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CHANGES IN THE GLYCOGEN CONTENT OF THE BRAIN DURING EXPERIMENTAL ISCHEMIA *

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Quantitative determinations of the glycogen content were carried out in the brains of 10-week-old Wistar rats subjected to bilateral ligation of the common carotid arteries. Determinations of glycogen were carried out 6, 12, 24, 48, 72 and 120 hours after arterial ligation.

Biochemical investigations were supplemented with histological and electron microscopic investigations and with histochemical determination of glycogen. The following conclusions have been reached:

1. Bilateral ligation of the common carotid arteries leads to a considerable rise in the glycogen content in the brain without evidence of morphological changes in its structure. This rise is statistically significant as compared with the control group examined 12 hours after ligation and reaches its peak value 48 hours after ligation. During 5 days after ligation the glycogen level fails to return to its initial value.

2. Histochemically detectable abnormal deposits of glycogen in the nervous tissue are localized in the gray matter of the brain, mainly in the cortex and to a lesser extent and at earlier periods only in the basal ganglia. Deposit formation in the white matter occurs very rarely. Electron microscopic investigations demonstrate that polysaccharide deposits accumulate exclusively in the cytoplasm of astrocytes and in their processes.

3. General anesthesia with ether leads to a slight but statistically significant rise in the glycogen content of the brain exclusively within 12 hours after administration of the anesthetic agent. Later, this effect disappears completely.

The authors suggest that accumulation of glycogen under conditions of partial ischemia of the brain is due to an impaired consumption of glucose by the nervous tissue.

The deposition of glycogen in the central nervous system is a common occurrence in lesions of various kinds. Abnormal deposits of this polysaccharide resulting usually from disturbances in glucose metabolism occur most frequently either in the vicinity of foci of irreversibly damaged nervous tissue, e.g. around necrotic foci independently of their origin (*Shimizu, Hamuro* 1958, *Klatzo et al.* 1961, 1970, *Guth, Watson* 1968) or in tissues without any histological evidence of damage (*Miquel et al.* 1963, *Mossakowski et al.* 1968, *Long et al.* 1971, *Mossakowski* and *Zelman* 1971). Because of that, the deposits of histochemically

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detectable glycogen may be regarded as morphological evidence of metabolic disturbances in the nervous tissue and its minimal damage, undetectable by routine histological methods.

Among various noxious factors leading to the accumulation of glycogen in the central nervous system under experimental conditions a considerable group of factors have the common feature of reduced oxygen uptake by the nervous tissue. *Mossakowski et al.* (1968) described profuse accumulation of glycogen during perinatal asphyxia in newborn monkeys. The same change, resulting from moderate ischemia of the central nervous system, was observed by *Long et al.* (1971) and *Ibrahim et al.* (1970). These authors observed accumulation of glycogen in the nervous tissue following experimental simple anoxia. *Crowell et al.* (quoted after *Klatzo et al.* (1970) found analogous changes during transient cerebral anoxia in monkeys.

Most investigations, apart from those of *Rivera et al.* (1970) concerning the model of perinatal anoxia, were conducted using histochemical methods which restrict, of necessity, the possibilities of quantitative assessment of the changes.

The purpose of our investigations was a quantitative analysis of the changes in the glycogen content of rat brain during partial ischemia and an attempt at evaluating their dynamic development.

MATERIAL AND METHODS

The experiments were carried out on Wistar rats of both sexes aged 10 weeks. Under ether anesthesia the experimental animals underwent bilateral ligation of the carotid arteries. The control animals were subjected exclusively to ether anesthesia for the time corresponding to the duration of the operation on the experimental animals *.

The animals in both groups were sacrificed by decapitation after 6, 12, 24, 48, 72 and 120 hours from ligation of the carotid arteries or the end of anesthesia and their brains were taken for biochemical or histological and histochemical investigation.

An additional control group comprised rats which had not been subjected to any operation.

