THE QUANTITATIVE HISTOCHEMISTRY OF THE BRAIN*

V. ENZYMES OF GLUCOSE METABOLISM

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The importance of glucose for nervous function has long been recognized. This paper is a report of the histochemical distribution in certain parts of the brain of seven enzymes which are known to act upon hexose or hexose phosphates. The distribution of these seven enzymes might be expected to signify for each histological element the relative capacity and possible importance of four main channels of glucose utilization: (*a*) primary glucose esterification (hexokinase[1]), (*b*) glycogen metabolism (phosphorylase and phosphoglucomutase), (*c*) glucose metabolism via the Embden-Meyerhof system (phosphoglucoisomerase or isomerase, phosphofructokinase, and aldolase), and (*d*) oxidation via the "shunt" (glucose-6-phosphate dehydrogenase).

The distribution of the seven enzymes was measured among ten histologically segregated zones of the central nervous system of the rabbit: two molecular layers, a layer of dendrites, two layers of packed cell bodies, and five myelinated fiber tracts.

It was not practical to obtain large samples of these zones. Therefore it was necessary to elaborate microchemical methods in the case of the five enzymes for which sufficiently sensitive methods were not already available. The resulting procedures require only 0.1 to 5 γ of dry brain and have been validated by a variety of tests, including the recovery of the activities of purified enzyme preparations when added to whole homogenates and the assessment of the stability of these enzymes in rabbit brain to freezing, drying, and storage.

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[1] The abbreviations used are Tris, tris(hydroxymethyl)aminomethane; P_i, inorganic phosphate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotides; ATP, adenosine triphosphate; HK, hexokinase; PGM, phosphoglucomutase; PFK, phosphofructokinase; G6DH, glucose-6-phosphate dehydrogenase; AMP, 2-amino-2-methylpropanediol; LDH, lactic dehydrogenase; and PHRL, glycogen phosphorylase.

At least some of the procedures developed might find application for more general purposes, since they are rather convenient and quite reproducible. The isomerase method is based on the measurement of the F6P formed. Changes made in the fructose method of Roe *et al.* (1) seem to constitute a substantial improvement. For the measurement of PGM, conditions were established for hydrolysis of G1P and measurement of the

TABLE I

Incubation Conditions for Six Enzymes Measured

	pH	Buffer		Substrate		Other additions
			M		*mM*	
Hexokinase	8.0	Hydrazine	0.04	Glucose	7	Mg 5 mM; KH_2PO_4 0.015 M; purified isomerase + PFK + aldolase
				ATP*	3	
Phosphoglucoisomerase	8.0	Tris	0.1	G6P	90	
Phosphofructokinase	8.0	K_2HPO_4	0.04	F6P	7	Hydrazine 0.03 M; Mg 6 mM; $(NH_4)_2HPO_4$ 1.3 mM; crystalline aldolase 0.01%; crystalline bovine albumin 0.02%
				ATP	15	
Phosphorylase	6.7	Substrate		G1P	30	Glycogen 0.5%; 5′-adenylic acid 1 mM; cysteine 15 mM; NaF 50 mM
Phosphoglucomutase	7.6	Tris	0.02	G1P	1	Mg 1.5 mM; BAL 2 mM; glucose 1,6-diphosphate, 2×10^{-6} M
G6P dehydrogenase	8.9	AMP†	0.1	G6P	5	Mg 10 mM; Versene 1 mM; TPN^+ 2 mM

* It would be preferable to increase ATP to 10 mM.

† 2-Amino-2-methylpropanediol.

P_i in one step. For the measurement of phosphorylase, improved conditions were found for measuring P_i in the presence of G1P.

Materials and Methods

The analyses were all performed on histologically characterized fragments dissected from frozen-dried sections of rabbit brain or spinal cord. The preparation and weighing of such fragments before analysis have been described (2). The general techniques and tools for measuring enzymatic activities on the required microscale, as well as a description of the aldolase method, have been given in detail (3, 4). The method for G6DH is taken

from an unpublished procedure of Dr. A. W. Albers. After incubation under the conditions given in Table I, protein is precipitated by adding 0.33 M KCl in 67 per cent ethylene glycol monomethyl ether, and the TPNH formed is measured by its absorption of light at 340 mμ. The lipide was measured by loss of weight upon extraction with alcohol and hexane (2).

