Glycogen and Phosphorylase Activity in Rat Brain During Recovery from Several Forms of Hypoxia

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GLYCOGEN ACCUMULATES IN THE MAMMALIAN BRAIN in a variety of pathologic conditions. Recovery from severe hypoxia is accompained by increased deposits of glycogen in adult rat brain,[1,2] adult cat brain [3] and neonatal monkey brain;[4] the mechanism of glycogen accumulation in these hypoxic states has not been elucidated. Prolonged anesthesia also leads to increases.[5] Glycogen accumulation in the mammalian brain directly damaged by a stab wound [6–9] or by ionizing radiation [10] has been attributed, in part at least, to a derangement of aerobic metabolism. Furthermore, Ibrahim *et al* [11] have suggested that in addition to inhibition of anaerobic glycolysis, there may also be an increase in glycogenesis in the irradiated brain.

The present communication is, therefore, devoted to the histochemical study of glycogen and certain enzymes related to its metabolism in the brain of rats exposed to various forms of hypoxia. Pertinent morphologic alterations will be described and an attempt will be made to define the mechanism by which glycogen accumulates. It is hoped that this may throw more light on the problem of glycogen increase in the pathologic brain in general.

Materials and Methods

About 100 young adult, male Sprague-Dawley rats, each approximately 250 g in weight, were used. The animals were divided into five groups and processed as follows:

Hypoxic Hypoxia (Hypoxia) Group. Each animal was exposed individually to 7.5% oxygen–92.5% nitrogen by Levine's [12] technic. When the animal became hyperexcitable, the flow of gas was stopped and pure nitrogen was introduced very cautiously in small spurts until the animal lost consciousness. This state of unconsciousness was maintained for 5–10 min.

Unilateral Ischemic Hypoxia (Unilateral Ischemia) Group. Under pentobarbital anesthesia, the left common carotid artery was exposed and ligated.

Bilateral Ischemic Hypoxia (Bilateral Ischemia) Group. Both common carotid arteries were ligated at the same time. The majority of animals survived this pro-

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cedure when it was carried out under ether anesthesia; under sodium pentobarbital anesthesia, the mortality rate was very high.

Ischemic-Hypoxic Hypoxia (Ischemic-Hypoxic) Group. Ligation of the left common carotid was performed, and on the same, or the following, day the rats were exposed to the hypoxic atmosphere as described for the hypoxia group.

Histotoxic Hypoxia (Cyanide) Group. The animals were exposed to hydrogen cyanide as in the static method of Levine,[13] kept unconscious for 20–30 min, then brought to air.

To process tissues for histochemical study, the animals were sacrificed by exsanguination under light anesthesia at times ranging from immediately after the hypoxic procedure to 7 days after. A control rat was sacrificed along with each animal in Groups 1, 3 and 5. In Groups 2 and 4, the hemisphere on the unoperated side served as a control, but sometimes control animals were used. Previous experiments have shown the importance of processing control and experimental tissues simultaneously.[14–16] A standard coronal block, bounded by the optic chiasm anteriorly and the pituitary stalk posteriorly, was cut from each brain and processed.[14] The remaining tissue was usually fixed in Rossman's fluid at 4 C, and paraffin sections were stained for glycogen by the periodic acid-Schiff (PAS) technic after treatment with dimedon according to Bulmer.[17] α-Amylase digestion was sometimes performed to confirm the identity of the glycogen deposits. Counterstains, when applied, were hematoxylin or gallocyanin.

In all instances, the following enzymes were investigated: total and active (without AMP in the incubation medium) phosphorylases,[14,18,19] phosphorylase plus branching enzyme [20] and glycogen synthetase. For the last enzyme, the modification developed for liver by Sie *et al* [21] of Takeuchi and Glenner's method [22] was used. Sections were stained with dimedon-PAS; the reaction was too weak when Gram's iodine was used.

Succinate, lactate, glucose-6-phosphate and 6-phosphogluconate dehydrogenases and cytochrome oxidase were demonstrated by methods found in Pearse.[23] For hexokinase activity, the method of Wegmann and Gerzeli [24] was used with the addition of KCN as inhibitor, and for glyceraldehyde-3-phosphate dehydrogenase, the method of Himmelhoch and Karnovsky.[25]

Phosphorylase activity was assayed biochemically by the method of Cori *et al* [26] to authenticate the results found histochemically. Controls, and some animals 24 hr after bilateral common carotid artery ligation, were etherized and, while still alive, the brain, without olfactory bulbs and cerebellum, was removed and dropped into liquid propane. Total phosphorylase activity was measured in duplicate in all brains, active phosphorylase only in some.

