricarboxylic acid cycle; this fits in with our demonstration of irreversible inhibition of succinic dehydrogenase activity during oxygen intoxication. This causes a shift in the equilibrium of the reactions: α -keto-glutaric acid $\rightleftharpoons$ succinic acid, and succinic acid $\rightleftharpoons$ fumaric acid, towards the accumulation of α -keto-glutaric acid.

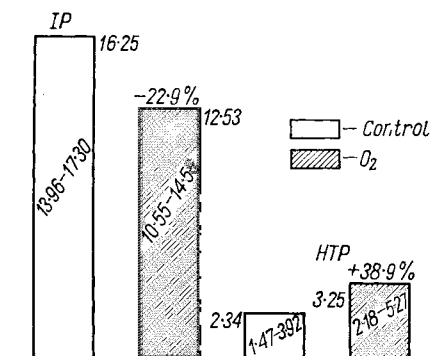


FIG. 6. Oxidative phosphorylation of brain sections under the influence of increased oxygen pressure (phosphorus in μg per 100 mg tissue).

Another indication of the possibility of α -keto-glutaric acid accumulation is the decrease in activity of co-enzyme A by almost 30 per cent due to increased oxygen pressure shown by Baron (31), and the change in properties of its apo-enzyme which would hinder further transformations of keto-acids (Gershenovich and Poverennyi).

It thus appears that α -keto-glutaric acid is of great importance in the reactions with ammonia.

1. α -keto-glutaric acid + ammonia $\rightleftharpoons$ imino-glutaric acid
2. Imino-glutaric acid + $\text{DPNH}_2 \rightleftharpoons$ glutamic acid + DPN
3. Glutamic acid + ammonia + $\text{ATP} \rightleftharpoons$ glutamine + $\text{ADP} + \text{P}$

The fact that considerable amounts of ammonia and glutamic acid are found in the brain at the same time can be explained by a disorder in glutamine synthesis with inhibition of ATP utilization, or activation of the glutaminase by increased oxygen pressure.

Free glutamic acid and glutamic acid in proteins both play a part in the formation and binding of ammonia. Vrba (32) did an extensive series of experiments showing that during stimulation changes take place in the brain proteins. In his experiments rats swimming for $4\frac{1}{2}$ hr served as models for functional activity. Under these conditions, no changes in the brain ammonia could be found. The author attributed this to the possibility that during physiological excitation *in vivo* ammonia accumulation does not occur in the brain, due to a simultaneous increase in the processes binding ammonia. In fact, estimations have shown that in the protein fraction of the brain the total nitrogen decreases corresponding to a decrease in amino-nitrogen. At rest the reverse process takes place.

Vrba believes that stimulation is related to a process leading to the breakdown of high molecular weight nitrogen-containing substances like proteins

and nucleoproteins. In a state of rest the processes of assimilation predominate and there is a decrease in the concentration of free glutamine in the brain with a simultaneous increase in the high molecular weight nitrogen-containing compounds.

The glutamic acid-glutamine system represents the link in the processes of stimulation and inhibition, as it regulates protein breakdown and synthesis. This hypothesis of a "glutamic acid cycle" suggested by Vrba puts into perspective the very important role of glutamic acid in brain metabolism.

Brain proteins are denatured (33) and their lability (judged by incorporation of ^{35}S -methionine) is increased when the animals are exposed to increased oxygen pressure (Gershenovich and Krichevskaya (34)).

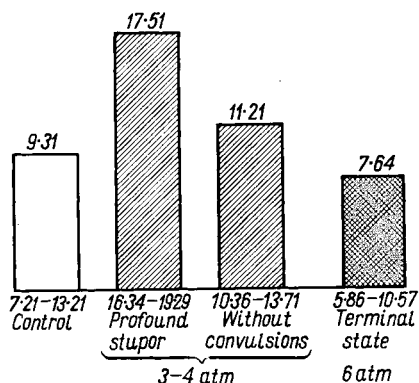


Fig. 7. Glutamine of brain proteins in rats under the influence of increased oxygen pressure (in $\mu\text{moles per g dried protein}$).

Estimations of glutamine in brain protein are shown in Fig. 7, and it can be seen that there is a correlation between the state of the animal and the amount of amino-protein present. Whereas during sleep the amino groups are almost double the controls, they gradually decrease as the intoxication proceeds, to reach a peak with the convulsions and then fall in the "terminal" state.

We are led to the conclusion that ammonia is not the *cause* of different functional states of the brain but a result of the chemical processes which take place in the dicarboxylic acid system and the proteins of the brain. If the functional activity of the brain is increased, carboxyl groups break off from the protein and this changes the configuration of the molecule. Thiol groups are liberated during denaturation and this probably has some functional importance (Nasonov and Alexandrov (35), Koshtoyants (36), Ungar (37)).

The liberation of carboxyl groups from proteins during the formation and splitting off of amides may lead to changes in the electrical charge which may cause denaturation of the protein. During functional "rest" the carboxyl groups of the protein are "shielded" by amino groups and when the former are split off the protein becomes activated. It would then appear that the ammonia accumulating in nervous tissue is an index of the liberation of protein carboxyl groups.

The breakdown of amides is phylogenetically a very ancient process. The younger an organ of a land animal is, phylogenetically, the better developed are its ammonia binding mechanisms, as in nervous tissue.

The breakdown of amino groups is also important energetically. In oxygen intoxication the capacity of the brain to use energy from ATP is disturbed and it is possible that the large amounts of ammonia formed are a result of the "compensatory" use of energy liberated by the breakage of amino links.

SUMMARY AND CONCLUSIONS

1. In oxygen intoxication the amount of free ammonia present in the brain shows a considerable increase with a simultaneous decrease in the free glutamine.

2. The amide content of the protein fraction of the brain depends upon the state of the animal. It increases in a state of sleep and decreases during convulsions as well as in the "terminal" state.

3. The rate of oxidative phosphorylation in the brain does not change during oxygen intoxication *in vivo*. *In vitro*, however, stimulation of oxidative phosphorylation occurs.

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CHANGES IN THE CEREBROSIDES OF THE BRAIN DURING EXCITATION AND INHIBITION INDUCED BY TOXINS AND DRUGS

M. SH. PROMYSLOV

Institute of Normal and Pathological Physiology, Soviet Academy of
Medical Sciences, Moscow

STUDIES of the cerebrosides began with Thudicum (1) who, in 1874, isolated sphingosine and neurokeratin from human brain. Fifty years later Klenk (2) established that nervous tissue contains two more compounds of a group he called nervone and oxynervone. Further studies to characterize the cerebrosides more precisely were done by Klenk (3), Carter *et al.* (4) and Lahut (5).

We know much less about the biological role and metabolism of cerebrosides in the central nervous system than about their chemical properties. Our knowledge of enzymes which act upon cerebrosides is also very limited. The only two publications, Thannhauser (6) and Fujino (7) both show that cerebrosidase is practically inactive. For activity it requires an activator containing an SH group.

Sloane-Stanley (8) reports changes in the amounts of cerebrosides in the nervous tissue in various pathological conditions. He quotes references for a decrease of cerebrosides during insulin coma in patients, although galactose appeared in their cerebral venous blood after injection of desoxy-corticosteroids, which may be connected with the breakdown of cerebrosides.

At the first symposium on neurochemistry in 1954, Klenk reported that in certain diseases an increase in the lipids and, in particular, of the cerebrosides, could be observed (9). Such conditions are Gaucher's disease, Niemann-Pick's disease, hereditary idiotism, and others. Thannhauser also describes an increase in cerebrosides in these neurolipidoses (10).

We were able to show that in rabbits, tetanus and gas gangrene caused a decrease in the amount of cerebrosides in the brain (11). Cerebrosides were estimated by the galactose found after hydrolysis of lipid extracts from the brain.

The results of these experiments made us wonder whether these two toxins have a similar action. Although gas gangrene and tetanus are different clinically, they both show a general increase in reflex excitability. If their chemical actions were the same, we might have expected similar changes in other substances in the central nervous system.

If a lethal dose of strychnine was administered subcutaneously to a rabbit there was an increase in reflex excitability, followed by marked contractions of the limb muscles and then the whole trunk. Generalized convulsions ensued and the animals died in 15 to 20 min. In the brains of these animals we did not find changes in the content of cerebroside; the same was true of generalized tetanus. If such a dose of toxin was injected that the animal died in 4 to 5 days, we could not detect changes in the brain. Only if the animals died in 7 or 8 days was there a decrease in brain cerebroside; the same applied to gas gangrene; a decrease in the amount of cerebroside could only be detected 30 hr after the injection of toxin.

TABLE 1

Weight of rabbits (kg)	Weight of fresh brain (g)	Weight of dried proteins (mg)	Galactose (mg)	Cerebroside (mg)	Per cent of galactose from proteins	Per cent of cerebroside from proteins
Normal conditions						
2.300	9.2	880.6	19.26	86.67	2.19	9.84
2.400	10.7	994.7	26.19	117.85	2.63	11.84
2.300	9.1	919.4	22.16	99.72	2.41	10.85
2.400	9.7	978.3	22.91	103.95	2.34	10.60
2.250	9.2	947.4	22.55	101.47	2.38	10.71
After administration of strychnine						
2.450	8.6	935.9	13.73	61.78	1.47	6.60
2.250	8.5	955.0	14.92	67.14	1.56	7.03
2.200	8.7	910.2	14.58	65.61	1.60	7.20
2.300	9.1	981.2	18.59	83.65	1.89	8.52
2.250	9.3	944.5	12.94	58.23	1.37	6.16

We had to choose a dose of strychnine which caused a long-lasting period of convulsions in the rabbits. After injection of 1 mg of strychnine nitrate in 0.5 ml of distilled water into an adult rabbit, convulsions occurred in 15 to 20 min and generalized tetanus supervened. The rabbit remained in that state for a day and then it was killed. Table 1 shows that there was a marked decrease in the cerebroside of the brain, both in general tetanus and gas gangrene. We, therefore, concluded that a decrease in

the amount of cerebrosides is related to a similar functional state of the central nervous system in these cases. We suggest that brain cerebrosides may be used as an energy substrate for the activity of the nervous system in a state of marked excitation. The amount of cerebrosides in the brain in generalized tetanus, gas gangrene and strychnine decreases because, after a time, the sugar which is a constituent of this compound is used by the brain as a substrate.