All of the analyses to be presented here were made with tissues from a single rabbit. However, the results have been confirmed by less complete data from other rabbits, and whole brain homogenates have been analyzed upon innumerable occasions for all of these enzymes. The disparity among the animals was found to be remarkably small.

All of the analyses were performed in the same manner. The dissected and weighed samples (1 to 2 γ) were placed in tubes in racks and stored at −20° until they could be analyzed. At this time the racks were placed in an ice bath, and a measured volume of complete buffer-substrate reagent was added to each tube. The entire rack of tubes was then incubated at 38°, and the action was arrested in an ice bath before subsequent steps.

All of the methods have been tested extensively with whole brain homogenates for effects of variations in pH, substrate, or coenzyme concentration, time of incubation, and activity with different dilutions of homogenate. Unless otherwise noted, the incubation conditions were such as to give maximal activity and proportionality between colorimeter readings and either the amount of enzyme or the time of incubation up to at least 1 hour. With reasonable care, the methods are all reproducible to the limits of the spectrophotometer and, in order to conserve space, this fact will not be documented. A more practical test is provided by the over-all reproducibility of the histochemical values to be presented.

Phosphofructokinase

Principle—The reagent contained added crystalline aldolase which converted hexose diphosphate as fast as it was formed to triose phosphates. These were trapped by hydrazine and measured colorimetrically with dinitrophenylhydrazine.

Procedure—Dry samples weighing 1 to 2 γ were incubated with 18 μl. of complete reagent for 30 minutes at 38°. This substrate reagent was made freshly before use (Table I). The aldolase, prepared according to Taylor *et al.* (5), was recrystallized twice, and the wet crystals were stored at −20°. A 1 per cent solution was prepared as needed. The reaction was permanently arrested with 3 μl. of 30 per cent trichloroacetic acid. The samples were centrifuged, but this is probably unnecessary because of the small amount of protein involved. However, acidification cannot be omitted. Aliquots of 10 μl. were analyzed for triose phosphates by a

published procedure (4) adapted from the aldolase method of Sibley and Lehninger. The final volume for reading was 345 μl. For calculation, it was assumed that the extinction coefficient corresponding to 1 mole of F6P converted to triose phosphate would be 47,800. This is based on the value obtained with G6P in the similar system used for the assay of hexokinase (see below). Neither F6P nor hexose diphosphate was available in sufficient purity to use as a standard. A sample calculation is as follows. A particle of tissue weighing 1.5 γ was incubated for 30 minutes as detailed above. The observed net optical density was 0.250. Therefore the activity was calculated to be (21 λ/1.5 γ) × (345 λ/10 λ) × (0.250/47,800) × (60/30) × 1000 = 5.05 moles per kilo per hour.

Comment—Rabbit brain PFK is exceedingly unstable in water homogenates, but may be protected if fresh brain is homogenized (1:10) in 0.2 M phosphate buffer at pH 7.8.

Frozen-dried brain sections, however, appear to be stable almost indefinitely if they are kept cold. Samples from the stratum radiatum of Ammon's horn after 4 years storage at −20° had 85 per cent of the activity of samples stored for 1 month (from another rabbit).

Rabbit brain PFK was purified about 20-fold by the procedure of Muntz (6). When this was mixed with a crude homogenate, the activities were additive. (Observed net optical densities were 0.627, 0.469, and 1.085 for samples containing, respectively, purified enzyme, whole homogenate, and both. The calculated sum was 1.096.)

Hexokinase Assay

Principle—The G6P formed was converted to triose phosphate by added PFK, phosphoglucoisomerase, and aldolase. Hydrazine was used to trap the triose phosphates and to prevent the action of triosephosphate isomerase. The triose phosphates were measured colorimetrically with dinitrophenylhydrazine.

Procedure—The substrate reagent was prepared freshly before use (Table I). However, all components other than ATP and PFK may be combined and stored at −20°. The auxiliary enzymes were prepared as a single mixture (PFK) from skeletal muscle by the procedure of Taylor (7) as modified by Crane and Sols (8). The amount used was sufficient to give not less than 90 per cent conversion of G6P to triose phosphates in 5 minutes.

Dry samples weighing 1 to 2 γ were incubated with 5 μl. of complete reagent for 30 minutes at 38°. The reaction was permanently arrested with 1 μl. of 30 per cent trichloroacetic acid. After centrifugation, 5 μl. were analyzed for triose phosphates, as in the case of PFK, except that all volumes were reduced by half. Standards consisted of 5 μl. samples of 1

mM G6P made up at the last minute in the complete substrate reagent, and these were carried through the entire procedure.

