A Hexokinase Inhibitor in Nerve.* (18806)

L. G. Abood and R. W. Gerard.
From the Department of Physiology, University of Chicago.

Whole frog nerve was found(1) to possess a considerable endogenous anaerobic glycolysis, much increased by addition of glucose to the medium. We have obtained comparable results with whole frog or rat sciatics. Homogenates of such nerves, however, exhibit little glycolytic activity; nor was this increased significantly by addition of glucose or by fortification with any combination of cofactors, etc., including ATP, DPN, Mg^{++}, F^-, and pyruvate. These findings suggested that an inhibitor of glycolysis is formed or released when normal structure is disrupted. A number of inhibitors of glycolysis is present in normal tissue. Sodium has been found to inhibit purified hexokinase (18% by .06M) (2); and to decrease glycolysis also by decreasing ATP, due to increased breakdown or decreased formation from AA and phosphopyruvate(3). Mouse brain homogenates inactivate phosphoglyceraldehyde dehydrogenase; the inhibitor resembling a cathepsin obtained from beef spleen and, like it, counteracted by certain amides and amino acid esters(4). Another potent hexokinase inhibitor, sorbose-1-phosphate, is formed under the action of aldolase by condensation of l-glyceraldehyde and dihydroxyacetone phosphate(5). Of special interest, is the hexokinase inhibitor obtained from the anterior pituitary; a protein fraction enhanced in activity by adrenocortical extract and counteracted by insulin(6). The inhibitor in nerve homogenates is a protein, which acts specifically on hexokinase and resembles the pituitary inhibitor.

Methods. Anaerobic glycolysis of rat cerebral cortex homogenates was measured by the method of LePage(7), but with the final concentration of ATP increased to 10^{-3}M. (The ATP, 90% pure, and crystalline zinc insulin were kindly supplied by the Armour Research Laboratory.) Rabbit brain hexokinase was prepared and its activity measured according to Colowick, Cori, and Slein(6), glucose disappearance proving a more reliable index of activity than either CO_2 liberation or lactic acid production. More consistent results were obtained when the reaction was carried out in the Warburg apparatus, where shaking insured optimal mixing. Sciatic and brachial nerves of adult Sprague-Dawley and Wistar rats of both sexes were used as the source of inhibitor. They were removed within 5 minutes, without cooling, after the animal was stunned by a sharp blow on the back of the head and exsanguinated. The nerves were washed free of blood, stripped of surrounding connective tissue, and homogenized in ice cold distilled water. From 100 to 150 mg of whole nerve were usually obtained per animal. The inhibitory protein was partially purified by isoelectric precipitation. 5% water homogenates were adjusted to pH 6.0 with 0.02N H_2SO_4, in an ice bath, kept standing for 3 minutes, and centrifuged at 5000 x g for 5 minutes. The supernatant was then adjusted to pH 5.4, allowed to stand 5 minutes at 0°C, and centrifuged at 10,000 x g for 5 minutes. The second sediment after one washing with ice cold distilled water and resuspension in 0.15M KCl, was ready for use. From 200 mg of fresh nerve about 3 mg of protein with an equal amount of lipid were obtained.

Results. Nerve homogenates can inhibit the anaerobic glycolysis of rat brain homogenates by an average of over 70% for 60

* Performed under contract between the Office of Naval Research and the University of Chicago.