This view is consistent with data in the literature. Energy liberated by carbohydrates under aerobic and anaerobic conditions is used for the activity of the nervous system. During excitation an increase in the activity of the enzymes breaking down sugar in the brain and a decrease in the carbohydrate occurs. Similar observations have been made in tetanus and strychnine poisoning. Jadassohn and Streit (12) showed a lowering of sugar in the brain during tetanus. Takahashi (13) investigated the brain of a person who died during tetanic convulsions and found a decrease in the glycogen compared with the brain of a person who had died from tuberculosis. Martino described similar effects of strychnine poisoning (14).

At the International Symposium at Oxford in 1954 Gerard (15) suggested that Abood and Geiger's demonstration that in the brain of cats perfused with a glucose-free solution the activity was maintained for several hours was probably due to a considerable breakdown of phospholipids. Gerard's view was supported by Himwich (16). At the same symposium, Klenk (17) cited the experiments of Sperry and Waelch, who showed an interrelation between the carbohydrate and lipid metabolisms of the brain. Finally, Winterstein and Hirschberg (18) had shown that frog brain loses glycogen as well as cerebroside sugar after various forms of stimulation. It appears that under certain conditions, the isolated brain of a frog obtains energy from cerebroside sugar.

To test this hypothesis, the most obvious way was to measure the cerebrosides in the brain of animals during prolonged drug-induced sleep. Sleep was caused for 48 hr by a mixture of urethane and sodium barbitone, and the animals were killed and their brains investigated. Table 2 shows that there was very little difference in the amount of brain cerebrosides between the narcotized and control rabbits.

This confirmed our assumption that cerebrosides are only used by the brain in some functional states. The decrease in rabbits during generalized tetanus could not be observed until 7 days after the injection of toxin. The same applies to other agents causing a decrease in brain cerebrosides. It seems that energy is derived from these sources only when the nervous system is completely exhausted. To test this, we carried out experiments

TABLE 2

Weight of rabbit (kg)	Weight of fresh brain (g)	Weight of dried protein (mg)	Galactose (mg)	Cerebro-sides (mg)	Per cent of galactose from proteins	Per cent of cerebro-sides from proteins
Control						
2.300	8.0	841.1	20.35	91.58	2.42	10.88
2.350	9.1	930.4	25.21	113.44	2.71	12.19
2.250	9.3	927.2	21.05	94.73	2.27	10.21
2.400	9.7	969.2	22.68	102.06	2.34	10.53
2.400	8.8	879.9	23.05	103.73	2.62	11.78
Sleep						
2.300	7.9	830.4	22.26	100.17	2.68	12.06
2.400	8.9	980.9	29.56	133.02	3.01	13.56
2.200	8.8	931.5	19.33	86.99	2.07	9.34
2.250	9.1	976.5	21.77	97.97	2.23	10.03
2.300	9.0	889.2	25.14	113.13	2.82	12.22

TABLE 3

Weight of rabbits (kg)	Weight of fresh brain (g)	Weight of dried proteins (mg)	Content of galactose (mg)	Content of cerebro-sides (mg)	Per cent of galactose from proteins	Per cent of cerebro-sides from proteins
Normal conditions						
2.300	9.3	900.2	24.44	109.98	2.71	12.21
2.400	9.1	734.0	19.06	85.77	2.59	11.68
2.200	8.5	871.4	20.88	91.26	2.44	10.47
2.250	8.7	971.0	22.21	99.95	2.28	10.29
2.300	9.3	962.0	20.21	90.94	2.10	9.45
General tetanus						
2.250	9.7	1006.0	16.03	72.14	1.59	7.17
2.350	8.1	825.3	15.23	68.54	1.84	8.30
2.100	8.8	987.2	17.89	80.50	1.87	8.15
2.450	9.2	865.3	14.22	63.99	1.62	7.39
2.400	9.4	897.2	15.09	67.91	1.57	6.56
General tetanus+sleep						
2.150	9.2	868.3	21.38	96.21	2.47	11.08
2.300	8.9	927.3	22.48	101.16	2.42	10.90
2.350	9.1	891.3	22.90	103.05	2.57	11.56
2.250	8.8	830.2	18.57	83.29	2.23	10.03
2.300	9.5	925.7	21.29	95.80	2.30	10.34

with rabbits giving them a lethal injection of tetanus toxin. On the 5th day, when all the animals had generalized tetanus, some were anaesthetized with a mixture of urethane and sodium barbitone. They slept for 2 days and when the animals who had remained awake were just dying, both groups of animals were killed and their brains investigated (Table 3).

In the conscious rabbits there was a considerable decrease in the amount of cerebroside in the brain, whereas the anaesthetized ones had a normal level. Drug induced inhibition of higher nervous activity in tetanus seems to protect the brain against the increased consumption of energy substances and such reserve stores as cerebroside are not exhausted. The galactose of brain cerebroside compensates for the deficiency of other carbohydrates.

Finally, in further experiments brain tissue was directly exposed to the action of tetanus toxin. Brain tissue from healthy rabbits was left to autolyse with a suspension of tetanus toxin at a temperature of 37°C for 24 hr. Table 4 shows that the amount of cerebroside was the same as without toxin.

TABLE 4

Weight of rabbits (kg)	Weight of fresh brain (g)	Weight of dried proteins (mg)	Galactose of brain cerebroside (mg)	Brain cerebroside (mg)	Per cent of galactose from proteins	Per cent of cerebroside from proteins
Normal conditions						
2.400	9.2	998.6	21.90	99.55	2.19	9.86
2.300	9.5	998.4	22.00	99.00	2.20	9.91
2.250	9.0	897.6	23.80	107.15	2.65	11.93
2.300	9.1	967.1	22.94	103.24	2.37	10.77
2.150	8.9	829.0	19.25	89.78	2.41	10.83
Autolysis of the brain						
2.400	9.1	1000.1	23.13	104.08	2.31	10.30
2.400	8.3	800.1	19.80	89.10	2.47	11.16
2.350	9.3	947.4	22.55	101.47	2.38	10.70
2.250	8.5	874.2	23.60	106.20	2.70	12.14
2.500	9.6	968.9	24.12	108.54	2.49	21.10
Autolysis of the brain after addition of tetanus toxin						
2.700	9.3	883.5	20.57	92.56	2.32	10.47
2.700	9.3	793.4	22.24	100.08	2.80	12.61
2.400	8.8	932.2	20.97	94.37	2.25	10.12
2.500	9.4	912.1	24.71	111.19	2.72	12.19
2.300	8.7	898.7	23.36	105.12	2.60	11.68

The tetanus toxin does not seem to possess enzymatic properties causing the breakdown of cerebroside, nor does the toxin activate enzymes which decrease them, but its effect on the central nervous system would appear to be a specific functional stimulation. As can be seen from Table 4, autolysis of brain tissue does not itself decrease brain cerebroside compared with the amount found in the brains of freshly killed animals. This indicates that these compounds are very resistant to change, and the decrease we show is a characteristic of the nervous system of a live animal in a particular functional situation. It seems that cerebroside in which the nervous tissue is particularly rich are not only a component of myelin but can also take an active part in nervous metabolism when necessary.

TABLE 5

Weight of rabbits (kg)	Weight of fresh brain (g)	Weight of dried proteins (mg)	Galactose (mg)	Per cent of galactose from proteins	Galactose (mg)	Per cent of galactose from proteins	Per cent of total galactose from proteins
Before hydrolysis					After hydrolysis		
2.200	9.0	893.8	19.70	2.20	11.5	1.28	3.48
2.350	8.8	992.7	20.96	2.11	12.40	1.25	3.36
2.350	9.5	100.1	24.99	2.49	12.24	1.23	3.72
2.200	8.3	833.6	16.35	1.96	11.07	1.33	3.29
2.250	8.8	772.6	16.80	2.19	11.13	1.44	3.63

We have established that the brain contains free as well as protein-linked cerebroside. Table 5 shows the relative amounts of galactose and brain cerebroside. Before hydrolysis the galactose from cerebroside is extracted from brain tissue by organic solvents. The residue after extraction is hydrolysed with a sulphuric acid-ethanol mixture, and the so-called "bound cerebroside" can then be extracted and the galactose estimated. Approximately one-third of the cerebroside of the brain is present in a bound form and two-thirds are free, and the changes observed in our experiments refer to the free cerebroside.

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RESPIRATION AND GLYCOLYSIS DURING INFECTION WITH POLIOMYELITIS VIRUS

R. N. ETINGOF, V. N. GERSHANOVICH AND N. N. GOLUBEVA

Institute for Poliomyelitis Research, Academy of Medical Sciences, Moscow

WE HAVE been studying aerobic and anaerobic glycolysis, oxygen uptake, succinic-dehydrogenase activity, aldolase and adenosinetriphosphatase activity of brain homogenates of healthy cotton voles and of rats infected with a new type of poliomyelitis virus (IV type strain AB) isolated in this country in 1956 by Chumakov and Voroshilova (6). In another series we studied the same systems in 1-2, 5-7, 14-16-day-old, and adult rats. Young rats were used, as 1-4-day-old rats are most susceptible to the virus in question.