Comment—The removal of G6P as fast as it is formed has the advantage of preventing the marked product inhibition which would otherwise occur (8, 9). Consequently, the color produced was proportional to enzyme concentration to within 5 per cent over a 4-fold range. Also, observed rates were nearly linear with time (10 per cent less at 90 than at 20 minutes).

The color produced per mole of glucose phosphorylated is about 6 times that given by 1 mole of glucose in the usual colorimetric reduction methods. Furthermore, the method is direct and can be employed on a scale which would be troublesome for glucose measurement.

On a semimicroscale, parallel measurements of glucose disappearance and triose phosphate formation gave identical results. Hydrazine was omitted in the first instance because this would have interfered with the glucose measurement.

A sample of brain hexokinase purified 50-fold was generously provided by Dr. Robert K. Crane and Dr. Alberto Sols. By the given procedure, the activities of this sample and of whole brain homogenate were additive. (Optical densities separately were 0.306 and 0.383, respectively; the optical density of the mixture was 0.685 observed, and 0.689 calculated.) Therefore, there were no obvious complications arising from the use of crude brain samples.

Rabbit brain hexokinase is unstable in aqueous homogenates, particularly when highly diluted, and is not preserved by freezing. However, the stability is much better in phosphate buffers at pH 7.5. Even so, attempts to dry small aliquots of frozen homogenates have resulted in loss of over 50 per cent of the activity. This loss may be, in part, the result of failure to disperse aggregated particles for the analysis (Crane and Sols (8)). In spite of the low values with frozen-dried homogenates, it is believed that HK is completely preserved in frozen-dried tissue sections. The values obtained for dried sections from various parts of the brain fall within the range expected from the average for whole brain, and frozen-dried sections stored for 4 years (at $-20°$) have been found to be at least 90 per cent as active as fresh frozen-dried sections.

Phosphoglucoisomerase

Procedure—Dry samples weighing 1 to 2 γ were incubated for 30 minutes at 38° in 10 μl. of reagent (Table I). Aliquots of 7 μl. were transferred into 1 ml. of color reagent in a 3 ml. test tube and, because of the viscosity of the reagent, were vibrated or tapped vigorously in order to mix them. Color was developed by heating the mixture 20 minutes in a water bath at

60°, and readings were made at 500 mμ. Standards, consisting of 10 μl. of reagent containing 5 mM fructose, and blanks were included in all the sets of samples. F6P appeared to give about 80 per cent as much color as fructose, and this value was used in the calculations.

The color reagent consisted of a fresh mixture of 40 volumes of 20 N H_2SO_4 with 1 volume of glacial acetic acid containing 0.4 per cent resorcinol and 1 per cent thiourea.

The color of samples is almost insensitive to light, and readings may be made at any time up to 24 hours. However, blanks and standards must

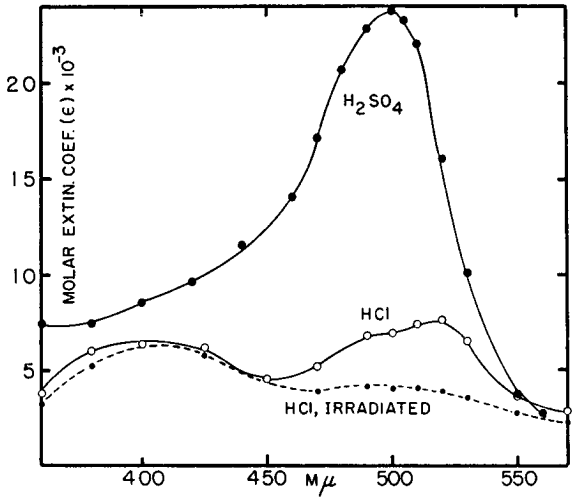

FIG. 1. Absorption spectra of fructose heated with resorcinol in H_2SO_4 and HCl. The conditions of reaction in H_2SO_4 were those proposed in this paper. The conditions of reaction in HCl were those prescribed by Roe *et al.* (1). Irradiation consisted of 60 minute exposure 12 inches from a 100 watt tungsten lamp.

be run at the same time because the color may change slightly, and the blank increases progressively as a result of the G6P.

Comment. Fructose Measurement—The method of Roe *et al.* (1) presented difficulties on a microscale. The HCl concentration is quite critical, and during the heating step (80°) HCl volatilized somewhat from microsamples and changed the composition and the final color in an erratic manner. The sensitivity to light of the color produced was an added source of error. This second difficulty could be largely avoided by reading at 400 instead of at 520 mμ, because the peak at the shorter wave length changes much less upon irradiation (Fig. 1).