Biochemical investigations

Both cerebral hemispheres were taken for glycogen determinations. The tissue was immediately frozen in a mixture of methyl alcohol and dry ice (−70°C). The time from decapitation to freezing did not exceed 1·5 min. The frozen

* In was found in preliminary experiments that there is no difference in the glycogen content of the brains between the animals undergoing ether anesthesia solely and the animals undergoing a sham operation with incision of skin and bilateral exposure of the vessels and nerves without ligation of the carotid arteries.

parts of the brain were weighed on a torsion balance and put into test-tubes containing boiling-hot 30% KOH (3 ml). Brain tissue was hydrolysed on a boiling-hot water bath for 45 min. Then 3·6 ml ethanol was added to each test tube to precipitate glycogen from the fluid and the test tubes were left overnight in a refrigerator. Glycogen was removed by centrifugation. The glycogen precipitate was resuspended in a mixture of alcohol and chloroform (5 ml) and heated in an ultrathermostat at 50°C for 5 min. After that the fluid was centrifuged again. Extraction was repeated two times. The purified glycogen was suspended in 1 n HCl and hydrolyzed for 3 hours in a boiling water bath. The hydrolyzed glycogen solution was neutralized and its volume was brought to 7 ml. Glucose determination was done by the colorimetric method of Nelson (1944). The color intensity was read using a Spekol spectrophotometer at 540 mμ wavelength. Glucose concentration was read from a standard curve corresponding to 10, 20, 40, 80 and 100 μg glucose. The initial solution contained 100 μg glucose in 1 ml. The results of determinations were expressed in mg of glucose calculated for 100 g of brain tissue. The statistical analysis was carried out by Student's t-test.

Morphological investigations

Morphological investigations were carried out on 18 rat brains including 8 brains of experimental animals and 10 of control animals. The sections were stained routinealy with hematoxylin and eosin and glycogen was determined histochemically.

After removal of the brain from the skull it was divided with two cuts in the frontal plane (1 — frontal lobes at the level of tuber cinereum, 2 — posteriorly to the mamillary bodies) and the blocks were fixed in Rossman fluid, embedded in paraffin and cut with a microtome into sections 10 μ thick.

Determinations of glycogen were carried out in sections using the PAS reaction with a simultaneous blockage of the reaction with dimedone according to the method described by *Bulmer* (1959). The specificity of the histochemical reaction was checked by digestion with diastase.

Electron microscopic investigations

Three rats were used for electron microscopic investigations (2 rats after ligation of both common carotid arteries and 1 rat after ether anesthesia). All animals survived 48 hours after these procedures. The rats were sacrificed by decapitation, their skulls promptly opened and small sections of tissue obtained from the fronto-parietal areas of both cerebral hemispheres. The obtained material was fixed for 2 hours in a 4% solution of glutaric aldehyde n phosphate buffer at pH 7·3, and later in a 2% solution of osmium tetroxide. ihe fixed tissue was dehydrated in ethanol of rising concentrations and embedded

in Epon 812. The material was cut on a Reichert ultramicrotome. Ultra-thin sections were stained with uranyl acetate and lead citrate and were examined under a JEM-7A electron microscope.

RESULTS

Clinical observations

None of the experimental animals showed any evidence of damage to the central nervous system after the disappearance of the effects of anesthesia, apart from bilateral ptosis observed in most animals which may be related to injury to the sympathetic carotid plexus during ligation of the common carotid arteries.

Quantitative determinations

The results of glycogen determinations in the group of animals with bilateral arterial ligation as well as in the group of control animals undergoing only ether anesthesia are shown in Table I.

Table I

Glycogen content in brains of rats at various time periods after bilateral ligation of carotid arteries and anesthesia

Time after ligation	Glycogen content mg per 100 g of tissue		t	p
	experimental animals (carotid ligation)	control animals (anesthesia)		
6	47·9* ± 5·2** (5)***	38·6 ± 7·5 (5)	2·3	0·1 > p > 0·05
12	55·9 ± 4·8 (7)	45·3 ± 4·6 (7)	4·2	0·02 > p > 0·01
24	53·4 ± 2·5 (5)	30·9 ± 4·0 (5)	10·7	< 0·001
48	73·0 ± 4·9 (6)	38·3 ± 3·3 (6)	14·5	< 0·001
72	70·5 ± 5·3 (5)	29·2 ± 3·7 (5)	5·4	0·02 > p > 0·01
120	63·2 ± 2·3 (4)	32·0 ± 1·7 (4)	21·8	< 0·001

* arithmetical mean
** standard deviation
*** number of animals in group
t — random variable in the test of Student
p — probability

The glycogen content of the brains of experimental animals in all survival groups was higher than in the controls. In the first survival group studied, that is in rats sacrificed 6 hours after arterial ligation, the differences in the glycogen content between the experimental and the control group were not statistically significantly different.