Animals 1 or 2 days old were infected with type IV poliomyelitis virus of titre 10^{-3} by intracerebral injection of 0.02 ml of a 10 per cent brain suspension prepared in normal saline. When the typical clinical picture of the illness had developed—about the 4th day after inoculation—the animals were killed, their brains taken out, homogenized, and diluted with normal saline. Controls 1 or 2 days old received intracerebral injections with brain suspensions made from healthy animals. In addition, we studied the brain of 1-2, 5-7, 14-15-day-old rats and of adult rats.

Anaerobic and aerobic glycolysis was estimated by the increase in lactic acid measured by the method of Barker and Summerson (7) after incubation of the brain homogenate in a Warburg apparatus in an atmosphere of nitrogen or air with Ringer's bicarbonate or Ringer's phosphate solution with glucose. The oxygen consumption was estimated in a Warburg apparatus, the succinic-dehydrogenase activity was estimated by the method of Thunberg, and the aldolase activity by the method of Tovarnitskii (8). Adenosinetriphosphatase activity was estimated by the liberation of inorganic phosphate following the incubation of the brain homogenate for 20 min at 37°C in borate buffer (pH 7.4) in the presence of sodium adenosinetriphosphate and magnesium ions. Each estimation was done with the corresponding controls.

Experiments on glycolysis in Table 1 show that both anaerobic and aerobic glycolysis occurs less intensively in animals suffering from the illness than in controls. (5-7-day-old animals were used as controls as the inocu-

lations were done on the 1st to 2nd days and the young rats were killed after 3 or 4 days.) Anaerobic and aerobic processes were both equally but only slightly inhibited. There was considerable variation in the degree of inhibition. Similar findings were made by Racker (1) when he used the type II virus.

TABLE 1. GLYCOLYSIS IN HOMOGENATES OF THE BRAIN OF HEALTHY COTTON VOLES AND OF VOLES INFECTED WITH POLIOVIRUS

Groups of animals	Aerobic glycolysis		Anaerobic glycolysis	
	Accumulation of lactic acid in 2 hr (μ g)	Changes compared with the control group (%)	Accumulation of lactic acid in 2 hr (μ g)	Changes compared with the control group (%)
Healthy Control	386*	—	479*	—
	379*	—	482*	—
Infected with poliovirus	357	— 6	357	—26
	108	—72	336	—30
	300	—21	470	—2
	312	—18	—	—
	240	—37	—	—
	300	—21	360	—25
	302	—20	312	—35
	256	—32	256	—45

* Average of six experiments.

In our experiments the rate of glycolysis was relatively low because the Ringer's bicarbonate buffer and glucose in which the homogenates were incubated was not an optimal medium. As, however, we were mainly interested in comparison of glycolysis in the brains of healthy animals and those infected with poliomyelitis, and in animals of various ages, we thought it inadvisable to enrich the medium.

Table 2 shows that the aldolase and adenosinetriphosphatase activity in animals infected with poliomyelitis virus is decreased by about 20–30 per cent. The fact that in infected animals glycolysis is inhibited is interesting in view of the inhibition of aldolase, which is one of the glycolytic systems.

The fact that type IV poliomyelitis virus just as type II poliomyelitis virus (Lancing-strain) inhibits glycolysis shows the similar changes engendered in animals by two different types of poliomyelitis virus.

Table 3 shows the dehydrogenase activity of brain homogenates from healthy young rats (5–7-day-old) (inoculation was done on the 1st or 2nd day and the rats killed after 3 to 4 days), from control animals into whom

TABLE 2. THE ALDOLASE AND ADENOSINETRIPHOSPHATASE ACTIVITY OF HOMOGENATES OF THE BRAIN OF HEALTHY VOLES AND VOLES INFECTED WITH POLIOVIRUS

Group of animals	Aldolase activity		Adenosintriphosphatase activity	
	Extinction $\times 100$	Changes compared with the control group (%)	Quantity of phosphorus split off (μ g)	Changes compared with the control group (%)
Healthy Control	59*	—	108*	—
	57*	—	106*	—
Infected with poliovirus	48	—16	76	—28
	48	—16	70	—34
	35	—39	84	—21
	50	—12	74	—30
	41	—27	72	—32

TABLE 3. THE DEHYDROGENATING ACTIVITY OF BRAIN HOMOGENATES FROM HEALTHY COTTON VOLES AND VOLES INFECTED WITH POLIOVIRUS

Group of animals	Dehydrogenation of succinic acid		Dehydrogenation of endogenous substrates	
	Time required for decolorization of methylene blue (min)	Changes compared with the control group (%)	Time required for decolorization of methylene blue (min)	Changes compared with the control group (%)
Healthy Control	16**	—	56**	—
	17*	—	53*	—
Infected with poliovirus	8	+53	23	+57
	10	+41	17	+68
	12	+29	45	+15
	10	+41	—	—
	12	+29	27	+49
	8	+53	47	+11

* Average findings from five experiments.

** Average findings from six experiments.

a healthy brain suspension had been injected, and from rats infected with the virus. Table 3 shows that the activities of succinic dehydrogenase and endogenous dehydrogenases are considerably increased in rats infected with the virus. There is also an increase in the rate of oxidation of succinic

acid under aerobic conditions (see Fig. 1). At the same time the oxygen consumption remains unchanged in the three groups of animals (Fig. 2).

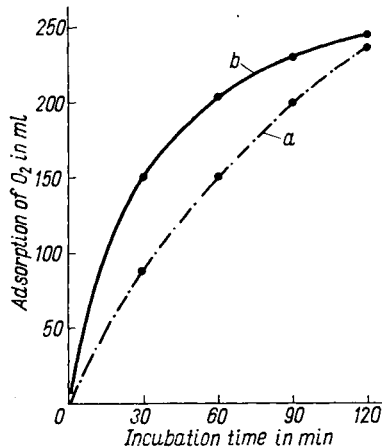


FIG. 1. Oxidation of succinic acid by brain homogenates from healthy cotton voles (a), and voles infected with poliovirus (b).

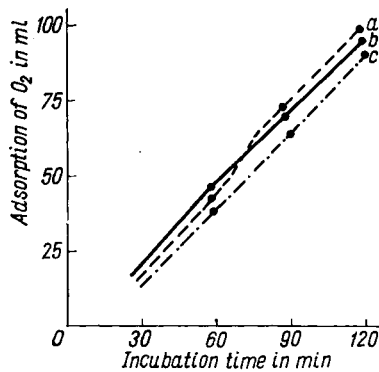


FIG. 2. Oxygen consumption by homogenates of the brain of healthy cotton voles (a), infected with poliovirus (b), and rats of the control group (c).

The considerable activation of the system which transforms succinic acid in the brain of animals infected with poliomyelitis indicates the importance of the tricarboxylic acid cycle in the metabolism of the virus (9).

Table 4 shows the aldolase, adenosinetriphosphatase, succinic dehydrogenase and endogenous dehydrogenase activity of brain homogenates of normal rats of different ages. The enzymic activity of brain homogenates

is, as a rule, lower in 1-2-day-old animals than in adults, which confirms the findings of other authors (10). By the 5th to 7th day of life, the aldolase and adenosinetriphosphatase activities reach steady levels. The activity of the dehydrogenases, on the other hand, becomes stable much later, by the 14th-16th day of life. Particularly marked changes can be observed in the activity of endogenous dehydrogenases, a fact which suggests either lack of substrates to dehydrogenate or impaired activity of these enzymes during the first two weeks of life.

TABLE 4. THE ENZYMATIC ACTIVITY OF BRAIN HOMOGENATES OF COTTON VOLES OF DIFFERENT AGES

Age of the animals (days)	Enzymatic activity*			
	Aldolase (extinction $\times 100$)	Adenosinetriphosphatase (quantity of phosphorus split off in μg)	Succinic dehydrogenases	Dehydrogenases of endogenous substrates
			time of decolorization of methylene blue (min)	
1-2	32	48	30	59
5-7	61	108	16	56
14-16	57	—	12	29
Adult rats	59	100	11	27

* All indices represent the average results of 5-6 experiments.

TABLE 5. GLYCOLYSIS IN BRAIN HOMOGENATES FROM COTTON VOLES OF DIFFERENT AGE*

Age of animals (days)	Glycolysis	
	Aerobic	Anaerobic
	Accumulation of lactic acid in 2 hr (μg)	
1-2	455	588
5-7	386	479
14-16	378	484
Adult rats	363	451

* All indices represent the average results from 7 experiments.

The findings for succinic dehydrogenase were confirmed by experiments on the oxidation of succinic acid under aerobic conditions (Fig.3). Homogenates of brain on the 1st-2nd and 5th-7th days of life had less succinic acid oxidative activity than brain homogenates from adult rats. This can

be seen if one compares oxygen uptakes during the first 30 min of the experiment.

These results for succinic dehydrogenase and succinic oxidase of the brain in cotton voles are in agreement with Potter's finding that these enzymes increase during the morphological differentiation of the brain in voles, when they are 10–12 days old (11).

From Table 5 it can be seen that both aerobic and anaerobic glycolysis is more intensive in young animals than older ones, as was also found by Tyler and van Harreveld (12).