The problem of volatility, however, was more troublesome, and H_2SO_4 was therefore substituted for HCl with considerable advantage. The

color produced was two or three times as great as in HCl, was stable to light, and could be developed in 20 minutes at 60° in 20 N H_2SO_4 or in an hour at 38° in 25 N H_2SO_4. The only disadvantage was the viscosity of the sulfuric acid, which was moderate at 20 N or below, but which increased much more rapidly above 20 N. Curiously, a 0.1 volume of glacial acetic acid used as a medium for adding thiourea and resorcinol increased the viscosity of 20 N sulfuric acid by nearly 50 per cent. Therefore, the proportion of acetic acid was reduced from 10 per cent, as used by Roe *et al.*,

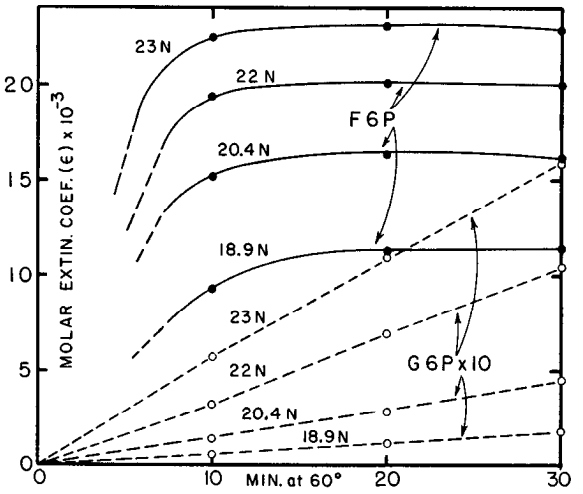

FIG. 2. Extinction coefficients at 500 mμ with F6P and G6P heated for various times with resorcinol in sulfuric acid of indicated concentrations. Note that the scale for G6P is expanded 10-fold.

to 2.5 per cent, with a compensatory increase in the concentration of thiourea and resorcinol in the acetic acid.

A record of the effects of changes in time of heating and sulfuric acid concentration on the color produced with F6P and G6P may be useful (Fig. 2). It is interesting that the color produced in H_2SO_4 differs from that obtained in HCl (Fig. 1). The HCl product is somewhat fluorescent and becomes very fluorescent on irradiation, whereas the H_2SO_4 product is only moderately fluorescent and is not enhanced by irradiation.

Isomerase Properties—Rabbit brain isomerase has a pH optimum at 8, with a decrease to 80 and 88 per cent of maximal activity at pH 7 and 8.5, respectively. The buffer used (Tris) is probably not inhibitory since about the same activity was obtained with half or twice the concentration. The K_m is probably small since the velocity was as fast with 2.5 mM substrate as with 90 mM, provided that the same small fraction of substrate

was isomerized. The velocity decreases as the fraction of the substrate is converted about as much as predicted for a reversible reaction. Consequently, for accurate analytical results, the fraction of substrate converted must be kept small, or a suitable correction curve must be used.[2] Because of the small K_m, the substrate concentration may be adjusted as necessary to keep the fraction of substrate converted within suitable limits.

Brain isomerase withstands freezing and drying without loss. There is a loss of about 50 per cent after a week at 25° in the dry state. At −20°, frozen-dried sections seem to be stable indefinitely (for years).

Isomerase was purified 25-fold from rabbit brain. An extract of brain made with 2 volumes of 0.03 N KOH was combined with a water washing of the residue. This was fractionated with ammonium sulfate at pH 7.5 between 1.6 and 2.4 M, and refractionated between 2.0 and 2.2 M. The product had an activity of about 8000 moles per 100 kilos of protein per minute. A recovery experiment was performed in which this preparation was added to whole brain homogenate. The recovery averaged 120 per cent. The extra activity is attributed to protection of the purified enzyme by the protein of the crude brain. It has since been found that at very high dilutions it is necessary to add inert protein, such as bovine plasma albumin, in order to avoid some loss of activity.

Phosphoglucomutase

Principle—The disappearance of G1P is measured after incubation in a single analytical step in which the Cori ester is hydrolyzed and the liberated phosphate produces maximal color with molybdate and ascorbic acid.