1. Gerard, R. W., and Meyerhof, O., *Biochem. Z.*, 1927, v191, 125.

2. Wiebelhaus, V. D., and Lardy, H. A., *Arch. Biochem.*, 1949, v21, 321.

3. Utter, M. F., *J. Biol. Chem.*, 1950, v185, 499.

4. Krimsky, I., and Racker, E., *J. Biol. Chem.*, 1949, v179, 903.

5. Lardy, H. A., Wiebelhaus, V. D., and Mann, K. F., *J. Biol. Chem.*, 1950, v187, 325.

6. Colowick, S. P., Cori, G. T., and Slein, M. W., *J. Biol. Chem.*, 1947, v168, 583.

7. LePage, G. A., *J. Biol. Chem.*, 1948, v176, 1009.

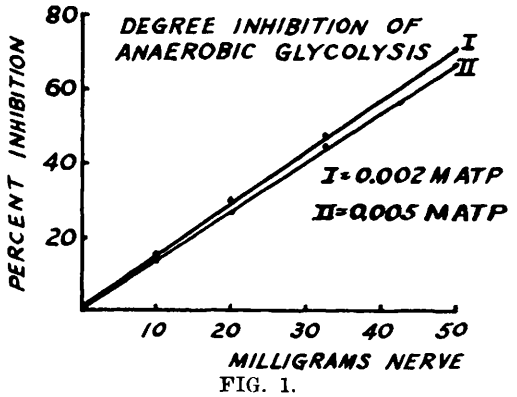

FIG. 1.

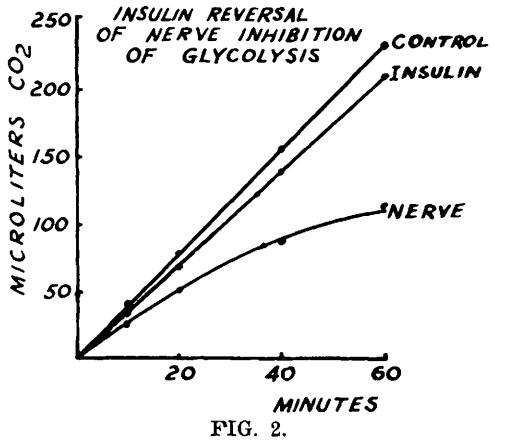

FIG. 2.

minutes, 100% at the end of this time. The amount of inhibition is linear with amount of nerve homogenate, from an average of 15% with 10 mg to 70% with 50 mg (Fig. 1). Most experiments were done with 40 mg of fresh nerve (30 mg of brain) which gave about 50% inhibition. The inhibition could be almost completely reversed by 0.5 mg of insulin (Fig. 2).

Glycolysis was at a constant rate, during the 60 minute period, in the control experiments and in those with nerve and insulin; but it dropped off rapidly in the presence of nerve homogenate only. This progressive fall of glycolytic activity cannot be due to an increased destruction of ATP by the nerve homogenate; for, at each nerve content, the inhibition of glycolysis was unchanged when the ATP was increased from .002M to .005M. (Fig. 3). Moreover, when brain and nerve homogenates, or brain hexokinase and nerve protein, were mixed an hour before adding substrate the inhibition was maximal from the start. Addition of insulin to a mixture with inhibition developed did not reinitiate glycolysis; fresh brain added to such a mixture showed at first its normal glycolytic rate. The evidence, therefore, favors a progressive inactivation of the enzyme (whether by chemical or physical change remains unknown) rather than a progressive activation of the inhibitor.

The step in the glycolytic sequence inhibited by nerve is that from glucose to hexose-6 phosphate, dependent on hexokinase. This is demonstrated by the failure to obtain inhibition of brain homogenate glycolysis with any hexose phosphate substituted for glucose as substrate (Table I; with fructose, inhibition by nerve is about half as great as with glucose), and also by the inhibition of separated hexokinase from brain. The inhibition of brain hexokinase, like that of brain homogenates, increases from 10 to 60 minutes, average inhibition for the hour being 40% with 30 mg nerve and 10 mg of enzyme (Fig. 3). Liver hexokinase is not inhibited, while that from yeast is actually accelerated by nerve homogenate.

Preliminary experiments showed the nerve inhibitor to be highly labile, so isoelectric precipitation was explored as a means of purification. Over two-thirds of the activity of the whole nerve homogenate appeared in a protein fraction isoelectric at pH 5.3 to 5.5. Two mg of this protein (not corrected for lipids present) gave a 23% inhibition of 10 mg of brain hexokinase activity; 5 mg gave almost 60%; and 0.2 mg of crystalline insulin

TABLE I. Nerve Inhibition of Brain Glycolysis with Various Substrates.