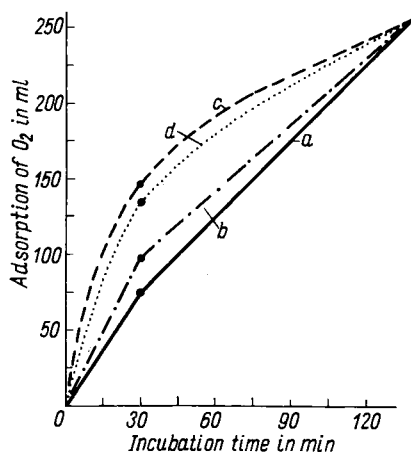


FIG. 3. Oxidation of succinic acid by brain homogenates of voles of different ages: a—1–2-day-old voles; b—5–7-day-old voles; c—15-day-old voles; d—adult voles.

The enzyme systems of the brain investigated by us alter in activity in various periods of the life of cotton voles; particularly marked differences were found in the enzymatic activity of 1–2-day-old voles. The final levels of activity became stabilized when the animals were 14–16 days old.

SUMMARY

1. Aerobic and anaerobic glycolysis, succinic dehydrogenase, aldolase and adenosinetriphosphatase activities in the brain of healthy cotton voles and voles infected with type IV poliomyelitis virus were investigated.

2. After infection of the animals with the virus, a marked activation of enzymes acting on succinic acid was seen.

3. Anaerobic and aerobic glycolysis in brains of infected animals is almost always lower than in healthy controls. The degree to which glycolysis is

suppressed varies and is not always very high. The nature of these changes agrees with others reported in the literature for effects of the type II virus.

4. The enzyme systems of the brains investigated by us possess different activities at different times in the life of cotton voles; the activity of different enzymes reaches a stable level at different times. The aldolase and adenosinetriphosphatase reach a constant level at 5–7 days of age and thereafter remain steady. Dehydrogenase activity becomes stabilized between 14–16 days. Aerobic and anaerobic glycolysis is more intensive in young animals; the relative proportion of lactic acid under aerobic and anaerobic conditions becomes fixed very early on.

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BIOCHEMICAL CHANGES DURING EXPERIMENTALLY INCREASED INTRACRANIAL PRESSURE

A. YA. MESTECHKINA

Institute of Neuro-Surgery, Ministry of Health, Ukraine, Kiev

CEREBRAL oedema is an important clinical phenomenon and there are many views in the literature of the relationship between cerebral oedema and increased intracranial pressure (1,3-6). Arutyunov (1) believes that increased intracranial pressure is not only a result of the brain swelling, but in some situations causes the swelling itself.

We have previously shown that when the intracranial pressure slowly increases the brain swells and undergoes morphological and biochemical changes that are ultimately fatal (2). We also concluded that the changes brought about by slow artificially increased intracranial pressure were similar to those in human beings due to cerebral tumors.

The present studies were designed to relate, if possible, the rate of increase of intracranial pressure to the degree of biochemical and morphological changes produced. The experiments were performed on dogs whose intracranial pressure was slowly increased by the method of Virozub and Sergiyenko (7). Animals were killed at the end of the experiment by sodium thiopentone injection which does not cause cerebral swelling according to White, *et al.* (8). Some of the animals died before sacrifice and their brains were analysed 1 to 16 hr after death, otherwise the brains were examined immediately after sacrifice. The cerebral hemisphere on the other side from that in which the cannula was used to increase the intracranial pressure served as a control. The white and grey matter were investigated separately. Degree of swelling was assessed by water content, local cholesterol inorganic phosphate, "soluble" phosphate, creatine, non-protein nitrogen and total nitrogen. Water content was measured by the method of Okun' (9); the cholesterol by the micromethod of Gorodisskaya (10); the total phosphate and nitrogen by ashing the tissues and spectrophotometric assay; the acid-soluble phosphate, non-protein nitrogen and the creatine by ashing the deproteinized tissue supernatant followed by spectrophoto-

metric assay. Simultaneously, nucleic acids, phospholipids and phosphoproteins were assayed, and in the latter two compounds the specific activity was measured after injection of ^{32}P . The radioactive phosphorus was injected intravenously 24 hr before death at a dose of $0.2\text{--}0.25\text{ }\mu\text{g/kg}$ body weight. Nucleic acids were measured by Schmidt and Thannhausers' method as modified by Chepinoga, Skvirskaya and Rukina (11).

RESULTS

The results are expressed for fresh and dried tissue. The tissue was sampled at various phases of increased intracranial pressure as defined by Virozub and Sergiyenko (7). The criteria on which these phases were distinguished were: the clinical appearance of the animal both *in vivo* and at the time of death, the CSF pressure and the time elapsing after the insertion of the cannula. From these criteria four phases were distinguished: (1) compensation, (2) subcompensation, (3) decompensation, and (4) terminal phase.

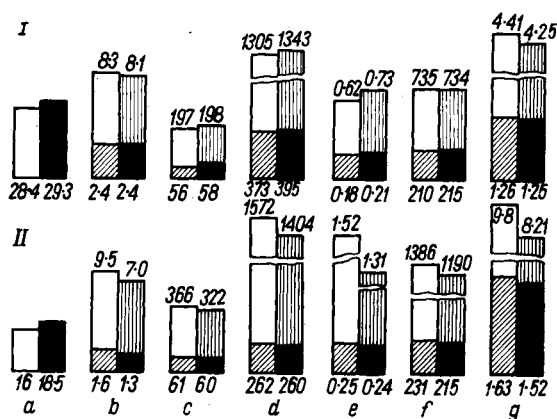


FIG. 1. The results of biochemical investigation of the white (I) and the grey (II) matter of the brain of dogs in the phase of clinical compensation to increased pressure: a—content of dry residue (in %); b—cholesterol (in %); c—acid-soluble phosphate; d—total phosphate (in mg %); e—creatine (in %); f—non-protein nitrogen (in mg %); g—total nitrogen (in %).

I. Compensation phase. No swelling could be observed in this phase. Figure 1 gives the results of the biochemical investigations, each column pair representing the control (left) and treated cerebral hemisphere (right). The lower columns give the values expressed per unit wet weight and the upper ones per unit dry weight of tissue.

In a few cases during the compensation phase the water content of the pressurized hemisphere was, surprisingly, lower than the control. The cholesterol, acid-soluble phosphate, total phosphate, creatine, protein and total nitrogen values were the same in both hemispheres whether calculated per unit wet or dry weight of tissue, and these results are in agreement with the absence of tissue swelling during this phase.

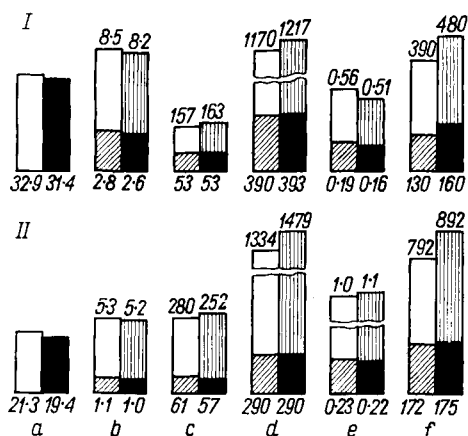


FIG. 2. Result of biochemical investigations of the white (I) and grey (II) matter of the brain of dogs in the phase of subcompensation of increased pressure. Legend as in Fig. 1.

II. *Subcompensation*. Again no swelling of the brain could be observed post mortem and results of biochemical studies are given in Fig. 2. These show no significant differences in the two hemispheres and once more are in agreement with absence of tissue swelling.

III. *Decompensation*. Post mortem examination revealed tissue swelling which was accompanied by an extensive increase in the bulk of white matter which invaded the grey matter. The results of biochemical studies are given in Fig. 3.

These show that the water content of the white matter of the pressurized hemisphere was higher by 3.5–8.6 per cent. Along with the increased water content of the white matter, the cholesterol, acid-soluble phosphate and total phosphate, creatine and total nitrogen content decreased as calculated per unit wet weight of tissue, but it should be noted that the decrease for each substance varied widely. Recalculating these values per unit dry weight of tissue eliminates this variation and for certain substances pressurization causes a slight increase of content per unit dry weight of tissue.

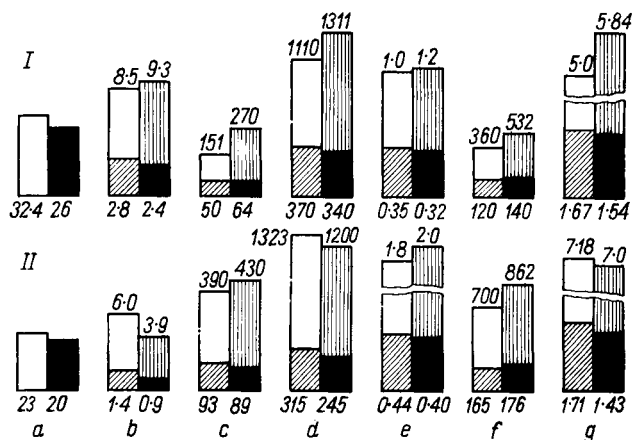


Fig. 3. Results of biochemical investigations of the white (I) and grey (II) matter of the brain of dogs in the phase of decompensation of increased pressure. Legend as in Fig. 1.

The deviations in the content of substances in the white matter found by calculation for the wet tissue are usually regarded as evidence for oedema and swelling of the white matter. In the grey matter the content of water and other substances did not show wide variations following a definite trend whether calculated per unit dry or wet weight of tissue.

It should be noted that during this phase the degree of swelling of the white matter did not always correlate with the clinical symptoms and changes in CSF pressure.