Procedure—Samples of 1 to 2 γ of dry weight were incubated for 20 minutes at 38° in 7 μl. of the substrate solution indicated (Table I). This substrate solution may be stored in a frozen state without the BAL or glucose diphosphate, which can then be added separately as needed. The BAL is conveniently prepared by shaking a 10 per cent solution in peanut oil with 10 volumes of water, which extracts almost 90 per cent and yields a 65 mM solution that is stable in a frozen state for at least a week. The reaction was permanently arrested with 7 μl. of 10 per cent trichloroacetic acid. After centrifugation to remove protein, a 10 μl. aliquot was transferred to a tube of 3 mm. inner diameter (50 or 60 mm. long), and 100 μl. of phosphate reagent were added. The tubes were heated for 45 minutes in a water bath at 50° (well capped with Parafilm to prevent evaporation), and the absorption was measured at 820 mμ. The phosphate reagent is prepared by mixing, just before use, 5 volumes of 10 per cent ascorbic acid

[2] Recently Alvardo and Sols have found that the F6P may be readily trapped with borate without inhibition of isomerase (10). In consequence, the reaction can be made linear until nearly all of the G6P is gone.

with 10 volumes of 1.7 per cent ammonium molybdate in 3 N H_2SO_4. The ascorbic acid solution is stored in a frozen state.

G1P is completely hydrolyzed by the procedure given, and the phosphomolybdate is reduced with a molar extinction coefficient of about 27,000. G6P is about 0.5 per cent hydrolyzed. The reaction can be standardized by the substitution of 7 μl. of 1 mM P_i for the substrate reagent.

Since a decrease in sample reading is the measure of enzyme action and since the reaction departs from linearity if too much substrate is consumed, an effort is made to convert not less than 20 or more than 50 per cent of the G1P to G6P. The size of the sample, the volume of the reagent, and the incubation time may be varied for this purpose. The substrate concentration may also be increased if necessary.

Comment—The only definite change in the assay system as compared to those of previous investigators was the substitution of BAL for cysteine or other metal-binding agents (11). With 0, 0.6, 1.2, 2.2, and 3.4 mM BAL, relative activities were 17, 87, 100, 100, and 100 per cent, respectively. With 12 mM cysteine the activity was 10 per cent less, possibly as a result of the increase in total salt concentration.

With 0, 1.5, and 15 mM Mg, activity was 18, 92, and 100 per cent of that with 4 mM Mg. The activity without added Mg is ascribable to Mg in the brain homogenate, and the data are consistent with a K_m for Mg of about 4×10^{-5} M.

Without added glucose diphosphate, activity was very low and varied with tissue dilution. At whole rabbit brain dilutions of 1:10,000 and 1:1000, activity was 10 and 40 per cent, respectively, of the activity with 2×10^{-6} M glucose diphosphate. With added coenzyme, activity was closely proportional to both time and concentration of brain until 50 per cent of the substrate was converted.

A pH optimum near 7.5 was confirmed for brain PGM. Activities were, respectively, 82 and 90 per cent of the maximum at pH values of 7.2 and 8.0. Because of the sensitivity to pH, dilute buffer was used in the reagent, in spite of the known inhibitory effect of salts.

The activity of brain enzyme at 1° and 25° was found to be 2.1 and 37.1 per cent of that at 38°.

In order to test for possible inhibitory or enhancing factors in whole brain homogenates, PGM was purified 25-fold from rabbit brain and mixed with a crude homogenate. The respective activities were additive to within 2 per cent when assayed in combination. The purification was accomplished with ammonium sulfate fractionation at pH 7.5 of an alkaline brain extract. The fraction, which precipitated between 40 and 60 per cent saturation, was again precipitated between 55 and 60 per cent saturation.

The activity of PGM is stable in frozen-dried sections stored at −20° for at least a year.

Color Development—G1P was hydrolyzed and the color developed from the liberated P_i in one step. This was accomplished at a moderate temperature by taking advantage of the catalytic activity of molybdate on the hydrolysis (12) and by increasing the sulfuric acid concentration to 2 N. As the acidity increases, phosphomolybdate becomes increasingly difficult to reduce. This is offset by the following changes, in comparison to a previously published method for P_i of similar sensitivity which was performed with 0.9 N H_2SO_4 (3). Ammonium molybdate is increased from 0.25 to 1 per cent, ascorbic acid from 1 to 3 per cent, and the temperature is raised from 38–50°. The extinction coefficient is about 6 times that obtained by the method of Fiske and Subbarow. The procedure is clearly useful only in special situations since many phosphate esters would be partially hydrolyzed under the analytical conditions. Under the prescribed conditions, P_i gives 80 per cent full color in 10 minutes, and G1P 80 per cent full color in 25 minutes. If desired, the time for color development may be decreased from 45 to 20 minutes by heating at 57° instead of at 50°.