Results

Hypoxia

Immediately after exposure to hypoxia, no obvious histologic abnormalities could be detected in brain tissue. Cytochrome oxidase activity was slightly diminished. No obvious change was visible in either the branching enzyme or glycogen synthetase. The only marked histochemical change was a considerable diminution in both total and active phosphorylase activity in the entire gray and white matter; often this was more noticeable in the hippocampus (Fig 1).

A dramatic reversal in phosphorylase activity occurred from about 6 hr after exposure; the entire grisea then showed abnormally high activity, being maximal in the neocortex, pyriform cortex, amygdaloid area, dorsomedial thalamus and hippocampus—the "sensitive" areas. An increase in histochemically demonstrable glycogen was also observed, particularly in those areas showing maximal phosphorylase activity. In severe hypoxia, loss of enzyme activity (mainly phosphorylase) occurred in Layers 2 and 3 of the neocortex and in the thalamus. In such cases, the remaining areas of the brain showed increased phosphorylase activity, particularly in the above-mentioned sensitive areas. Of the other enzymes investigated, none showed a detectable change. No glycogen deposits or increase in phosphorylase activity could be found in the brain stem or cerebellum.

Thereafter, the findings varied according to the depth and duration of hypoxia. After exposure to moderately severe hypoxia, both phosphorylase activity and glycogen returned to normal by 24 hr. After more severe hypoxia, necrosis occurred in the sensitive areas, accompanied by loss of enzymic activity and glycogen (upper half of brain in Fig 2). Abutting tissue showed abnormally large amounts of glycogen and enhanced phosphorylase activity; the stronger the phosphorylase activity, the heavier the glycogen deposits. The rest of the brain showed a slight increase in phosphorylase activity, more pronounced in the cingulate gyrus and the molecular layers of the dentate gyrus and subiculum in the hippocampal area; loss of activity occurred only in the lateral extremity of the hippocampus (upper half of brain in Fig 2). Changes in other enzymes are very similar to those to be described in the ischemic-hypoxic group.

Unilateral Ischemia

The only observed change was a transient slight diminution of phosphorylase activity on the ligated side of the brain about 1 hr after operation.

Bilateral Ischemia

An immediate marked loss of phosphorylase activity in both gray and white matter was superseded after 16–24 hr by an abnormally intense activity (Fig 3), except in the necrotic areas found in a few brains. By 48 hr, the phosphorylase activity had returned to normal in the non-necrotic brains. Glycogen accumulation accompanying the increased phosphorylase activity occurred in the same areas as in the hypoxic group but only in the more severely affected brains. A moder-

ate increase in lactate dehydrogenase activity by 24 hr was the only other enzymic change observed.

Ischemic-Hypoxic

After an initial loss of phosphorylase activity on the ischemic side, both phosphorylase and branching enzyme increased slightly 1 hr after exposure. However, the lateral tip of the hippocampus usually showed a slight reduction. None of the other enzymes tested was visibly affected.

After 4–6 hr, phosphorylase hyperactivity extended to the entire brain, but was more marked on the ischemic side. In mild cases, slight increase of activity was visible only on the hypoxic-ischemic side in the hippocampus, with the exception of the lateral tip. In more severe cases, diminished activity was found also in the middle of the cerebral cortex on the ischemic side, with the rest of the hemisphere hyperactive; the less damaged sensitive areas, as usual, contained the strongest activity. Glycogen synthetase was slightly increased in all areas demonstrating phosphorylase hyperactivity. Cytochrome oxidase and succinate and lactate dehydrogenases were diminished in the more severely damaged areas.

One day after exposure, lesions were maximal, and subsequent alterations were towards ultimate healing. Lesion size was determined by the severity and duration of the hypoxic episode. Phosphorylase activity was most marked in the non-necrotic portions of the sensitive areas, and in tissue adjacent to necrotic zones (compare Fig 4 and 5, and the upper half of the brain in Fig 3). This activity was within the protoplasmic astrocytes and was especially clear in the molecular layers of the hippocampus (Fig 6) and cerebral cortex. Activity was also diffusely dispersed in the "neuropil" of the gray matter; no hyperactivity was noted in the neurons. In the white matter, increased activity was found in the fibrous astrocytes, oligodendroglia, axons and neuropil (Fig 6 and 7).

All other enzyme activities investigated were slightly decreased in the necrotic zones, but moderately increased at the borders, except for succinate dehydrogenase. Most enzyme activity was associated with both astrocytes and oligodendroglia, but localization of lactate dehydrogenase and cytochrome oxidase activities was uncertain. In the rest of the ischemic hemisphere, the only enzymic alterations noted were increases in hexokinase (Fig 8) and 6-phosphogluconate dehydrogenase (Fig 9) activities in those areas showing enhanced phosphorylase activity.