In all subsequent survival groups starting with that sacrificed 12 hours after ligation these differences were already highly significant statistically. The rise in the glycogen content occurred fairly gradually (diagram 1). The maximum increase was found 48 hours after the experiment remaining at the same level

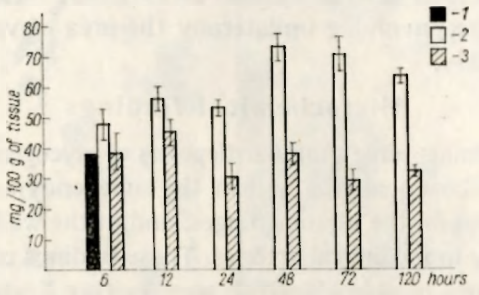

Diagram 1. Differences in glycogen content in the brain tissue of rats at various time intervals following bilateral carotid arteries ligation and ether anesthesia, compared with that in normal animals: 1 — normal animals, 2 — operated animals, 3 — anesthetized animals.

Table II
Comparison of glycogen content in brains of non-experimental and anesthetized rats

Rat group		Glycogen content in mg per 100 g of tissue			t	p
Intact rats		37·9*	±1·2**	(6)***	—	—
Anesthetized rats, after	6 hours	38·5	±5·6	(8)	0·29	0·8>p>0·7
	12 hours	44·4	±4·7	(7)	3·3	<0·01
	24 hours	30·9	±4	(5)	2·27	0·05>p>0·02

* arithmetical mean
** standard deviation
*** number of rats
t — random variable in the test of Student
p — probability

for the further hours and being still significantly different from the glycogen content in controls 120 hours after arterial ligation.

The glycogen content in the brains of control animals changed slightly during the first 24 hours after ether anesthesia as compared with the glycogen content in the brains of animals which had not been subjected to any experimental procedures. However, in relation to the starting level these changes were not statistically significant in the 6th and 24th hours of the experiment (Table II). Only a slight rise in the glycogen content at the border of statistical significance was observed 12 hours after anesthesia.

Morphological findings

The histological appearance of the brains of animals with bilateral ligation of common carotid arteries was not essentially different from that in the control animals, independently of the time lapse after the procedure. In only one rat sacrificed 120 hours after arterial ligation an extensive focal necrosis was found in the cerebral cortex involving unilaterally the area of vascularization of the middle cerebral artery.

Histochemical findings

In the control animals some granular deposits of glycogen were present in the cells of the ventricular ependyma and in the subependymal layer, in the cells of the chorioid plexus, in the leptomeninges, and in the walls of certain cerebral arteries, particularly in meningeal arteries. These findings corresponded to these observed in the brains of animals which were neither ligated nor anesthetized.

In the experimental group the presence of abundant granular deposits of glycogen was observed in the cerebral hemispheres. Apart from the sites of their typical localization they were present in the cerebral cortex and less frequently in the basal ganglia. In some rats they were also found in the subcortical white matter. These deposits appeared as minute granules scattered in the neuropil of the gray matter and grouped in larger amounts around the blood vessels (Fig. 1), or around the glial nuclei, especially the astroglial nuclei (Fig. 2), their arrangement there suggesting the intracytoplasmic localization. Sometimes, larger accumulations of the polysaccharide filling the perikaryon as well as the processes of astrocytes showed their silhouettes in a way resembling the appearance of glia after Cayal's impregnation (Fig. 3). Cortical glycogen deposits usually occupied the whole thickness of the cortex, less frequently they had a laminary arrangement, or were scattered as patches in apparently unchanged tissue.

In the above mentioned rat with necrosis of the cerebral cortex very large glycogen deposits, perivascular in the astrocytes and loosely scattered in the neuropil, formed a broad band around the necrotic focus (Fig. 4).