Glycogen Phosphorylase—The reagent was a fresh mixture of equal volumes of a solution (*a*) containing, at double strength, all of the ingredients indicated (Table I) except cysteine, and a fresh, neutralized (pH 6.7), 0.03 M cysteine solution (*b*) prepared from 0.3 M cysteine hydrochloride. Solution *a* was stored at −20°.

Each tissue sample (1 to 2 γ) was incubated with 10 μl. of complete reagent for 30 minutes at 38°. The reaction was permanently arrested with 1 μl. of 55 per cent trichloroacetic acid with prompt centrifugation in the cold to minimize the acid hydrolysis of G1P. The tubes were kept in ice water as much as possible. Aliquots of 10 μl. of supernatant fluid were promptly added to 50 μl. of phosphate reagent. This consisted of a fresh mixture of 1 ml. of 2.5 per cent ammonium molybdate, 7 ml. of H_2O, 0.02 ml. of 1 per cent $CuSO_4 \cdot 5H_2O$, and 1 ml. of a fresh 6 per cent solution of a powder composed of 95 per cent $NaHSO_3$, 4.5 per cent Na_2SO_3, and 0.5 per cent 1-amino-2-naphthol-4-sulfonic acid (13).

The samples were read at 700 mμ after 10 minutes. Standards consisted of 10 μl. of reagent which was 0.5 mM in KH_2PO_4. Standards and blanks were treated as nearly as possible in the same way as the samples.

Comment. Measurement of Inorganic Phosphate Released—The P_i is measured with a reagent similar to that of Fiske and Subbarow (13), except that sulfuric acid is omitted, and the only acid is the trichloroacetic acid used to precipitate protein (final concentration about 0.05 N). Since the final pH is about 2.5 (instead of 0.6), there is practically no hydrolysis

of G1P, and the color from P_i does not increase with time (Furchgott and deGubareff (14)). Copper is added to accelerate color development, as suggested in 1949 by Dr. C. S. Hanes (personal communication).

If more color is desired, the molar extinction coefficient, ϵ_m, can be increased from about 4000 to 24,000 by using a reagent which contains (at final dilution) 0.2 N H_2SO_4, 0.1 per cent ammonium molybdate, and 3 per cent ascorbic acid. Reading is made after 60 minutes at room temperature (25–28°) or after 20 minutes at 38°. The reagent has some disadvantages. Approximately 1.5 per cent of the G1P is hydrolyzed in this length of time, and the blank increases a little with time, even without G1P present.

Rabbit Brain Phosphorylase Properties and Validation of Procedure— With dilution of rabbit brain 200-fold or more, P_i formation in the absence of added glycogen was not over 2 per cent of that with 0.5 per cent glycogen present. This indicated that, even though the assay was made on whole brain, there were no significant sources of P_i other than that of the phosphorylation reaction. Half maximal activity was obtained with 0.013 per cent glycogen.

When G1P concentration was varied, the activities indicated a K_m of 7.2 mM. Similarly, the activities with different levels of adenylic acid were compatible with a K_m of about 0.05 mM. Activity was enhanced 25 per cent by cysteine, 15 mM, but a lower level of BAL (1.5 mM) was not stimulatory. Versene will not replace cysteine with the brain enzyme. Activity was relatively independent of pH between 6 and 7.

Altogether, brain phosphorylase in the whole tissue assay system behaved about as might be expected from the studies of Cori *et al.* (15) with highly purified preparations.

Phosphorylase in brain homogenates withstood freeze-drying; in fact, the activity increased 35 per cent, probably as a result of better disruption of the tissue. There is no evidence of destruction of activity during storage in the dry state at −20°. A brain stored at −20° for a year without drying retained 60 per cent of the activity of a fresh brain.

P_i liberation was closely proportional to the time of incubation and the amount of brain, provided that not more than 10 per cent of the substrate was used.

RESULTS AND DISCUSSION

The values have been calculated on a lipide-free basis since the wide range of lipide might obscure the significance of the results. The lipide-free dry weight has been found to be a nearly constant fraction (10 to 12 per cent) of the wet weight of various parts of brain, in spite of extreme differences in lipide content.