Abnormal accumulation of glycogen was observed in those areas showing increased phosphorylase activity (Fig 10), but, as expected, none was present in necrotic zones. The glycogen granules were usually scattered in the "neuropil" of the grisea, occasionally in protoplasmic astrocytes, and rarely in oligodendroglia. No glycogen increase was seen in nerve cells. The neuropil of white matter contained glycogen granules, as did the cytoplasm of fibrous astrocytes and oligodendroglia near necrotic areas. Although white matter reacted less intensively than gray matter, thin layers of white matter adjacent to reactive grisea showed abundant glycogen (Fig 11). Marked edema and myelin disintegration were seen in severely injured white matter while in areas showing only myelin pallor and separation of the sheaths, phosphorylase hyperactivity and glycogen accumulation occurred. Damage of the corpus callosum was seen in one case.

Animals surviving longer than 3 days usually showed a marked increase in the oligodendroglial population of the white matter "islands" of the corpus striatum at levels anterior to that of the thalamus. The proliferated and slightly hypertrophic oligodendroglia had somewhat enlarged and pale nuclei and showed increased activity of NADP-linked enzyme, as did astrocytes of the gray matter surrounding the islands. Some of these astrocytes contained glycogen granules but the proliferated oligodendroglia did not. Although the pale myelin in these islands was apparently intact, at about 1 week after exposure it was severely damaged and had lost its affinity for luxol fast blue.

Cyanide

Only the changes observed between 24 and 48 hr will be described here, since prior and subsequent observations will constitute a separate communication. In general, the observations paralleled closely those of the ischemic-hypoxic animals but they were bilateral (Fig 12). Also, in the corpus callosum, necrosis, which could extend a small distance into the callosal radiations, was a common feature. Glycogen, absent from the severely damaged areas, was abundant in border zones, including the angles of the callosal radiations. The granules were mainly in the neuropil but occasionally were clearly related to astroglia and oligodendroglia. Moderate glycogen accumulation was observed in the gray matter of the corpus striatum and the griseal elements of the rhinencephalon if these were not severely injured.

Characteristic oligodendroglial proliferation was seen in the islands of pale white matter of the corpus striatum in the more severely in-

jured brains, but, as in the ischemic-hypoxic brains, these cells did not contain any demonstrable glycogen.

Biochemical Assay of Phosphorylase Activity

Total phosphorylase activity of brain tissue from 6 normal rats released 251 ± 27.8 μM of phosphate/hr/g tissue. Sixteen to twenty-four hours after bilateral common carotid artery ligation, brain tissue showed 129–179% of the control activity, the variable increase probably depending on the degree of the brain insult at the time of sacrifice of the rat.

The proportion of active phosphorylase after bilateral carotid ligation remained unchanged from that in control brains, about 84%

Discussion

Our discussion will be limited to changes during recovery from hypoxia in glycogen and the enzymes involved in its metabolism. Extensive studies are available on the normal distribution in brain of glycogen [27,11] and phosphorylase plus branching enzyme,[28,29] and sequential changes of other enzymes in hypoxia.[30–32]

After a hypoxic or ischemic episode, increased glycogen was detected in brain after 6 hr of reoxygenation. After a similar period, glycogen accumulation results from direct brain injury [6] and also brain irradiation with x and γ rays.[13] This similarity of response to diverse conditions may reflect a common focus of biochemical injury or a specific response of brain tissue to certain types of insult.

Definition of the mechanism of post-hypoxic glycogen accumulation is hindered because most pertinent biochemical studies on hypoxia were terminated before glycogen accumulated [33–35] or were performed on peripheral nerve.[36, 37] These and other experiments, however, showed the following changes in metabolite concentrations *during* the hypoxic or ischemic episode: decreases in phosphocreatine, glycogen, glucose, glucose-6-phosphate, fructose-6-phosphate and ATP, and increases in ADP, AMP, inorganic phosphate, fructose-1,6-diphosphate, α-glycerophosphate, lactate and also acetyl coenzyme A.[38] In general, these changes point to hyperactivity of the Embden-Meyerhof pathway and possibly also to inefficiency in the citric acid cycle.

Return of these metabolites to normal values occurs at different rates.[36] Recovery of altered ion transport across cell membranes is slow, and the resulting diminution of intracellular potassium concentration and increased sodium are probably involved in the edema observed.[3, 39,40] Diminution of potassium, which is known to maintain

high brain respiration by acting on the Embden-Meyerhof pathway,[41] may limit activity of this pathway. Also, accumulation of lactic acid causes an intracellular drop in pH which itself may stimulate glycogen formation.[42]

Hyperglycemia accompanying hypoxic states [43] may be an additional factor, as it increases brain glucose.[44,45] Furthermore, hypoxia is said to stimulate glucose phosphorylation.[46] Therefore, increased glucose in a brain with defective glycolysis probably enhances the accumulation of glycogen.[8,47,48] The late increase observed in hexokinase activity may reflect increased glucose transport while the increase in 6-phosphoglyconate dehydrogenase activity may represent a compensatory reactivity of the pentose shunt to reduced glycolysis. It seems, therefore, that glycogen accumulation represents not only diminished glycolysis but also an actual increase in synthesis.