Glycogen deposits of the same character and distribution in the brain were found in all groups of experimental animals independently of the length of survival after arterial ligation. The only detectable difference in the distribution of deposits was their presence in the neuropil of the basal ganglia in animals sacrificed 6 and 12 hours after the procedure while in the remaining groups they were restricted essentially to the cerebral cortex.

Electron microscopic findings

In the electron microscopic investigations of the brains of animals after arterial ligation a very marked accumulation of glycogen was observed in the astrocytes, in the first place in their perivascular processes. Around the capillaries the

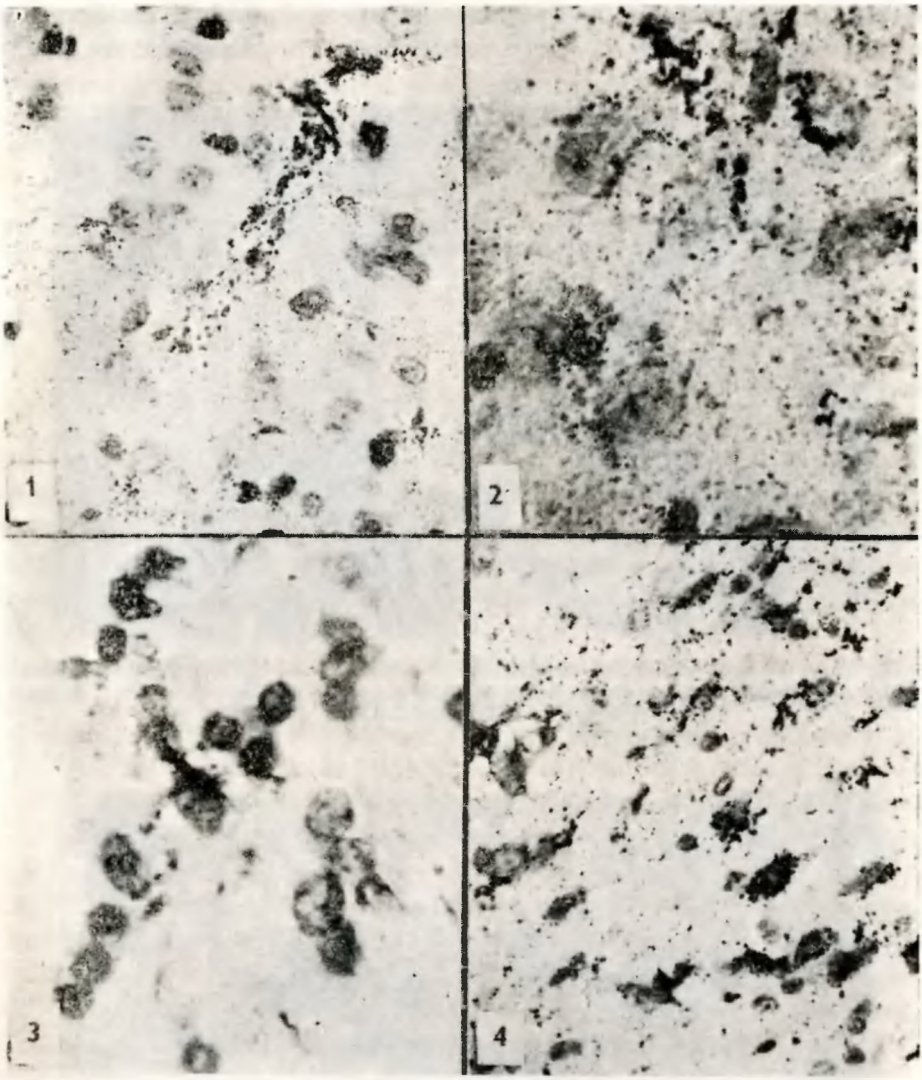

Fig. 1. Glycogen granules scattered in the neuropil and grouped around vessels in cerebra cortex of rat 24 hours after bilateral ligation of common carotid arteries. PAS-dimedone. Magn. × 400.

Fig. 2. Granular glycogen deposits scattered in neuropil and grouped around astrocyte nuclei in cerebral cortex of rat 48 hours after bilateral ligation of common carotid arteries. PAS-dimedone. Magn. × 1200.

Fig. 3. Glycogen deposits in cytoplasm and processes of an astrocyte in subcortical white matter of rat brain 72 hours after bilateral ligation of common carotid arteries. PAS-dimedone. Magn. × 1200.