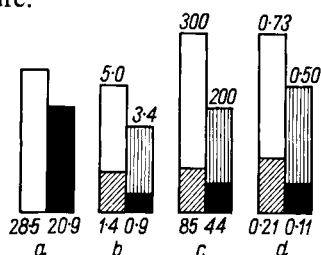


FIG. 4. Results of biochemical investigation of the white matter of the brain of dogs in the terminal phase of increased pressure. Legend as in Fig. 1.

IV. *Terminal phase.* Post mortem examination revealed extensive swelling of the tissue, and an increase in the water content of the white matter. Biochemical changes (see Figs. 4 and 5) resulted in a decrease of the cholesterol, acid-soluble and total phosphate, creatine and total nitrogen of the white matter as expressed per unit weight of wet tissue. Expressed as proportions of the dry weight these decreases were nullified and even reversed

for one or two substances. These changes observed in white matter were not as profound as in the grey matter; there was a slight increase in water content, along with a slight decrease in the proportion of cholesterol, total nitrogen and phosphate expressed per unit wet weight of tissue.

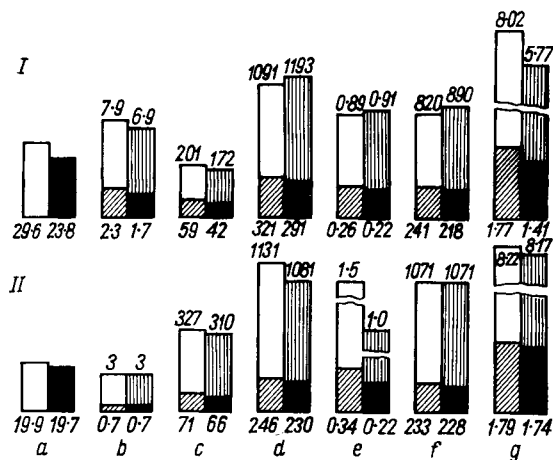


FIG. 5. The results of biochemical investigations of the white (I) and grey (II) matter of the brain of dogs in the terminal phase of increased pressure. Legend as in Fig. 1.

Hence at the terminal phase there is a marked swelling of the brain accompanied by distinct biochemical changes and furthermore these symptoms are correlated with changes in the clinical appearance.

Thus, well defined swelling and water uptake by the white matter occurs only during decompensation and terminal phases. The grey matter remains unaffected despite the CSF pressure or changes in the white matter.

MEASUREMENTS OF NUCLEIC ACIDS, PHOSPHOLIPIDS AND PHOSPHOPROTEINS

The levels of these substances as well as their ^{32}P turnover were measured up to the end of the decompensation phase. Owing to the fact that the isotope was injected only once during the course of the experiment the results can only be considered to be exploratory.

Table 1 gives the results for RNA,* DNA, PI and PP calculated both per unit dry and wet weight of tissue.

During compensation and subcompensation the levels of these substances did not change in the white matter, but the RNA, DNA and PI did decrease during decompensation and the early terminal phase. Expression of

*RNA = ribonucleic acid; DNA = deoxyribonucleic acid; PI = phospholipid; PP = phosphoprotein.

TABLE 1. THE CONTENT OF RNA, DNA, PHOSPHOLIPIDS AND PHOSPHOPROTEINS IN THE COMPRESSED HEMISPHERE COMPARED TO THE CONTROL HEMISPHERE (in mg %)

White matter				Grey matter				Phases of increased pressure
in moist tissue		in the dry residue		in moist tissue		in the dry residue		
Control	Compressed hemisphere	Control	Compressed hemisphere	Control	Compressed hemisphere	Control	Compressed hemisphere	
RNA								
23.2	23.5	81.2	80.0	29.2	27.2	175.2	155	Compensation
36.6	34.1	110.0	120.0	30.0	29.2	147.0	162	Subcompensation
22.9	19.3	75.5	73.4	32.2	36.6	154.5	181	Decompensation
19.2	14.0	55.6	51.8	24.2	36.0	111.2	180	Terminal phase
DNA								
5.9	6.1	20.7	20.8	7.6	6.7	45.0	36.4	Compensation
4.0	4.5	12.0	15.8	4.0	4.4	19.6	24.6	Subcompensation
8.5	6.8	28.0	25.8	5.7	8.5	27.4	42.5	Decompensation
7.4	5.6	21.4	20.7	7.6	6.8	35.5	32.6	Terminal phase
Phospholipids								
325	312	1140	1002	197	197	1185	1066	Compensation
245	250	730	875	160	145	784	812	Subcompensation
310	262	1032	987	160	160	770	800	Decompensation
265	180	768	666	197	190	908	912	Terminal phase
Phosphoproteins								
1.6	1.6	5.67	5.34	1.8	2.0	10.4	11.4	Compensation
1.8	2.1	5.46	5.45	2.8	2.8	13.6	15.7	Subcompensation
0.9	0.8	2.93	3.20	0.8	1.0	2.8	3.6	Decompensation
1.4	1.2	3.9	4.4	1.2	1.4	5.7	6.4	Terminal phase

the results per unit dry weight of tissue showed the same trends. Levels of these compounds did not change in the grey matter in either of the phases studied, except for a slight increase in RNA during the terminal phase.

Changes in the ^{32}P Turnover

Table 2 gives the changes in relative specific activity expressed, as a percentage thus

$$RSA = \frac{\text{Value for compressed hemisphere} \times 100}{\text{Value for control hemisphere}}$$

TABLE 2. CHANGES IN THE SPECIFIC ACTIVITY OF RNA PHOSPHOLIPIDS, AND PHOSPHOPROTEINS IN THE COMPRESSED HEMISPHERE COMPARED TO THE CONTROL HEMISPHERE (in %)

White matter	Grey matter	Phases of increased pressure
RNA		
+18	+29	Compensation
+26	+20	Subcompensation
+63	+6	Decompensation
Phospholipids		
+11	+12	Compensation
+14	+14	Subcompensation
+36	+11	Decompensation
Phosphoproteins		
+20	+13	Compensation
+6	+12	Subcompensation
+28	+29	Decompensation

This table also shows that the relative activity in both grey and white matter of RNA, PI and PP tends to increase during compensation, subcompensation and decompensation. These increases could, according to the theories of Palladin and other authors (13-20) indicate increased functional activity of the brain. We consider that this interpretation is indeed correct for the compensation and subcompensation phases where the brain reacts to the insertion of the cannula, but during decompensation when the animal is in an apparent state of inhibition the above changes could hardly be due to enhanced functional activity.

Oedematous conditions of the brain are usually thought to be due to increased vascular permeability (4, 21) and since swelling of the tissue is well established at decompensation, the biochemical changes also may be due to increased vascular permeability. Table 3 gives values for the RSA of the acid-soluble phosphate in the white and grey matter (referred to the blood phosphate specific activity.)

During compensation, the ratio of tissue acid-soluble phosphate specific activity in either hemisphere to that of the blood are the same, indicating normal permeability. Any increase in total tissue radioactivity due to pressurization would result from an increased turnover rate of the phosphorus compound.

During subcompensation and decompensation however a higher RSA is observed in the white matter from the pressurized hemisphere than the

TABLE 3. THE RELATIVE SPECIFIC ACTIVITY OF THE ACID-SOLUBLE PHOSPHATE FRACTION IN THE COMPRESSED HEMISPHERE AND THE CONTROL HEMISPHERE RESPECTIVELY

White matter		Grey matter		Phases of increased pressure
Control	Compressed hemisphere	Control	Compressed hemisphere	
5.08	4.9	25.6	26	Compensation
5.67	9.9	17.3	73.5	Subcompensation
5.4	10.4	20.1	20.2	Decompensation

control side. Since the RSA is referred to the blood ^{32}P level such an increase suggests a disorder of vascular permeability. The increased specific activities of RNA, PI, and PP from the white matter of the pressurized hemisphere are therefore most likely to be due to disordered vascular permeability rather than increased metabolism.

Such a hypothesis is supported by data described previously in this report. Where tissue swelling was well established the contents of various phosphorus and nitrogen containing compounds, cholesterol and phospholipids apparently fell when expressed per unit wet weight of tissue but when corrected to unit dry weight these apparent decreases were much smaller and sometimes reversed, probably due to disordered vascular permeability. What does remain unexplained is the fact that during subcompensation the RSA of the acid-soluble phosphate from the compressed hemisphere increases although no tissue swelling can be observed. Also unexplained is the increase in the specific activities of RNA, PI, and PP of the grey matter from the compressed hemisphere when no tissue swelling or signs of disordered vascular permeability could be observed.

Much therefore remains to be understood of the biochemical changes immediately preceding the appearance of brain swelling and oedema.

CONCLUSIONS

1. Swelling of the brain is not responsible for increased intracranial pressure; in some cases the swelling may precede this increase. Swelling in response to increased intracranial pressure is indistinct and variable during subcompensation and decompensation; only in the terminal phase is this response well defined.

2. Swelling in response to artificially raised intracranial pressure occurs only in the white matter of the hemisphere in which the cannula is inserted. The grey matter remains unaffected.

3. White matter from compressed and swollen hemispheres is characterised by an increased water content and decreased cholesterol, phospholipid, acid-soluble and total phosphate, RNA, DNA and phosphoprotein phosphate and sometimes non-protein nitrogen. These decreases were calculated per unit wet weight of tissue but if expressed per unit dry weight, they largely disappeared. This is probably due to infiltration of the grey by white matter and increased vascular permeability.

4. The increase in RSA of acid-soluble phosphorus from swollen white matter is considered indicative of disordered vascular permeability.

