THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 244, No. 13, Issue of July 10, pp. 3647-3653, 1969
Printed in U.S.A.

Effects of Changes in Brain Metabolism on Levels of Pentose Phosphate Pathway Intermediates*

(Received for publication, March 19, 1969)

FREDERICK C. KAUFFMAN, JOSEPH G. BROWN, JANET V. PASSONNEAU, AND OLIVER H. LOWRY

From the Department of Pharmacology and the Beaumont-May Institute of Neurology, Washington University Medical School, St. Louis, Missouri 63110

SUMMARY

Several metabolites of the pentose phosphate pathway, together with related intermediates of the Embden-Meyerhof pathway, were measured in cerebral tissue of mice after periods of ischemia, anesthesia by phenobarbital, hyperthermia, hypothermia, and iodoacetate and fluoroacetate poisoning. Normal levels (micromoles per kg) were found to be 6-P-gluconate, 10; ribulose-5-P, 7; xylulose-5-P, 14; and erythrose-4-P, less than 2. The sum of ribose-5-P and sedoheptulose-7-P was 64 μmoles per kg.

During 5 min of complete ischemia, 6-P-gluconate decreased 30% and xylulose-5-P fell 80%. Ribulose-5-P was also sharply reduced. Anesthesia resulted in increases of 40 to 50% for ribulose-5-P and xylulose-5-P without change in 6-P-gluconate. Anesthesia also prevented or greatly diminished the changes in phosphate intermediates induced by ischemia.

Hypothermia resulted in decreases in ribulose-5-P and xylulose-5-P, and an increase in 6-P-gluconate. Conversely, hyperthermia exactly reversed the pattern of these changes.

Iodoacetate intoxication resulted in marked increases in ribulose-5-P and xylulose-5-P, but glucose-6-P and 6-P-gluconate fell below control values. There did not appear to be any direct correlation between the magnitude of the pentose phosphate increases and those of the triose phosphates. In fluoroacetate-treated mice glucose-6-P levels increased approximately 3-fold, whereas intermediates in the pentose phosphate pathway and triose phosphates remained near control levels or fell slightly.

The results are compatible with a regulatory mechanism at both of the dehydrogenase steps with some effect of glyceraldehyde-P and fructose-6-P concentrations on the levels of intermediates of the pentose pathway below 6-P-gluconate.

To date regulatory mechanisms associated with the pentose phosphate pathway in cerebral tissue have not received much attention. Studies of this route of metabolism have been based almost exclusively on measurements of the conversion of differentially labeled glucose-^{14}C to $^{14}CO_2$ *in vitro* (1–4) and on determinations of one or more of the enzyme components (5–8). Data obtained from isotope studies *in vitro* suggest that only a minor amount of glucose is catabolized via the pentose phosphate pathway in brain (1–4). Nevertheless, it seems clear that control of the pentose phosphate pathway must be geared to regulatory processes operative in the Embden-Meyerhof pathway since glucose-6-P, fructose-6-P, and glyceraldehyde-P are intermediates common to both metabolic routes.

This study is a report of the normal tissue levels of some of the pentose phosphate pathway intermediates in brain and of the changes in these levels when metabolic activity of the brain is altered by various experimental stresses. The results are believed to constitute a step toward an understanding of the interrelationships between this route of metabolism and the Embden-Meyerhof glycolytic pathway.

EXPERIMENTAL PROCEDURE

Preparation of Animals and Brain Samples

Adult, male Swiss-Webster mice weighing 20 to 25 g were used. The animals had unrestricted access to food and water up to the experimental period. After the desired treatment, whole animals, or the severed heads in the case of the ischemia study, were rapidly frozen in Freon 12 (CCl_2F_2) maintained at −150° by liquid N_2. The tissue was stored at −80° until it could be prepared for analysis.

Ischemia—Mice were decapitated and the heads were frozen after intervals of 6, 30, 60, and 300 sec. Until frozen, the heads were maintained at 38°. Zero time animals were decapitated directly into Freon or the whole mouse was frozen.

Anesthesia—Mice were anesthetized with a single intraperitoneal dose of phenobarbital (200 mg per kg) administered as an isotonic solution of the sodium salt. Anesthesia was maintained for 60 min after loss of the righting reflex before sacrifice.