Fig. 4. Abundant deposits of glycogen scattered loosely in cytoplasm of astrocytes in area surrounding necrotic focus in cerebral cortex of rat 120 hours after bilateral ligation of common carotid arteries. PAS-dimedone. Magn. × 200.

uniformly scattered glycogen granules accumulation in the markedly swollen processes of astrocytes (Fig. 5). The number of granules in the individual processes surrounding even one vessel varied considerably. The glial processes grouped

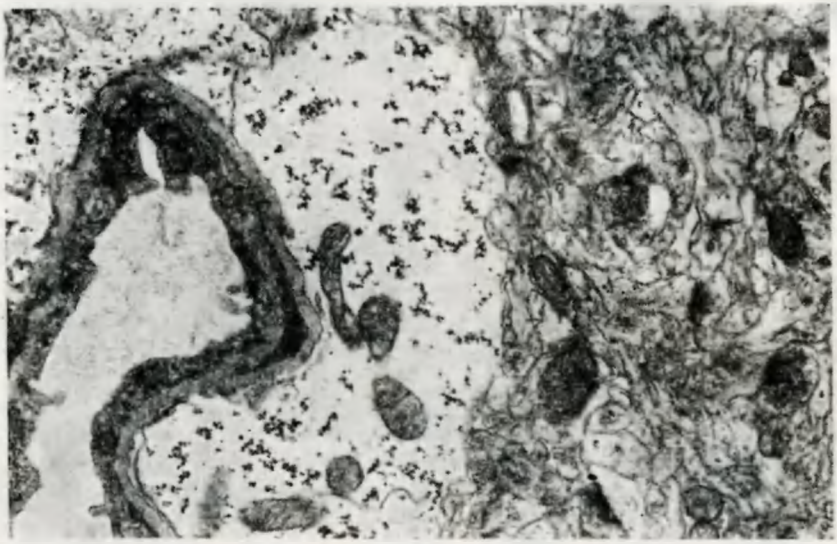

Fig. 5. Very marked swelling of perivascular processes of astroglia containing numerous granules of glycogen. Cerebral cortex of rat 48 hours after bilateral ligation of common carotid arteries. Electron microscope. Magn. × 18,000.

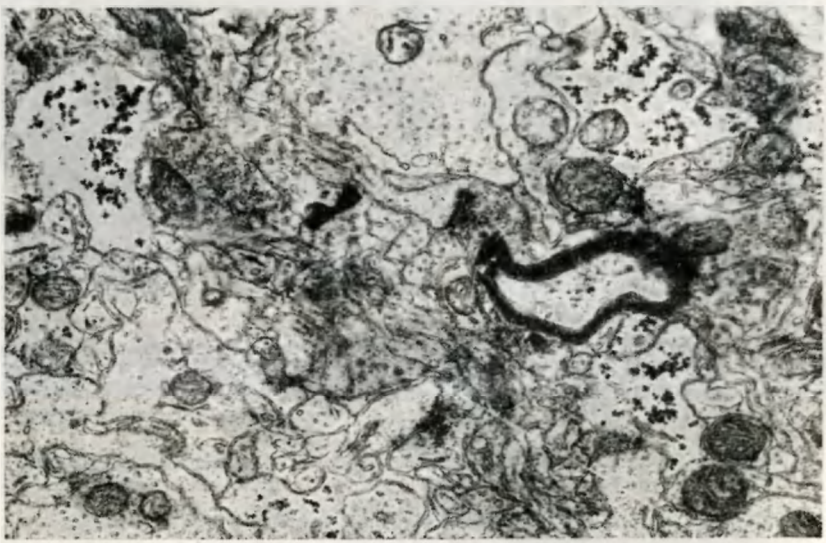

Fig. 6. Swollen processes of astroglia in cerebral cortex containing characteristic accumulations of glycogen granules. Rat killed 48 hours after bilateral ligation of common carotid arteries. Electron microscope. Magn. × 13,000.

around the precapillary vessels were less swollen or showed no evidence of swelling, and glycogen deposits accumulated in them in some places completely filled their cytoplasm.