The results will be examined with three purposes in mind: (1) to compare the absolute activity values for the seven related enzymes, (2) to contrast the histological distribution of enzymes concerned with alternative pathways of hexose utilization, and (3) to ascertain to what degree enzymes of the same metabolic pathway vary in parallel fashion as the metabolic pattern changes throughout the nervous system.

The enzymatic activities in whole brain were all measured under the near optimal conditions which are believed to make the enzyme in question strictly rate-limiting and which avoid significant destructive or inhibitory factors. Therefore, the absolute values may merit examination (Table II). Isomerase leads, with the enormous value of 150 moles per kilo of fat-free dry weight per hour, which is 100 times greater than the value of G6DH, the least active. It must be recognized that these are maximal velocities obtained with high substrate levels at optimal pH for each enzyme. Therefore, in interpreting these activities the actual pH and substrate conditions in the cells would have to be evaluated. For example, because of its small Michaelis constant, it may be that G6DH, in spite of its low absolute activity, may compete with some success with isomerase and PFK for G6P. It is clear that most of the seven enzymes operate at far less than maximal velocity since oxygen consumption of brain does not exceed that necessary to burn 0.2 mole of glucose per kilo of fat-free dry weight per hour, and maximal glycolysis is sufficient only to use 0.6 mole of glucose per kilo per hour (16).

There are modest but significant differences among the five non-myelinated layers (Table II). Nearly all of the enzymes tend to be a little lower in content in the cell body layers, whereas the dendrite layer is particularly rich in isomerase and PFK.

More striking are the differences between the gray layers and the myelinated tracts. Without exception, the white tracts are relatively poor in hexokinase and relatively rich in G6DH. The data suggest that white tracts are more capable of using glycogen than glucose, and that the direct oxidative shunt is a relatively important pathway in the use of G6P. Among themselves, the white tracts differ a good deal. There is indication of an inverse relationship between enzymes of glycolysis and G6DH (PFK *versus* G6DH in Table III), and a direct relationship between total lipide and G6DH (Table III).

Optic tract is exceptional in its high isomerase activity. It was previously found to be very rich in lactic dehydrogenase (17). The retinal ganglion cell layer, containing the cells of origin of the optic tract, is also rich in lactic dehydrogenase in the rabbit (17, 18). It is presumably rich in all enzymes of the glycolytic cycle. The optic tract is the richest in PFK of the five tracts, but by a much smaller margin than in the case of

isomerase or lactic dehydrogenase, and it is only second highest in aldolase activity. Possibly this means that the extraordinary levels of certain enzymes of glycolysis in the optic tract are the consequence of diffusion

TABLE II

Distribution of Seven Enzymes of Glucose Metabolism in Ten Regions of Rabbit Brain

All enzyme values are recorded as moles of substrate transformed per kilo of lipide-free dry weight per hour. The lipide is recorded as percentage of lipide-free dry weight. Each enzyme value represents the average of six analyses as a rule. The standard errors are given in bold-faced type. Lipide values are from earlier studies.

	Lipide	HK	Isomerase	PFK	Aldolase	PGM	PHRL	G6DH
Molecular layer, cerebellum	73	6.70	164	21.3	10.6	9.4	12.9	2.38
		0.42	**7**	**1.9**	**0.3**	**0.4**	**0.3**	**0.06**
" " Ammon's horn	63	6.42	116	15.9	8.4	10.3	8.9	1.30
		0.44	**4**	**1.2**	**0.3**	**0.3**	**0.6**	**0.08**
Dendrite layer, Ammon's horn	61	5.46	193	32.5	9.4	9.0	6.8	1.18
		0.21	**7**	**1.6**	**0.2**	**0.3**	**0.3**	**0.04**
Cell body layer, cerebellum	45	5.09	88	11.0	5.2	5.9	7.3	1.15
		0.20	**3**	**1.1**	**0.1**	**0.3**	**0.1**	**0.03**
" " " Ammon's horn	28	4.32	94	11.1	4.7	5.3	5.4	0.86
		0.15	**3**	**0.6**	**0.1**	**0.2**	**0.2**	**0.03**
White tract, Ammon's horn	161	2.43	82	13.0	5.0	6.0	6.4	1.85
		0.18	**3**	**0.4**	**0.1**	**0.3**	**0.3**	**0.05**
" " cerebellum	240	1.93	95	13.4	7.3	7.4	5.0	3.32
		0.17	**3**	**0.8**	**0.3**	**0.6**	**0.2**	**0.08**
" " optic	251	2.17	234	15.6	6.9	6.6	3.9	2.67
		0.14	**11**	**0.8**	**0.1**	**0.2**	**0.2**	**0.15**
" " dorsal columns	317	1.54	66	11.2	2.6	9.2	5.7	5.42
		0.08	**4**	**0.7**	**0.2**	**0.5**	**0.2**	**0.21**
" " dorsal spinocerebellar	324	1.38	62	8.4	3.0	6.5	4.4	5.25
		0.04	**4**	**0.4**	**0.1**	**0.5**	**0.4**	**0.21**
Average brain	100	6	150	16	6.5	10	7	1.5