Hypothermia—Mice were lightly anesthetized with ether and then chilled in crushed ice until body temperature reached 16–18° (measured rectally with a thermocouple). The animals were maintained at this temperature for exactly 5 min prior to sacrifice.

Hyperthermia—Animals were individually irradiated with an infrared lamp in an insulated chamber of approximately 4-liter

* This research was supported in part by grants from the United States Public Health Service (NB-05221 and NB-00434) and the American Cancer Society (P-78).

capacity. When rectal temperature reached 42–44° (3 to 4 min), the animals were plunged into the freezing medium.

Iodoacetate—Animals were anesthetized with phenobarbital (150 mg per kg), and the iodoacetate (100 mg per kg) was administered intraperitoneally as an isotonic solution at pH 7.0. The mice were sacrificed 60 min later.

Fluoroacetate—Mice were frozen 60 min after intraperitoneal injection of sodium monofluoroacetate (100 mg per kg).

Preparation of Brain Extracts—The frozen cerebral hemispheres and major portions of the midbrain were dissected and weighed in a room maintained at −20°. Approximately 200 mg of frozen brain were placed on top of 400 μl of 3.0 M $HClO_4$ which had been frozen at Dry Ice temperature in the bottom of 4-ml plastic centrifuge tubes. The tissue was powdered at this temperature with a flat-bottomed glass rod, after which the $HClO_4$ was allowed to thaw at −10° and the mixture worked to a smooth homogenate at this temperature. Each sample was subsequently diluted with 1 ml of 1 mM EDTA at 4° and centrifuged at the same temperature ($HClO_4$ concentration about 0.75 M).

The fluorescent blank contributed by the tissue extract was reduced by treating 1 ml of the supernatant fluid with 10 mg of dried, acid-washed charcoal (Norit). This procedure was necessary because of the low tissue levels of most of the metabolites measured (less than 50 μmoles per kg, wet weight). After addition of the charcoal the sample was brought to pH 6.8 with 0.24 ml of a solution containing 3 M KOH, 0.4 M imidazole, and 0.7 M KCl and filtered through glass fiber paper (Reeves-Angel). Treatment with charcoal removed over 90% of the tissue fluorescence and allowed complete recovery of the phosphorylated intermediates.

Materials

Enzymes—All enzymes except transketolase, transaldolase, pentose-P epimerase, and pentose-P isomerase were purchased from Boehringer (Mannheim Corporation, New York). Transketolase was prepared from baker's yeast according to Srere *et al.* (9) with the following modification: alcohol dehydrogenase, which was present in great excess over transketolase, could be completely removed by rechromatographing the preparation on DEAE-cellulose. One hundred milligrams of protein in 5 to 10 ml of 0.005 M phosphate buffer, pH 7.7, were placed on a column, 2.3 × 50 cm, which had previously been equilibrated with this buffer. The enzyme was eluted at 4° with the same buffer at a flow rate of 10 ml per hour; 4-ml fractions were collected. Transketolase was recovered between fractions 10 and 14. The 200-fold purified transketolase (0.54 mmole per mg of protein per hour) lost less than 30% of its activity after storage for 6 months as a suspension in 3.0 M $(NH_4)_2SO_4$.

Transketolase was assayed fluorometrically according to an adaptation of the spectrophotometric method described by De Lahaba, Leder, and Racker (10). The reagent used in this measurement consisted of 0.02 M imidazole-HCl (pH 7.6), 0.01 mM DPNH, 0.02 mM thiamine pyrophosphate, 0.2 mM $MgCl_2$, 0.01% bovine serum albumin, 0.5 mM ribose-5-P, 0.2 mM xylulose-5-P, and 5 μg of a mixture of glycero-P dehydrogenase and triose-P isomerase per ml.