Normally, glycogen synthesis is totally dependent on UDPG-glycogen synthetase, while glycogenolysis is effected by phosphorylase.[49] Thus, the parallel changes observed in glycogen and in phosphorylase, coupled with the slight and late increase in synthetase activity, require explanation. Within seconds of the onset of hypoxia, and while ATP levels are still normal, inactive phosphorylase *b* is transformed to the active *a* form, after activation of phosphorylase kinase by cyclic AMP.[15] This causes the initial rapid loss in glycogen, and the usual postmortem loss. Soon thereafter, ATP falls, and AMP and inorganic phosphate rise. We noted an initial (10 min) fall in phosphorylase but could not observe, histochemically, a decrease in the normally low level of glycogen. However, Lowry *et al*[35] were able to detect an initial glycogen fall in mice by biochemical determination.

When oxygenation has been reinstated for some time, ATP recovers and presumably restores phosphorylase activity to normal, then to supranormal values, as confirmed biochemically by us. Simultaneous increases in phosphorylase activity and glycogen and their equal duration could be partly an adaptive mechanism to catabolize the increased glycogen.[50]

Although increased, phosphorylase is apparently incapable of hydrolyzing all of the excess glycogen present, but the importance of factors other than phosphorylase activity alone in the control of glycogenolysis has been stressed.[14,51,52] Among these could be the molecular form of newly synthesized glycogen which, if of high molecular weight, would probably bind more phosphorylase, especially the active form, and more firmly.[11] Maintenance of glycogen deposits may also reflect a change in metabolism to greater dependence on anaerobic

pathways and more efficient utilization of glycogen rather than glucose, as can happen in the alloxan-diabetic rat.[50]

Involvement of glycogen synthetase in the parallel changes of glycogen and phosphorylase is less apparent, yet an increase in activity was observed at later stages when glycogen increase had already occurred. Furthermore, Mossakowski *et al*,[4] working on the asphyxiated neonatal monkey, did find an early increase within the astrocytes. Quantitative relationships between enzyme and substrate or product may be paramount, as the enzyme normally present in brain can theoretically synthesize 12 times the amount of glycogen present. Phosphorylase, in contrast, probably works close to capacity even under control conditions.[49] Structurally, intimate association of glycogen, phosphorylase and UDPG-glycogen synthetase as "metabolically active subcellular particles" was suggested by Selinger and Schramm [53] and our results support this close relationship.

The histopathologic findings generally conform to those described by previous workers.[12,13,32,39,54,55] According to Levine [12] and to Lucas and Strangeways,[56] the hippocampus is the structure most vulnerable to hypoxia, followed by the cerebral cortex, the white matter being the most resistant. Our findings indicate that, in addition, the amygdaloid complex and pyriform cortex are almost as vulnerable. These "sensitive" areas are also the first to show increased glycogen and phosphorylase activity when hypoxic damage is moderate and also the first to undergo necrosis when damage is severe. Remarkably, they are identical to the areas showing glycogen accumulation and increased phosphorylase activity under irradiation conditions [11] and in suitably situated direct injury.[2] It is more than probable that the same ultimate mechanism responsible for glycogen accumulation in hypoxia operates under different conditions, as Ibrahim *et al*[2] attempted to show.

In hypoxia-ischemia, the oligodendroglial proliferation, myelin pallor and, later, demyelination in the corpus striatum have also been seen in cyanide-intoxicated rats both in this and previous studies.[55, 57] This is of significance since Ibrahim and Adams[58] noted that one of the earliest reactions in multiple sclerosis is a proliferation of glial cells (mostly oligodendroglia).

Again, similarity between cyanide and hypoxic-ischemic lesions extends to their distribution (compare Fig 4, 5 and 12); occasional involvement of the corpus callosum occurs in hypoxia-ischemia but this is a regular finding in the cyanide brains. This similarity indicates that cyanide lesions may not be caused simply by inhibition of cytochrome oxidase [59,60] but may also involve an ischemic element, the mech-

anism of which is not at present clear. Levine and Klein [61] previously suggested that vascular factors may play a role in the localization of cyanide encephalopathy. Lastly, it should be mentioned that, as in the present report, Hirano *et al* [62] described glycogen accumulation in glia (unidentified) in the corpus callosum of rats 24 hr after single exposure to cyanide gas.