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THE FUNCTIONAL BIOCHEMISTRY OF THE BRAIN

COMMUNICATION TO THE XXth INTERNATIONAL CONGRESS
OF PHYSIOLOGY AT BRUSSELS, JULY 30th–AUGUST 4th, 1956

A. V. PALLADIN

Institute of Biochemistry, Ukrainian Academy of Sciences, Kiev

THE task of functional biochemistry of the brain is to reveal correlations between the specific functions of the nervous system and the chemical structure and metabolism of nervous tissue, as well as to understand the mechanisms by which the nervous system regulates metabolic processes in the body. Although these problems are at present receiving considerable attention all over the world, they are still at a comparatively early stage of development, and the results of workers of different disciplines are often conflicting.

In this lecture I want to discuss the results of investigations done under my directions mainly at the Biochemistry Institute of the Ukrainian Academy of Sciences, and work of other Soviet Scientific Institutes.

One of the most promising approaches in by the study of brain metabolism during experimental stimulation and inhibition, and another is the study of chemical change during growth and development of a species.

THE CHEMICAL COMPOSITION AND METABOLISM OF DIFFERENT FUNCTIONAL PARTS OF THE BRAIN

Proteins play an important part in the function of the central nervous system and their greatest concentration is in the cerebral cortex, then the white matter, then the spinal cord, and least in the peripheral nerves (1). Our own studies have shown that the grey matter of the cerebral hemispheres, which is the youngest developmentally and the most complex functionally, contains the greatest quantity of proteins; lesser amounts are found in cerebellar cortical grey matter, in sub-cortical grey matter, and least in spinal grey matter. This suggests that proteins play an important role in the function of the higher parts of the brain (2).

Proteins of the grey and white matter differ both qualitatively and quantitatively; for example, the grey matter contains more water-soluble and less insoluble protein residue than the white matter (3).

Recently we have used zonal electrophoresis to study the water-soluble proteins of the brain and were able to confirm that functionally different parts of the nervous system have different protein compositions (Palladin and Polyakova (4)). We have also used radioactive isotopes to study the rate of protein metabolism both in tissue preparations and in the intact animal.

We found that the rate of incorporation of ^{35}S -labelled methionine into the proteins of cat brains was highest in the cerebral grey matter and in the cerebellar grey matter, which are the youngest and most functionally developed parts of the brain. The lowest rate of protein turnover was found in the spinal cord which is the oldest phylogenetically. The rate of protein turnover in the white matter of the hemispheres approaches that of the spinal cord, and the medulla oblongata, the mesencephalon and the optic thalamus occupy an intermediate position (Palladin and Vertheimer (5)).

Similar results in grey and white matter of brain were obtained by Cohn, Gaitonde and Richter (6) who, using autoradiography after intraperitoneal or intracisternal injections of ^{35}S -methionine, found that the incorporation of ^{35}S takes place at a greater rate in the grey than in the white matter of the brain (7). Some authors have injected labelled methionine intravenously and found that its rate of incorporation into the brain was very low and that brain proteins were inert (8, 9, 10), which is, of course, in conflict with our findings.

To calculate the rate at which methionine was incorporated into the brain protein, Gaitonde and Richter estimated the specific activity of the acid-soluble fraction of the brain and compared it with the specific activity of the proteins (11). They showed that brain proteins possess a high metabolic activity, even higher than the liver (12).

Functionally different areas of the brain have different amounts of nucleic acids. We have shown that the cerebral cortex and the white matter of the cerebral hemispheres have similar total amounts of nucleic acid including ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) although the cortex contains a somewhat larger quantity of these substances (Skvirskaya and Silich (13)). The content of nucleic acids, particularly DNA, in the cerebellum is considerably higher than that in the grey matter and even more than in the white matter of the cerebral hemispheres. This is explained by the density of small cells in the cerebellum, and

the large concentration of nuclei, particularly in the granular layer. We found that in rabbits the rate of incorporation of labelled phosphorus into RNA in the grey matter of the brain is lower than in the white matter and in the cerebellum (13).

Similar results have been reported by Kreps in Leningrad (14). In dogs which have a higher degree of cortical function, the rate of RNA turnover is highest in the cerebral hemispheres. This also shows that nucleic acids play an important part in the function of the nervous system. The comparatively high RNA turnover in the white matter of the cerebral hemispheres indicates that white matter is by no means metabolically inert; the nervous pathways have the same basic nervous mechanisms as the nerve cells themselves. The white matter also contains a considerable amount of enzymes, particularly of the oxidative type (15).

The rate of incorporation of radioactive phosphorus into the phospholipids is highest in the grey matter of the cerebral hemispheres, then the cerebellum and lower in the white matter (13, 14). Glycolysis also takes place at different rates in different parts of the brain. We have found that the enzymes of carbohydrate metabolism—hexokinase, aldolase, phospholipase and adenosinetriphosphatase—are most active in the cerebral cortex and in the cerebellum (16, 17, 18, 19). Polyakova has shown by adsorption chromatography that the grey and white matter of the human cerebrum have different quantities of steroids, and that grey matter contains 7-oxysterol, which is not found in the white matter. Tracer studies show that the motor area has a higher rate of incorporation of phosphorus into RNA and into phospholipids than does the auditory area (14).

Palladin, Rashba and Shtutman developed a method for the isolation of the nuclei of cells from the cerebral cortex and cerebellum and showed that the nucleoproteins of the nuclei of grey matter of the cerebral cortex were 20–30 per cent RNA and 70–80 per cent DNA, while the nuclei of the cerebellum were 12–13 per cent RNA and 87–88 per cent DNA (21, 19).

Biochemical studies of particulate fractions are of great value (Flexner (22), Bodian (23), and Pope (24)). Abood and his co-workers fractionated the grey matter of the brain and the white matter of the spinal cord into a nuclear fraction, a mitochondrial fraction, and supernatant, and showed that dehydrogenase and phosphatase activities were mainly localized in the mitochondria and glycolytic enzymes in the cytoplasm. The lipids were also localized in the mitochondria which contained 50 per cent of all phosphatides; the DNA was localized in the nuclei and the RNA in the cytoplasm.

THE METABOLISM OF THE BRAIN DURING
ONTOGENESIS AND PHYLOGENESIS

Kreps and his co-workers (26, 27, 28, 29, 30) studied the biochemistry of the brain during the growth of the animal and during evolutionary development. They studied a number of enzyme systems in the brain and compared the biochemical development with the morphological and functional maturation of the central nervous system. Verzhbinskaya showed that in the evolution of vertebrate brains the rate of respiration increased, anaerobic glycolysis decreased and the activity of the whole cytochrome system increased (31, 32).

Skvirskaya and Chepinoga (33) estimated the nucleic acids and phosphoproteins in the whole brain of rabbits at various times during their foetal and post-natal development, and found that nucleic acids were high in the early stages of foetal development and gradually decreased. After birth the rate of this decrease became less and by 1 month of age it had reached the adult level. The phosphoprotein content of brain was also high in the early stages of development (109). Manukian showed the same general trend in different parts of the brain as in the whole brain during development (34, 35), but the cerebellum is an exception. Unlike other parts of the brain, the cerebellum increases its content of nucleic acids up to 5 days after birth and this high level is maintained until about the 17th day.

The phospholipid content of all parts of the brain increases with age, mainly due to myelination of nervous fibres. The phospholipids of the brain show a very high rate of turnover in the early stages of development. If one examines their turnover rates it is seen that there is a low phospholipid metabolism at that time when phospholipids are accumulating rapidly in the membranes of nerve fibres, which have low rates of metabolism.

Protein metabolism also undergoes changes with age. Gaitonde and Richter (77a) found that the rate of incorporation of methionine into the proteins of the brains of young rats was higher than in adults. Belik and Krachko (111), at our Institute, have studied the incorporation of radioactive sulphur into the proteins of the brain of newly born, 1 month old, and adult rabbits. They found that with increasing age, incorporation of ^{35}S into brain protein decreased and its incorporation into the lipid fraction also decreased.

Although species differences in nucleic acid, phospholipid and phosphoprotein turnovers cannot be found, their turnover rate is much higher in warm-blooded than in cold-blooded animals. This difference is particularly marked in the phospholipids (Gasteva (36)).

These data provide evidence for the close correlation between the level of energy metabolism and rate of turnover of phosphorus compounds of high molecular weight. In cold-blooded animals, the intensity of energy yielding processes in the brain is considerably lower than in warm-blooded animals, and the metabolism of their high molecular weight phosphorus compounds is much slower (37).

In all animals phospholipid turnover is much lower in the spinal cord than in the brain, possibly because there is a large quantity of metabolically inert lipids in the myelin.

Hyden (38) did some very interesting X-ray micrographs on frozen sections of nerve cells. He estimated the levels of lipids, ribonucleoproteins and proteins in single cells, and found that as they get older their nucleoproteins decreased and were replaced by lipoproteins. If the neurone was stimulated the nucleoprotein content decreased, but the protein residue was unaffected. It thus appears that there are two types of change in the nerve cells; slow changes due to ageing, and others involving the metabolism of nucleoproteins due to activity. Hyden regards the latter as the chemical basis for the function of the neurone.

Bulankin studied the brain proteins of rats and found that as the animals aged, the nucleic acids were replaced by lipid compounds. Similar changes occurred in the whole brain, with the nucleoproteins becoming richer in protein. The glycolipid content of the brain also changed with age. In human embryonic brain gangliosides occur, but they are later replaced by cerebroside (Svennerholm (106)).

METABOLISM DURING STIMULATION

Recently McIlwain, Weil-Malherbe and others have stimulated brain slices electrically *in vitro* and studied their metabolism under these conditions. It should however, be kept in mind that while the physiological and functional importance of metabolic processes can only be elucidated *in vivo*, *in vitro* experiments reveal the reactions which participate in metabolism.