Moreover, glycogen accumulation was observed also in the perikarya of the astrocytes in the cerebral cortex and in their processes showing no direct connection with the blood vessels. Somewhat greater accumulations of glycogen were observed in the processes of astrocytes surrounding the "dark" neurons.

In the ultrastructural investigations glycogen was present in the form of single granules lying loosely in the cytoplasm or in the form of typical rosettes. Besides that, polymorphous accumulations of up to twenty granules of glycogen were seen.

DISCUSSION

The observed rise in the glycogen content in the brains of rats with experimentally induced cerebral ischemia is a quantitative confirmation of the previous histochemical investigations carried out using the same experimental models by *Ibrahim et al.* (1970) and *Long et al.* (1971). The quantitative increase observed in these experiments has found confirmation in the results of the histochemical investigations reported here.

Abnormal glycogen deposits seen in histochemical preparations under the conditions of the at present applied experimental model appear 6 hours after the moment of ligation of both carotid arteries (*Ibrahim et al.* 1970, *Long et al.* 1971). After approximately the same time the earliest appearance of glycogen deposits in the astroglia was observed in perinatal asphyxia in monkeys (*Mossakowski et al.* 1968). In the present material the glycogen content in the brain was also observed to increase 6 hours after the onset of action of the noxious factor, but this increase was without statistical significance. This divergence in observations may be explained by assuming that the slight deposits appearing at that time in only several structures of the central nervous system are too small to significantly change the total amount of glycogen in the brain. A statistically significant rise in the glycogen content occurs only 12 hours after arterial ligation reaching its peak after 48 hours and remaining at this statistically significant level until the end of the experiment, that is until the fifth day after the procedure.

The dynamics of the changes in the glycogen content of the brain in the present material differed from the observations of *Rivera et al.* (1970) obtained in the experimental model of perinatal asphyxia in monkeys. These authors found that the maximum accumulation of glycogen in the brains of these animals occurred in the 12th hour of the experiment. After 48 hours the glycogen content fell to the control level while in the present investigations the peak value of this polysaccharide was found at that time. The source of these divergencies

may either be related to the species — specific differences since different animal species were used for these investigations, or to the age-dependent differences in the susceptibility of the central nervous system to the action of the noxious factor, or to the differences in the experimental models which were a transient, short-lasting asphyxia in the experiments of *Rivera et al.* (1970), and permanent ligation of carotid arteries in the present experiment. The role of species-specific, age-dependent or even topographic factors may be suggested among others, by the differences in the time of the appearance of histochemically detectable glycogen deposits under the conditions of the same experimental model. In partial ischemia of the spinal cord in cats the deposits of glycogen appeared at the earliest one hour after ligation of the abdominal aorta (*Long et al.* 1971).

Most authors working on the problem of the appearance of glycogen in the nervous tissue as a result of factors impairing its oxygen supply (*Mossakowski et al.* 1968, *Ibrahim et al.* 1970, *Klatzo et al.* 1970, *Long et al.* 1971, *Mossakowski, Zelman* 1971) pointed to the complete reversibility of the changes and stressed that the polysaccharide deposits are accumulated in morphologically undamaged tissue. In the present experiments the first of these observations could not be confirmed. Even after 120 hours from ligation of the carotid arteries the glycogen content in the brain considerably exceeded its level in the control animals. It can, however, be supposed that in the presently used experimental model (permanent ligation of carotid arteries) the return to norm occurs later. Abnormal glycogen deposits in the spinal cord after partial ischemia persisted for 7 to 10 days (*Long et al.* 1971). On the other hand, the accumulation of glycogen in the present material, similarly as in that of other authors, was observed exclusively in the cerebral structures showing no histological evidence of injury.