Summary

Young adult male Sprague-Dawley rats were exposed to different forms of hypoxia: hypoxic, unilateral ischemic, bilateral ischemic, ischemic-hypoxic and histotoxic (cyanide). Accumulation of glycogen granules was seen in the hypoxic, bilateral ischemic, ischemic-hypoxic and cyanide brains in "sensitive" areas—hippocampus, neocortex, amygdaloid area, pyriform cortex, dorsomedial thalamus—when these were not severely damaged. These same areas were the first to show glycogen loss and undergo necrosis when the damage was severe. The increased glycogen was detectable about 6 hr after exposure and was maintained for variable periods of time, according to the severity of the injury.

Simultaneously, total phosphorylase activity increased, but glycogen synthetase increased only later. Glycogen deposits and increased phosphorylase activity were generally closely linked in time and localization. Cytologically, these events were best seen in astrocytes, although less frequently in oligodendroglia also. Diminution in glycolytic activity, with increased glycogenesis in the presence of hyperglycemia was thought to be directly responsible for increased glycogen and indirectly for increased phosphorylase activity.

In severely damaged zones, normal enzymic activity was reduced; that of phosphorylase was reduced the most. Phosphorylase activity far surpassed that of some oxidoreductive enzymes as a marker for hypoxic damage.

The similarity between the ischemic-hypoxic and the cyanide brains is stressed and it is suggested that an ischemic factor, as well as the histotoxic, is probably operative in the etiology of cyanide encephalopathy.

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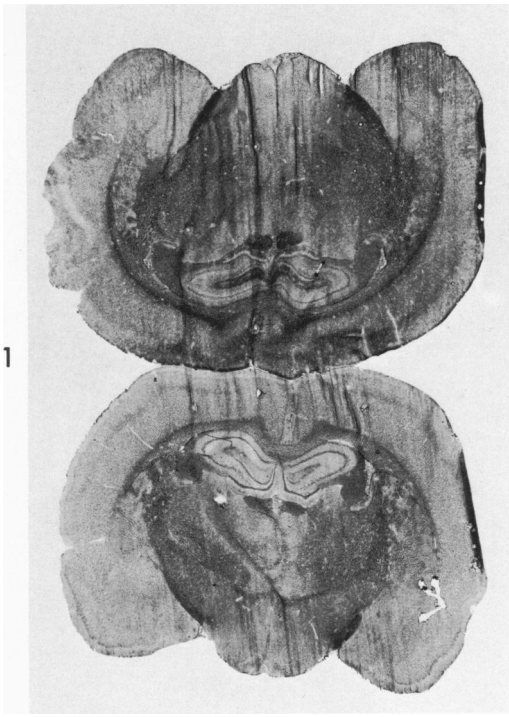
[*Illustrations follow*]



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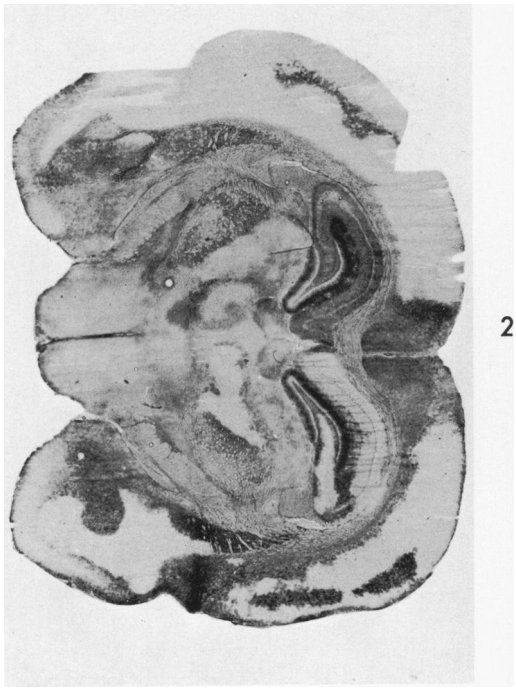

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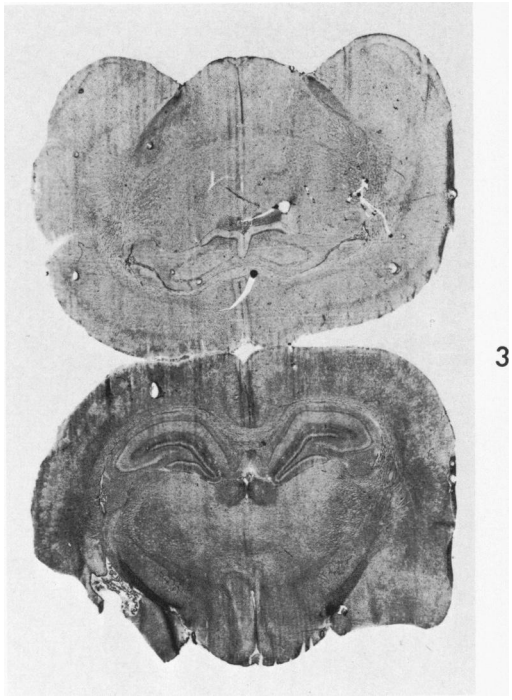
Fig 1. Brain of rat exposed to hypoxia for 5 min and sacrificed immediately afterwards. Phosphorylase activity reduced throughout brain *(lower section)*. Dilute Gram's iodine. X 4.