Cutting brain sections can lead to rapid breakdown of a number of compounds which are of fundamental importance to the activity of the brain. Furthermore, stimulation of nervous elements which are deprived of their natural connections with other parts of the brain may produce a different picture from that in the whole brain. For example, certain barbiturates were found to increase the phosphocreatine content of the brain *in vivo* (40, 41), while they decreased it *in vitro* (42). The inorganic phosphates

also showed opposite changes. On the other hand, sometimes parallel effects are seen. Narcotic drugs, for instance, inhibit the respiration of brain slices as well as whole brain (43, 44). We must, therefore, have reservations in applying the results of *in vitro* experiments to conclusions about the functional biochemistry of the brain.

We have investigated the labile phosphates in the brain during excitation and inhibition. Because the brain consumes about a quarter of the total oxygen consumed by a person, and its temperature is 0.5°C higher than arterial blood, its metabolic processes must always be very active. This considerable degree of activity is modified by excitation or inhibition.

When increased activity was induced by convulsions or electrical stimulation the authors found increased temperature, blood flow, oxygen consumption and electrical activity in the cerebral cortex; glycolysis was increased, lactic acid production was increased, and high-energy phosphates like ATP were broken down.

Olsen and Klein (45) found that convulsions caused by electrical stimulation to cats caused a decrease in the glucose, glycogen, ATP and phosphocreatine in the brain, with an increase in inorganic phosphate and lactic acid. Lactic acid also increased in convulsions caused by strychnine and camphor (46). Stone, using rapid freezing in liquid air, studied the effect of various convulsants on the brain lactic acid. After physical work the brain lactic acid rose in the same way as it did in convulsions induced by picrotoxin, sodium cyanide and metrazol (47). Stone, Webster and Gurdjian (48) showed an increased lactic acid and inorganic phosphate in the brain during metrazol-induced convulsions with a corresponding decrease in the brain phosphocreatine (48). Richter and Dawson demonstrated increased lactic acid in the brains of rats when they were excited emotionally (49).

It would thus appear that during increased activity the brain is unable to satisfy its increased requirements of oxygen and uses glycolysis as a source of energy, thus leading to increased lactic acid.

During excitation, phosphocreatine breakdown takes place very rapidly. Dawson and Richter (50) stimulated the skull of rats electrically for only 1 sec and found that 50 per cent of the phosphocreatine was broken down with a corresponding increase in the hexose phosphate content. The ATP decreased slightly as it was largely replenished by phosphocreatine. After electrical stimulation for 15–45 sec the phosphocreatine, ATP and phosphates gradually returned to normal.

Using a micro-incineration technique, it has been shown that in brain during convulsions potassium ions leave the nerve cells of the cerebral cor-

tex and sodium ions accumulate in them, as has been observed in peripheral nerve. This takes place whether the stimulation is caused by electrical, chemical or thermal stimuli (51).

Ayres and McIlwain developed a special apparatus to stimulate brain slices electrically in order to study their metabolism (52, 53). McIlwain and his co-workers have shown that stimulation of brain slices causes increased respiration and lactic acid formation (54) and a decrease in creatine phosphate with a rise in inorganic phosphate (55), as occurs *in vivo*; 2:4-dinitrophenol has a similar effect. He also showed that brain tissue in conditions which reproduced severe hypoglycaemia did not respond to electrical stimulation by increased respiration or lactic acid formation (56). McIlwain studied respiration and glycolysis in rabbit and guinea pig brain slices in media containing different glucose concentrations, and the effect of electrical stimulation on respiration and glycolysis (57). He found that electrical stimulation increased tissue respiration as it did in human brain sections.

Heald studied phosphocreatine in cortical slices during electrical stimulation, and showed that there was a maximal breakdown of phosphocreatine within 5 sec. The breakdown of phosphocreatine was preceded by a slight decrease in ATP which was restored as soon as breakdown of phosphocreatine began. If the stimulation lasted only 7 sec the creatine phosphate was resynthesized and returned to normal within 20 sec (58).

At the Biochemistry Institute of the Ukrainian Academy of Sciences, we have been studying brain metabolism in various functional states and under various influences for several years (59, 60). Recently we have been studying the effect of excitation and inhibition, which, according to Pavlov, are the main physiological processes characterizing higher nervous activity (61, 65, 110). In the first stages of this study we used drugs to induce these states and later turned to the use of physiological conditioned and unconditioned stimuli.

Gorodisskaya (66) studied the effect of natural stimulation on proteolytic processes in the visual area of the cortex and found that as the optic centres increase their activity, protein metabolism becomes more intensive.

Our own studies were devoted to the metabolism of nucleic acids, carbohydrates, adenosinetriphosphate, phospholipids and phosphoproteins during functional states in rabbits, dogs and rats, quick-freezing their brains in liquid air. Excitation was produced by injection of desoxyephedrine which is fairly similar to the stimulant amphetamine; we also used pentamethylenetetrazole which is in clinical use.

In our early experiments we caused prolonged excitation either with large doses of pentamethylenetetrazole or with electric currents and found a decrease in the glycogen and ATP content of the brain (67, 68). These results confirm the findings of Olsen and Klein (45) and others (40, 69).

In a more physiological series of experiments, excitation was produced by a single injection of 5–7 mg/kg desoxyephedrine or 50–70 mg/kg pentamethylene tetrazole, 4 hr before the animal was killed. However, the two stimulants cause different changes (67). Pentamethylenetetrazole produces a greater increase in lactic acid than does desoxyephedrine (70). While the former has no effect on the ATP and glycogen, the latter increases them. Desoxyephedrine excitation also causes a slight increase in RNA. It thus seems that desoxyephedrine increases the capacity of the nervous system to work, while pentamethylenetetrazole stimulates the cortex without increasing its working capacity.

Metabolic changes similar to those observed in convulsions (Khaikina and Goncharova (17)) were found in our laboratory in a state of "conflict" of nervous activity, as a result of two opposing stimuli—an elementary conditioned stimulus, a bell, and an unconditioned electrical stimulus. During this state of "conflict" the ATP content of rat brain decreased and there was an increase in inorganic phosphates, lactic acid and glycolysis.

During stimulation, tracer studies can reveal changes that are not normally detectable chemically. For example, desoxyephedrine stimulation does not show appreciable changes in the total phospholipids or their separate fractions (72), but using radioactive phosphorus we were able to show that the incorporation of phosphorus into both fractions of phospholipids was not normal.

Vladimirov (73) stimulated the skin of rats' paws electrically for 3 hr and studied their rate of incorporation of radioactive phosphorus. He showed an increase of 20 per cent in the RNA turnover, and of phospholipid turnover of 50 per cent. If one combines electrical stimulation with an indifferent stimulus and develops a conditioned reflex in the rat on the basis of an unconditioned defence reaction, conditioned stimulation will also cause an increase in RNA metabolism as judged by radioactive phosphorus turnover.

Another result of excessive nervous stimulation was caused in rats by two different methods: (i) electrical stimulation in a specially designed cell for 1 to 1½ months, and (ii) keeping the animal awake day and night in a rotating drum. The incorporation of ^{32}P into RNA and phospholipids of the brain takes place at a sub-normal rate during chronic over-stimula-

tion by electric current or artificial insomnia. This is probably due to exhaustion of the central nervous system and weakening of its activity as a result of excessive strain (74).

According to Vladimirov (107), excitation of the brain is associated with increased turnover of hexose phosphates, which is consistent with the other changes in carbohydrate metabolism.

Nechayeva (75) showed that in a state of excitation produced by electrical stimulation of the skin of the paws of rats, the rate of incorporation of ^{35}S into the brain protein increased. This increase, however, was only observed when the excitatory state was well marked. If signs of inhibition due to excessive stimulation occurred, the rate of protein sulphur turnover was approximately the same as that of animals in a state of rest. Similar results were obtained with ^{14}C -labelled glycine, when incorporation into rat brain proteins became more intensive during excitation (76).

Gaitonde and Richter (77, 77a), on the other hand, found that electrically induced convulsions caused a scarcely detectable decrease in the rate of incorporation of labelled methionine into brain proteins. This apparent discrepancy may be due to the different times which elapsed in the different experiments between the injection of the tracer and the sacrifice of the animal. For example, in our laboratory, Belik (78) was able to detect an increased rate of incorporation of labelled methionine into brain proteins $2\frac{1}{2}$ hr after injections of methionine, but at 12 hr no appreciable change in the rate of its incorporation could be observed.

Taking into account the fact that the effect of stimulation can vary with the nature and time of the stimulus, Palladin and Rybina (79) studied ATP metabolism in the brain 1, 2, and 4 hr after injection of desoxyephedrine or pentamethylenetetrazole. They showed that with desoxyephedrine hyperexcitability, the ATP content of rabbit brain fell during the first hour, gradually rose to normal during the second hour and increased above normal by 4 hr. The inorganic phosphate showed a reverse change (70).

In these studies very unphysiological stimuli were used and the animals were small and their cytoarchitectonics were insufficiently known to investigate functionally homogeneous areas of the cerebral cortex. For this reason Smirnov (80) studied phosphorus metabolism in the various parts of the cerebral cortex of dogs during increased but quite natural activity of one of the cortical analysing systems, the auditory area. The metabolism of RNA and phospholipids was studied with radioactive phosphorus against a background of a conditioned reflex developed in response to an auditory stimulus. The stimulation by electric bell, buzzer or whistle was accompanied by alimentary reinforcement.