Substrate	Nerve homogenate (mg)	Inhibition (%)
Glucose	25	32
Fructose	25	19
Fructose	40	30
Glucose-1-phosphate	30	0
Glucose-6-phosphate	30	0
Fructose-6-phosphate	30	0
Fructose-1,6-phosphate	30	0

Nerve incubated with brain 20 min. before reaction of 40 min. duration. Final concentration of ATP was 0.003M. Substrate concentration was 0.01M. Each figure is avg of 5 experiments. They agreed within 5%.

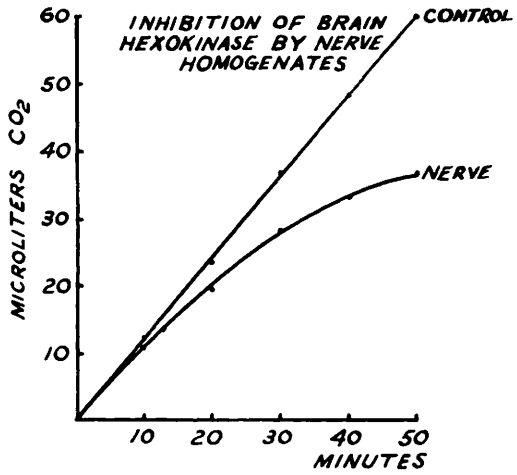

FIG. 3.

reduced this to only 20%. The values in Table II are statistically significant at the 5% level.

Heating whole nerve homogenates at 70°C (5 minutes was the minimal time tried) completely destroyed the inhibitor; even the isolated protein lost two-thirds of its activity when kept 12 hours at −5° (Table III).

Discussion. It appears conclusive from the above experiments that an inhibitor of hexokinase, a labile protein isoelectric at about pH 5.4, is present in nerve homogenates. The lability of the inhibitor, even at low temperatures, helps exclude such substances as sodium or sorbose-1-phosphate; and a spurious inhibition due to ATP-ase activity—present in the nerve protein as well as homogenate—is disposed of by addition of excess ATP. In fact, the considerable ability of crystalline insulin to reverse the inhibition tends to exclude loss of any cofactor and points to a direct action on the hexokinase enzyme.

Present evidence favors the existence of only one hexokinase in brain(8,9), so it is noteworthy that the inhibition of fructose utilization is significantly less than that of glucose. The rate of glycolysis in the presence of glucose is about 20% greater than that with fructose and is also more constant during the initial 60 minute period. These facts may relate to the difference in inhibition for the two hexoses.

Since glycolysis with fructose-6-phosphate as initial substrate is not inhibited by nerve, phosphofructokinase, which catalyzes the conversion of fructose-6-phosphate to fructose-1, 6-phosphate in the presence of ATP, is insensitive to the inhibitor.

The significance of a hexokinase inhibitor in nerve is highly conjectural. As indicated in the opening paragraph, the inhibitor seems to be formed or released when the integrity of nerve structures is destroyed. Yet some activity in whole nerve is not excluded, and this would at least fit with the 10-fold lower rate of glycolysis in nerve than in brain slices. Moreover, the inhibitor is present in the white matter of cord, at least, although less so than in nerve, while it is entirely absent from muscle and liver. The inhibitor of hexokinase obtained from nerve is strongly reminiscent of that found in the anterior pituitary by Colowick, Cori, and Slein(6). Particularly striking, is the similar isoelectric point of the nerve and pituitary factors, pH 5.4 and 5.3

TABLE II. Inhibition of Brain Hexokinase by Nerve Protein.

	No. determinations	Glucose utilized (micromoles)	Inhibition (%)
Control	10	5.6 ± .21*	
2 mg nerve protein	8	4.1 ± .20	23
5 mg " "	8	2.3 ± .13	59
5 mg " " and 0.2 mg insulin	8	4.5 ± .82	20

* Std dev. from mean. Each Warburg flask contained the following: 10 mg brain hexokinase, 1 mg glucose, .04M KF, .01M $KHCO_3$, .005M $MgCl_2$, .003M ATP. Final volume was 2.5 cc. Incubated 40 min. at 37°.