Fig 2. Ischemia combined with hypoxia. Hypoxic episode more severe than usual so that control side *(upper)* developed severe lesions (see text). Rat killed 2 days after hypoxia. Phosphorylase; dilute Gram's iodine. X 6.

Fig 3. Considerable increase in phosphorylase activity throughout brain of rat subjected to bilateral carotid ligation 16 hr before sacrifice *(lower section)*. The hypoxia was severe so that mottling due to patchy necrosis of the cerebral cortex is seen. Dilute Gram's iodine. X 4.



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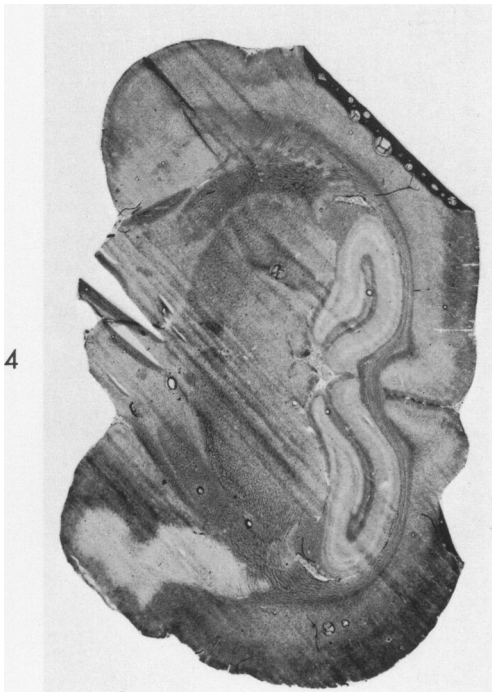

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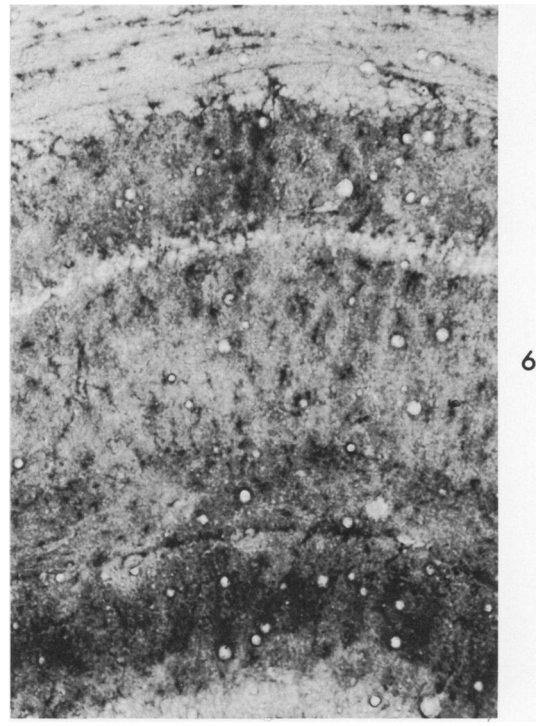

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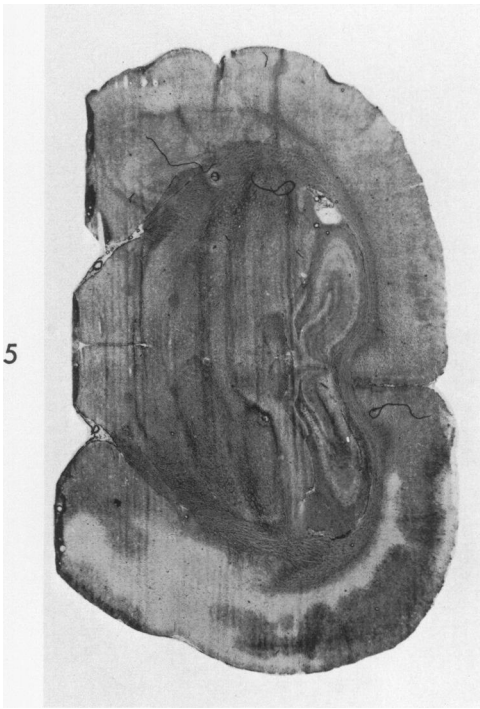

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Fig 4. Ischemic-hypoxic brain of animal killed 4 days after hypoxia. Note characteristic lesion in amygdaloid area, pyriform cortex and corpus striatum. Neocortex and rest of rhinencephalon of ischemic hemisphere *(lower)* show increased phosphorylase activity. Dilute Gram's iodine. X 6.