He showed that there was a considerable increase in the rate of turnover of RNA and phospholipid phosphorus in the auditory area as a result of sound stimulation, compared with controls. No changes in the phosphorus metabolism could be observed in the motor or visual areas of the cortex or in the medulla oblongata.

A number of authors have studied ammonia formation in the brain during excitation, and always found an increase of ammonia in the brain (Tashiro (81), Winterstein and Hirschberg (82), Gerard and Meyerhof (83), Pravdich-Neminsky 84)). Vladimirova found that camphor excitation caused an increase in brain ammonia, as did unconditioned painful electrical stimuli. According to Richter and Dawson (87) the ammonia content of the brain increases during picrotoxin convulsions, electrical stimulation and anoxia. The adenine system is not the only source of ammonia in the brain as it is in muscle (88) but probably more of it comes from breakdown of protein (108).

It thus appears that increased activity of the brain during excitation is accompanied by increased glycolysis, increased ammonia formation and increased turnover of proteins, phosphoproteins, nucleic acids, and phospholipids. The particular metabolic changes can vary with the type of stimulation and, where stimulation is excessive, it can lead to exhaustion and a state of inhibition.

THE METABOLISM OF INHIBITION

One can produce inhibition of the nervous system by different methods. Some of these cause a state similar to the inhibition of natural sleep, like small doses of narcotics, while others cause a more profound inhibition, as in anaesthesia. During anaesthesia there is a decrease in reflex activity, a fall in brain temperature, a decrease in brain electrical activity, a slowing down of the circulation and a lowering of oxygen and glucose consumption.

During barbiturate anaesthesia the phosphocreatine level in mouse brain increases and the inorganic phosphate and lactic acid decrease (Stone (91, 92)). Richter and Dawson (49) found a decrease in brain lactic acid during pentobarbitone anaesthesia as well as during sleep.

Inhibition also affects ammonia formation in the brain. A decrease in brain ammonia can be found in urethane narcosis (85), pentobarbitone narcosis (87) and thiopentone anaesthesia (67, 93), and also in other conditions which lead to a state of inhibition in the cerebral hemispheres. Vladimirova found that the brain ammonia of rats in deep sleep was only half that found when the animal was awake.

Ammonia is an excellent biochemical indicator of the functional state of the brain, as can be seen from Vladimirova's experiments (94). If the rat cannot escape from the electrical stimulation it moves for a few seconds and then stiffens for a long time in a characteristic position—standing on its hind legs. One can then tell whether excitation or inhibition is the predominating influence on the central nervous system by estimating the brain ammonia. During the first 15 sec the brain ammonia increases as it always does during excitation. After a minute the brain ammonia returns to normal and then falls below normal in 2 min. This is interpreted to mean that when the rat is standing stiffly on its hind legs inhibition has supervened upon excitation.

In our experiments small doses of barbiturates and urethane were given to rabbits and rats to induce a sleep which was similar to natural sleep; the animals still reacted to sound, touch and similar stimuli. In 4 hr of such sleep the intensity of glycolysis is hardly different from normal and the glycogen content increases, which suggests a decrease in consumption of carbohydrates. The adenosine triphosphate increases (67) and the glycogen turnover (76) and hexose phosphates decrease (107).

Dawson and Richter (95) found that during pentobarbitone narcosis the incorporation of radioactive phosphorus into the nucleoproteins and phospholipids of the brain decreased, which suggests that sleep inhibits their synthesis. We studied the incorporation of ^{32}P into ribonucleic acid phosphoproteins and phospholipids in rat brain during 24 hr of drug-induced sleep (Skvirskaya and Silich (96)) and showed that the turnover of RNA, phosphoproteins and phospholipids decreased by 23, 20 and 26.5 per cent respectively. Similar results for RNA and phospholipid turnover were obtained by Vladimirov (73) using other drugs to induce sleep. Changes in phospholipid metabolism could only be demonstrated by tracer techniques.

The decrease in lipid metabolism in amylobarbitone-induced sleep is confirmed by the fact that sulphatide-sulphur turnover decreases (75).

These are only effects of short-lasting sleep on metabolism. In longer experiments, where sleep was induced for 24 hr and 9 days, the decreased specific activity for RNA and phosphoprotein was more marked after 9 days than 1 day, whereas the phospholipid was less marked. The uptake of radioactive phosphorus from the blood into the brain was decreased in the first day and this decrease was more marked in 9 days.

The protein metabolism of brain does not escape the effect of inhibition. The rate of incorporation of ^{35}S -labelled methionine and carbon-labelled glycine is decreased (75, 76). Gaitonde and Richter (77a) found that the

incorporation of ^{35}S into brain protein was decreased during ether and pentobarbitone anaesthesia by 24–34 per cent

In view of the possibility that drugs had physiological effects other than producing sleep we decided to study animals during the natural inhibition of hibernation. Skvirskaya and Silich (97) studied the Siberian marmot in the following conditions: (i) awake, (ii) hibernation, (iii) having been awakened from hibernation 4 hr before, and (iv) in drug-induced sleep lasting 24 hr. They showed that radioactive phosphorus incorporation into phosphoproteins and phospholipids of the brains of these animals during hibernation was only a fraction of that found in the awake animals, and the rate of incorporation into RNA was practically zero. During narcotic sleep ^{32}P incorporation was also decreased but to a lesser extent than during hibernation. If the animals were artificially awakened the ^{32}P incorporation into RNA phosphoprotein and phospholipid was increased but did not reach anything like the levels of the naturally awake animals.

The rate of passage of phosphorus across the blood-brain barrier was also decreased during hibernation.

Ribonuclease—the enzyme responsible for the first stage of breakdown of RNA, and phosphoprotein phosphatase, had decreased activities during hibernation. If the marmots were artificially awakened, the ribonuclease and phosphoprotein phosphatase activity increased to the level of awake animals.

Smirnov showed that in natural sleep the turnover of phosphorus in phospholipids and RNA was considerably decreased in the motor and visual areas, with only a slight decrease in phospholipids and a very slight decrease of RNA in the auditory area. This suggests that in dog the auditory area of the cortex is less inhibited than the motor and visual areas. This is interesting when one recollects that a dog remains sensitive to auditory stimulation during sleep and reacts to it very rapidly.

In sleep, phosphorus metabolism decreases in the diencephalon as well as in the cerebral cortex, which confirms Pavlov's view that during sleep a state of diffuse inhibition spreads into the sub-cortical region. Comparison of phosphorus metabolism during natural sleep with amylobarbitone-induced sleep, in both of which cases the dog can be easily awakened, shows that there is a similar effect on phospholipid and RNA metabolism in the motor and visual areas. The auditory area is slightly depressed during narcotic sleep in regard to its phosphorus metabolism compared with natural sleep and, indeed, in narcotic sleep the dog does not respond to weak auditory stimuli.

In deep narcosis due to large doses of drugs, the phospholipid and RNA turnover was much lower in the diencephalon and all three zones of the cerebral cortex than in narcotic sleep, which suggests a more profound inhibition of the nervous system.

In summary, it can be seen that changes in brain metabolism caused by inhibition are opposite to those caused by excitation. In inhibition breakdown processes are slowed, ATP and glycogen increases, glycogen turnover decreases, ammonia content decreases and the rate of turnover of proteins, phosphoproteins, RNA and phospholipids decrease.

Changes in the functional state of the brain also occur in avitaminosis. Palladin and Vertheimer (5) studied the protein turnover with labelled methionine in vitamin C and E deficiencies. In vitamin C deficiency they found a slight decrease in turnover rate of brain proteins. In vitamin E deficiency the protein turnover rate in the cerebral hemispheres and the cerebellum decreased by 50 per cent.

HYPOXIA

In brain sections during anoxia and poisoning with substances suppressing oxidation, the incorporation of ^{32}P into phospholipids (Schachner, Fries and Chaikoff (99)), into phosphoproteins (Engelhardt and Lisovskaya, 100) and into nucleic acids (Findlay, Rossiter and Strickland, 101) almost stops. Kreps (102) repeated these investigations on intact animals and found that if rats are subjected to pressure changes, equivalent to being at an altitude of 9000–11,000 metres for 2–4 hr, the severe hypoxic conditions are not accompanied by appreciable changes in the incorporation of phosphorus into high molecular weight phosphates of the brain. The differences between the experiments on brain sections and intact animals is probably due to the possession of compensatory mechanisms in intact animals. If hypoxia is continued beyond the limits of a compensating mechanism, the oxygen tension in the nervous tissue falls and consequently the turnover of RNA, phosphate proteins, and particularly phospholipids, decreases even in the intact brain.

Decrease in phospholipid metabolism occurs at a level of hypoxia at which there is no appreciable decrease in the metabolic rate of nucleic acids and phosphoproteins (Chetverikov, 103). It seems that different levels of hypoxia have different effects, depending upon whether the phosphate metabolism is affected.

Recently Kreps (104) has studied the effect of hypoxia on the nucleoproteins in the cortical cells of rats, using ultra violet microscopy. During

hypoxia the nucleic acids and nucleoproteins decrease in the cytoplasm and nucleoli of the cortical cells and the cells of Ammon's horn; this decrease is due to rapid breakdown of nucleoproteins, intense elimination of nucleic acids from the cells and disorders in nucleic acid synthesis.

In my lecture I have not attempted to cover the whole field of the functional biochemistry of the brain, but I hope that I have shown some of the considerable advances that have been made in relating the chemistry and function of the brain, particularly during excitation and inhibition. There is, particularly for the joint study of biochemists and physiologists, a great amount of work to be done, and by the co-operation of scientists of all parts of the world progress can be achieved for the common good of all peoples.

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