TABLE III. Lability of Nerve Inhibitor of Brain Hexokinase.

Preparation	pH of precipitation	Protein wt (mg)	Inhibition (%)
Fresh nerve protein	6	20	20
	5.4	5	59
Nerve protein 12 hr at 0°	6	20	5
	5.4	5	20
Nerve heated at 70° for 5 min.	Whole homogenate	50	0

8. Meyerhof, O., and Wilson, J. R., *Arch. Biochem.*, 1948, v19, 502.

9. Slein, M. W., Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, 1950, v186. 763.

respectively. Both factors are also heat labile, and their effects can be counteracted by crystalline insulin. The pars distalis of the hypophysis receives unmyelinated fibers from the carotid plexus(10); it is barely possible that they constitute the source of the anterior pituitary factor. An effort is being made at present to further purify and identify the inhibitor of hexokinase found in nervous tissue.

Summary. 1. Nerve homogenates, but not those of muscle or liver, inhibit brain glycolysis, the inhibition being a linear function of the amount of nerve. 2. Two thirds of the inhibitor activity is in a protein and lipid fraction precipitated at pH 5.3 to pH 5.5. From 200 mg of fresh nerve, 3 mg of protein and a like amount of lipid are obtained. Even this semi-purified fraction loses activity slowly in the cold, almost at once on heating. 3. The inhibition is specific for brain hexokinase. The enzyme from yeast or liver is not inhibited; glycolysis of hexose phosphates by brain is not inhibited. Fructose loss is inhibited half as strongly as that of glucose. 4. Crystalline insulin can counteract the nerve inhibitor. This and its other properties are similar to those of the inhibitor from the anterior pituitary.

10. Maximow, A. A., and Bloom, W., Textbook of Histology, W. B. Saunders, Philadelphia, Pa., 1948.

Received June 7, 1951. P.S.E.B.M., 1951, v77.

Properties of a Chromoprotein Containing Extract of the Particulate Fraction of Rat Liver. (18807)

ERNEST KUN.†

*From the Department of Medicine, The University of Chicago.**

It is generally recognized that certain reactions of terminal electron transfer to molecular oxygen are catalyzed by enzymes structurally associated with the particulate fraction of tissue suspensions. One of the difficulties in studying these reactions is the inaccessibility of tissue granules to enzyme isolation procedures. Organic solvents have been used (1,2) for the separation of catalytically active chromoproteins, associated with insoluble cell particles. It was attempted in this laboratory to use alcohols and a protective colloid simultaneously for the extraction of enzymes bound to tissue particles. The following report contains a procedure for the extraction of a catalytic system from rat liver particles, which can transfer electrons from reduced pyridine nucleotides to cytochrome C. Some physical properties of this optically homogenous system are also discussed. While this work was in progress Morton(3) reported that he could successfully employ n-butanol for the extraction of enzymes hitherto considered to be inseparable from tissue particles.

Experimental. Preparation of extract. 50 g of rat liver were homogenized in a Waring blendor for 30 seconds in ice cold solution of 0.15 *M* KCl, brought to pH 7.6 with bicarbonate. The volume of KCl solution was 100 ml. The temperature of the suspension during blending was kept at 0°C by the addition of crushed ice particles (made of distilled H_2O). The liver suspension was centrifuged between 0-4°C on the Servall angle centrifuge (type SS-1A) at 15.000 x g for 30 minutes. The supernate was decanted and the sedimented particulate fraction removed with a porcelain spoon, leaving the layer of agglutinated erythrocytes on the bottom of the centrifuge tube. The particulate fraction, or

* Present address: The Tulane University of Louisiana, Department of Medicine and Biochemistry.

† This work was supported by a grant of the U. S. Public Health Service.

1. Straub, F. B., *Biochem. J.*, 1939, v33, 787.

2. Yakushiji, E., and Mori, T., *Acta. phytochim.* (Japan), 1937, v10, 113.

3. Morton, R. K., *Nature*, 1950, v166, 1092.