Fig. 5. Ischemic-hypoxic brain of animal killed 7 days after hypoxia, showing manner in which small lesion, seen in Fig 4, can be more extensive. Hippocampus and neocortex now also involved. Phosphorylase; dilute Gram's iodine. X 6.

Fig 6. Higher magnification of hyperactive area of hippocampus of hypoxic-ischemic side *(lower)* in Fig 2, showing considerably increased phosphorylase activity in all layers, especially molecular layer of dentate gyrus *(lower part)*. Neuroglial elements and neuropil are involved. Adjoining white matter *(top)* shows rows of hyperactive cells (oligodendroglia) and interspersed astrocytes. Dilute Gram's iodine. X 84.

Fig 7. *(bottom)* Higher power of area of white matter seen in Fig 6 to show hyperactivity in rows of oligodendroglial cells, fibrous astrocytes, axons (?) and neuropil. Hyperactive astrocytes in adjoining hippocampus also seen at left. *(top)* Almost the same area in normal control brain. Phosphorylase; dilute Gram's iodine. X 533.

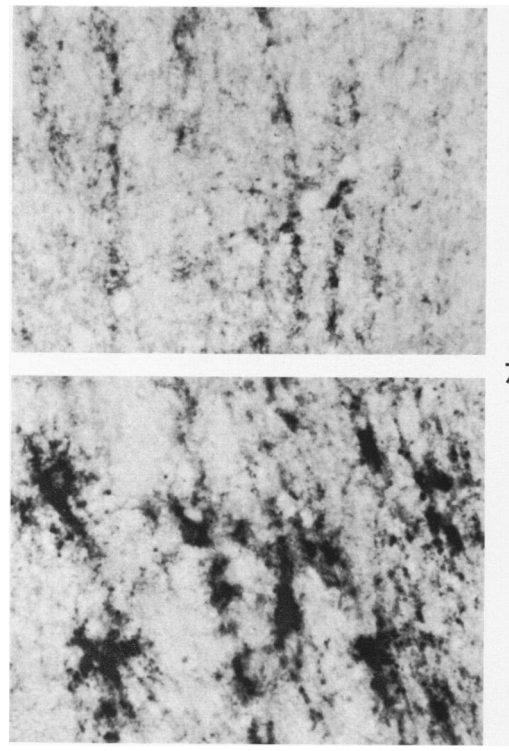

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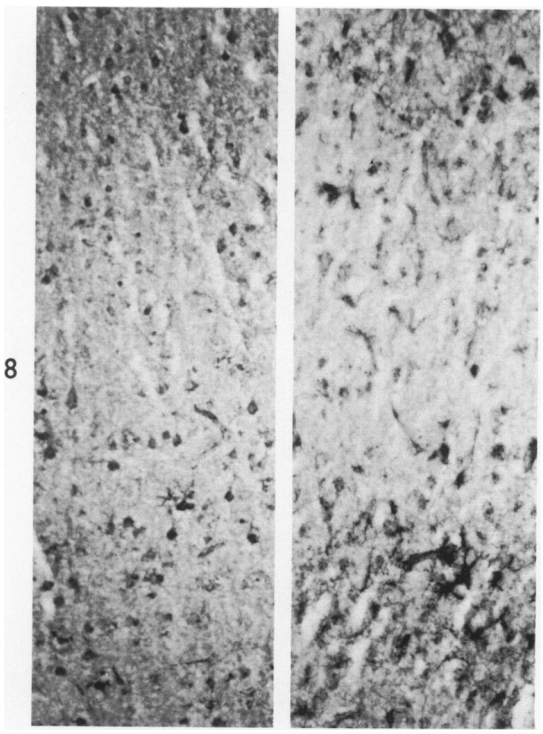
Fig 8. *(right)* Ischemic-hypoxic brain 2 days after hypoxia, showing hexokinase activity. Pale region is necrotic area in neocortex. Rest of cortex, especially at junction with necrotic area, shows hyperactivity in neuroglia and neuropil. X 50. *(left)* Same enzyme and area of opposite control hemisphere. X 150.

Fig 9. Similar section of that in Fig 4 incubated for 6-phosphogluconate dehydrogenase. Note absence of sharp outline of lesion seen in Fig. 4. Rest of rhinencephalon and neocortex show some increase in activity *(below)*. X 6.