The topographic distribution of glycogen deposits is a problem of special interest. These deposits appeared mainly in the cerebral cortex, and to a much lesser extent in the basal ganglia. The deposits in the white matter were of a very rare occurrence and they appeared there only at a later time period. The topographic distribution of deposits showed no essential changes during the observations after the application of the noxious factor. Histochemical investigations showed that the deposits were localized in the astrocytes and neuropil. The latter site of localization, as demonstrated by electron microscopic investigations, corresponded to the presence of glycogen in the swollen processes of the astrocytes. The polysaccharide deposits in the neurons have never been observed. The topographic distribution of deposits shows that they appear in those structures of the central nervous system which have the highest uptake of oxygen, and their intracellular localization is exclusively in the astrocytes; according to *Friede* (1954) and *Oksche* (1961) astrocytes play the basic role in the metabolism and transport of glucose in the central nervous system. This suggests that the

accumulation of glycogen in the nervous tissue resulting from a decreased oxygen supply to this tissue represents evidence of an impaired consumption of glucose by it. The impairment of glucose consumption under these conditions must involve, in the first place, the nerve cells since they are the main consumers of glucose and are particularly sensitive to hypoxia. In the present state of investigations the mechanism of the probable decrease in glucose consumption by the nervous tissue resulting from ischemia remains unexplained. It is possible that restriction in the utilization of glucose due a decrease of the pool of energy-rich compounds is connected with the reduced rate of protein synthesis as demonstrated by the investigations of *Yap* and *Spector* (1965).

When the glucose consumption by the nerve cells is reduced or when glucose is accumulated in excess in the nervous tissue due to hypoxia (*Thorn et al.* 1959, *Steward et al.* 1970, *Domańska-Janik* 1970), it can be accumulated as glycogen in the cytoplasm of astrocytes which participate in the transport of glucose in the nervous system (Hager 1966). The accumulation of glycogen in the central nervous system may be due either to its increased synthesis or to reduction in its breakdown. The investigations of *Klatzo et al.* (1970) have elucidated the problem in favor of the first mechanism. Hypoxia, as shown by *Passenneaue* and *Lowry* (1962) is a factor stimulating the phosphorylation of glucose. Glucose-6-phosphate in its turn activates UDP-glucose-glycogen glucosyltransferase (*Basu, Bachhawat* 1961, *Stossel et al.* 1970). It seems possible therefore, that glucose accumulating in the nervous system can by itself lead under these conditions to activation of the enzyme system synthetizing glycogen which is followed by deposition of this polysaccharide in the tissue. The histochemical investigations of *Mossakowski et al.* (1968), *Goldberg* and *O'Toole* (1969), *Ibrahim et al.* (1970) and *Long et al.* (1971) on the activity of enzymes participating in the metabolism of glycogen and on their connection with the accumulation of glycogen in the brain confirmed this possibility. This mechanism of glycogen deposition in the central nervous system is suggested also by the investigations of *Śmiałek et al.* (1971) on the activity of UDP glucose-glycogen glucosyltransferase under conditions of brain ischemia.

An important problem is the role of anesthesia as a factor leading to a rise in the glycogen content in the brain. The influence of this factor on the glycogen level in the nervous tissue was stressed by *Oksche* (1961) and *Goldfied et al.* (1966).

Rivera et al. (1970) found a slight rise in the glycogen content in the brains of the control animals during the first 6 hours of observation and suggested that these changes might be explained by the action of the anesthetic agent. In our investigations slight fluctuations in the glycogen level were observed during the same time period in the brains of control animals subjected to anesthesia. However, the statistical analysis demonstrated that these changes were not significant as shown by a comparison with the fluctuations in the glycogen

level in the brains of animals decapitated without any preceeding anesthesia or other procedures. It seems, however, that some effect of anesthesia on the glycogen content in the brains of animals examined 12 hours after it can be assumed to exist. Later this effect disappears completely.

CONCLUSIONS

1. Bilateral ligation of the common carotid arteries in 10-week-old rats causes a considerable rise in the glycogen content in their brains although morphological changes are still lacking. This rise is statistically significant in comparison to the control group 12 hours after ligation reaching its peak value 48 hours after ligation. During 5 days following this procedure the glycogen level fails to return to its initial value.

2. Histochemically detectable abnormal deposits of glycogen in the nervous tissue are localized in the gray substance of the brain, mainly in the cortex, and to a lesser extent in the basal ganglia, but they are present there only soon after the procedure. Deposits in the white substance are seen only rarely.

Electron microscopic investigations show that polysaccharide deposits accumulate exclusively in the cytoplasm of astrocytes and in their processes.

3. General ether anesthesia leads to a small but statistically significant rise in the glycogen content in the brain only during 12 hours after the administration of anesthetics. Later this effect disappears completely.

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