from the avascular ganglion cell layer of the retina rather than indicative of an unusually high glycolytic rate in the optic tract itself.

PFK and PHRL bear a nearly constant ratio in many of the regions of the brain examined (Table III). The ratios are less constant between PFK and HK. This might suggest that maximal capacity for a burst of glycolysis is related to ability to use glycogen rather than to capacity for direct utilization of glucose.

The above discussion presupposes that the local concentration of a particular enzyme is indicative of the capacity for metabolism along the pathway which contains that enzyme. If this is true, then all members of that pathway should vary in constant ratio. The ratios of Table III support this thesis, but only in part. Thus isomerase, PFK, aldolase, and lactic dehydrogenase in general vary in a like manner, but in certain cases there are rather large deviations from the mean ratios. These deviations could indicate some tolerance in the concentration of certain

TABLE III

Ratios between Various Enzymes of Glucose Metabolism in Rabbit Brain

Tissue	PFK / HK	PFK / PHRL	PFK / G6DH	Isomerase / PFK	PFK / Aldolase	Isomerase / LDH*	PGM / PHRL	G6DH −0.6 / Lipide†
Molecular layer, cerebellum	3.2	1.6	9	8	2.0	5.8	0.7	26
" " Ammon's horn	2.5	1.8	12	7	1.9	4.6	1.2	11
Dendrite layer, Ammon's horn	6.0	4.8	27	6	3.5	6.0	1.3	11
Cell body layer, cerebellum	2.2	1.5	10	8	2.1	4.1	0.8	14
" " " Ammon's horn	2.6	2.1	13	9	2.4	3.4	1.0	13
White tract, Ammon's horn	5.3	2.0	7	6	2.6	4.5	0.9	8
" " cerebellum	6.9	2.7	4	7	1.8	4.1	1.5	12
" " optic	7.2	4.0	6	15	2.3	4.2	1.7	9
" " dorsal columns	7.3	2.0	2.1	6	2.0	4.1	1.6	15
" " " spino-cerebellar	6.1	1.9	1.6	7	1.6		1.5	15
Average brain	2.3	2.3	11	9	2.5		1.4	20

* Calculated from data previously published (17) on lactic dehydrogenase.

† Gm. of lipide per gm. of lipide-free dry weight. This empirical relationship gives the best graphical fit of a linear plot of G6DH *versus* total lipide.

enzymes of a series, or might indicate a more complex situation than anticipated. Thus the high PGM to PHRL ratios found in most of the white tracts could mean that G1P has another use in these tracts, perhaps in the formation of uridine diphosphoglucose.

SUMMARY

1. New micromethods are presented for measuring hexokinase, phosphoglucoisomerase, phosphofructokinase, glycogen phosphorylase, and phosphoglucomutase in 1 or 2 γ of tissue (dry weight). The methods have been validated for use with frozen-dried brain that is either fresh or stored at $-20°$ for several years.

2. The new methods, together with existing procedures of similar sensitivity for aldolase and glucose-6-phosphate dehydrogenase, have been used to measure the distribution of seven major enzymes of hexose metabolism among five white tracts, two molecular layers, two layers of cell bodies, and a layer of packed dendrites from rabbit brain.

3. Cell body layers (of cerebellum and Ammon's horn) tend to be lower in content than other gray areas with respect to every enzyme measured. White tracts vary greatly among themselves. Heavily myelinated tracts are low in hexokinase activity and rich in glucose-6-phosphate dehydrogenase. In respect to the other five enzymes, all of the tracts examined are about as active on a fat-free dry weight basis as the cell body layers.

4. Enzyme members of the same metabolic path tend to vary in a similar manner, but the correspondence is by no means perfect.

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