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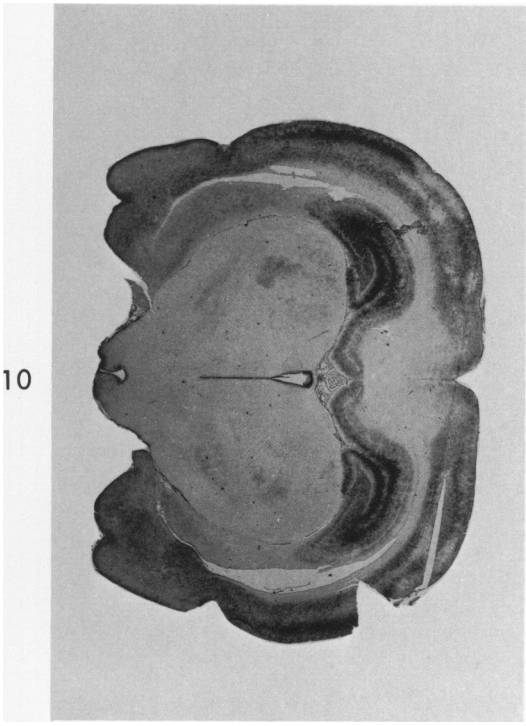

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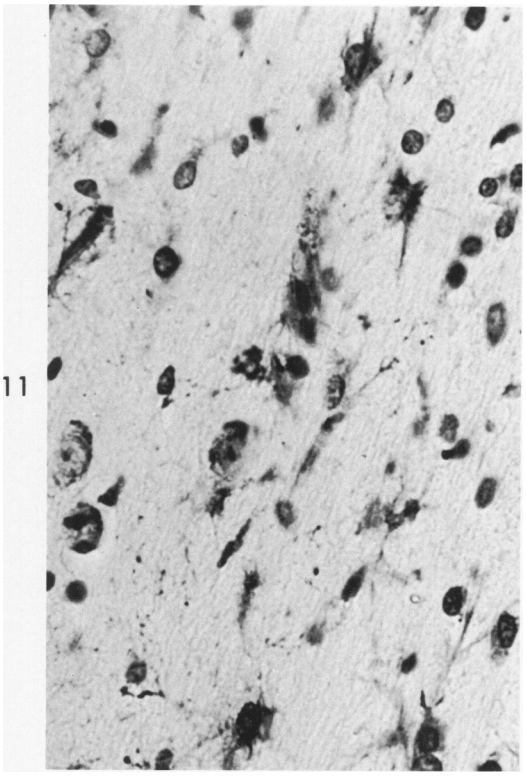
Fig 10. Paraffin section of ischemic-hypoxic brain, 24 hr after hypoxia, stained for glycogen. Hypoxic episode was severe and therefore control side *(notched)* also contains considerable glycogen. White matter and most of basal ganglia show practically no glycogen at this magnification. Dimedon-PAS without counterstain. X 6.

Fig 11. Inferior tip of callosal radiation of ischemic-hypoxic brain stained for glycogen. Fibrous astrocytes and some oligodendrocytes show some deposits. A few granules scattered in neuropil. Dimedon-PAS-hematoxylin. X 510.

Fig 12. Brain of animal sacrificed 2 days after exposure to cyanide gas *(lower section)*. Bilateral lesions in rhinencephalon and neocortex and lesion in corpus callosum are devoid of activity. Note similarity in distribution of lesions (except the callosal) to that in ischemichypoxic brains. Phosphorylase; dilute Gram's iodine. X 3.5.



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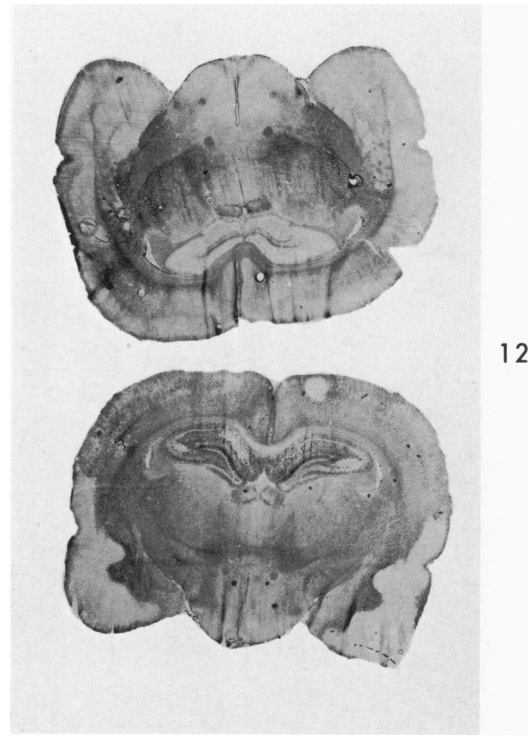

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