Transaldolase was purified 200-fold from *Candida utilis* according to Pontremoli *et al.* (11) with the following modification: after treatment of the preparation with calcium phosphate gel, transaldolase was precipitated with 3.2 M $(NH_4)_2SO_4$, dissolved in 0.005 M potassium phosphate (pH 7.6), dialyzed for 2 hours against the same buffer, and chromatographed on a column of Sephadex G-200, 2.5 × 48 cm, at 4°. Approximately 100 mg of protein were placed on the column in a volume of 5 ml. The enzyme was eluted with the same buffer at a flow rate of 8 ml per hour. Transaldolase was recovered between the 105th and 140th ml of eluent. The enzyme, purified 63-fold by this step, was precipitated and stored as a suspension in 3.5 M (NH_2)-SO_4 at 4°.

Transaldolase was measured fluorometrically according to an adaptation of a spectrophotometric assay reported by Venkataraman and Racker (12). The assay is based on measuring the formation of glyceraldehyde-P from fructose-6-P and erythrose-4-P, and it is carried out in 1 ml of reagent having the following composition: imidazole-HCl, pH 7.6, 0.05 M; bovine serum albumin, 0.02%; fructose-6-P, 0.5 mM; erythrose-4-P, 0.03 mM; DPNH, 0.01 mM; and 12.5 μg of a mixture of triose-P isomerase and glycero-P dehydrogenase per ml.

Pentose-P epimerase was purified from calf spleen according to Ashwell and Hickman (13). Separation of pentose-P epimerase and pentose-P isomerase was achieved by repeating the acetone fractionation a second time. Epimerase was recovered in the fraction precipitating between 50 and 80% (v/v) of acetone. The purified enzyme was dissolved in water and stored at −80°. The specific activity varied between 1.2 and 2.6 mmoles per mg of protein per hour.

Pentose-P epimerase was measured by a direct fluorometric assay in which ribose-5-P served as substrate and pentose-P isomerase, transketolase, triose-P isomerase, and glycero-P dehydrogenase were utilized as auxiliary enzymes. The assay is based on measuring the rate of formation of glyceraldehyde-P, which is directly proportional to the rate-limiting enzyme in this system, pentose-P epimerase. The assay was proportional over a range of product generated of at least 5 to 50 μmoles per liter per hour. The assay was carried out in 1 ml of reagent having the following composition: imidazole-HCl, pH 7.6, 0.05 M; ribose-5-P, 1 mM; DPNH, 0.01 mM; thiamine pyrophosphate, 0.02 mM; 12.5 μg of a mixture of glycero-P dehydrogenase and triose-P isomerase per ml; and pentose-P isomerase and transketolase, which were present at activities of 4.5 and 6 mmoles per liter per hour, respectively.

Pentose-P isomerase was purchased from Sigma and further purified by chromatography on a Sephadex G-200 column (2.5 × 50 cm). Approximately 100 mg of protein were placed on the column in a volume of 5 ml of 0.005 M PO_4 buffer, pH 7.6, and isomerase was eluted with the same buffer, with a flow rate of 8 ml per hour. Isomerase was recovered in the fractions between 135 and 185 ml. Fractions containing isomerase were concentrated by dialysis against powdered Sephadex G-200.[1] The concentrated fractions were stored at −80°.

Pentose-P isomerase was assayed by a direct fluorometric assay in a system which is essentially the same as that used to measure pentose-P epimerase. With epimerase present at an activity of 4 to 5 mmoles per liter per hour, and with pentose-P isomerase as the rate-limiting enzyme, the assay was proportional over essentially the same range as the epimerase assay. Prior to the analysis ribose-5-P was treated with 1 N NaOH according to Racker (14) to destroy ribulose-5-P, which was present in the ribose-5-P stock solution.

[1] Information supplied by Pharmacia, the manufacturer, Technical Data Sheet, No. 1.

TABLE I

Analytical conditions

Analyses were conducted in 1 ml of reagent in fluorometer tubes (10 × 75 mm) plus neutralized $HClO_4$ extract equivalent to the amount of brain indicated (wet weight). Reagents contained 0.005% bovine serum albumin. Tissue extracts were treated with 1% charcoal prior to analysis and used in an amount equivalent to about 10 mg of brain.

Metabolite	Reagent			$t_{\frac{1}{2}}$	Incubation
	Buffer	Other additions	Enzymes		
				min	*min*
6-P-gluconate	0.05 M Tris-HCl, pH 8.0	EDTA, 0.1 mM; dithiothreitol, 0.1 mM; ammonium acetate, 0.03 M; TPN, 0.02 mM	6-P-gluconate dehydrogenase, 0.5 μg/ml	2	20
Xylulose-5-P	0.05 M imidazole-HCl, pH 7.6	Thiamine pyrophosphate, 0.02 mM; $MgCl_2$, 2 mM; DPNH, 0.001 mM; erythrose-4-P, 0.015 mM	Glycero-P[a] dehydrogenase, 7 μg/ml; triose-P isomerase, 4 μg/ml; transketolase, 5 mmoles/liter/hr	2	20
Ribulose-5-P	Same	Same	Same, plus ribulose-5-P epimerase, 2 mmoles/liter/hr	4	30
Ribose-5-P plus sedoheptulose-7-P	Same	Same	Same, plus pentose-P isomerase, 5 mmoles/liter/hr	10	60
Erythrose-4-P	Same	Fructose-6-P, 1 mM; DPNH, 0.001 mM	Glycero-P dehydrogenase, 7 μg/ml; triose-P isomerase, 5 μg/ml; transaldolase, 2 mmoles/liter/hr	5	40
Glucose-6-P	0.05 M Tris-HCl, pH 8.0	Dithiothreitol, 0.2 mM; TPN, 0.02 mM	Glucose-6-P dehydrogenase, 1 μg/ml	½	5
Fructose-6-P	Same	Same	Same, plus P-glucoisomerase, 1 μg/ml	1½	15

[a] Dihydroxyacetone-P and glyceraldehyde-P were analyzed in the same sample following sequential additions of glycero-P dehydrogenase and triose-P isomerase.

Other Materials—Erythrose-4-P dimethylacetal dicyclohexylammonium salt was purchased from Calbiochem. Ribose-5-P, Na^+ salt, and glyceraldehyde-P diethylacetal, Ba^{2+} salt, were obtained from Schwarz BioResearch. Glucose-6-P, 6-P-gluconate, dihydroxyacetone-P, cocarboxylase, and pyridine nucleotides were purchased from Sigma. Ribulose-5-P and xylulose-5-P were generated by treating 50 mM ribose-5-P in 0.05 M imidazole-HCl, pH 7.6, with a mixture of pentose-P isomerase and pentose-P epimerase according to Ashwell and Hickman (13). After an equilibrium mixture of pentose phosphates had been formed (usually within 30 min at 38°), the reaction was stopped by adjusting the pH to 4.5 with HCl and heating 3 min at 100°. The precipitated protein was removed by centrifugation, and the mixture was stored at −80°.

Analytical Methods

Fluorometry—All of the substrate analyses were made fluorometrically by measuring the disappearance of DPNH, or the appearance of TPNH. Fluorescence of reduced pyridine nucleotides was measured in test tubes (10 × 75 mm) with a Farrand model A fluorometer as previously described (15). In general, 1 ml of reagent having the composition indicated in Table I was placed in each fluorometer tube, the brain extract was added, and the fluorescence was read at an appropriate sensitivity. Necessary analytical enzymes were then added in small volumes (1 to 10 μl). The tubes were read again when the reaction was complete (usually 6 to 10 half-times). Prior to each series of analyses, one blank, one standard, and one tissue sample were read at short intervals throughout the course of the reaction to determine the proper time at which final readings should be made for the bulk of the extracts (15, 16). Standards, in volumes of 10 μl or less, were added directly to reagent in the fluorometer tubes together with an amount of blank solution simulating the tissue samples in volume and composition ($HClO_4$ neutralized in the same manner as for the tissue samples).

6-P-gluconate—This was measured with 6-P-gluconate dehydrogenase from yeast. The commercial crystalline preparation is essentially free of glucose-6-P dehydrogenase and was found quite satisfactory for measuring the low concentration of 6-P-gluconate present in tissue extracts. Addition of glucose-6-P in 10-fold excess did not interfere with the assay.

Xylulose-5-P, Ribulose-5-P, and Sedoheptulose-7-P plus Ribose-5-P—These substrates were measured in the same tissue sample. The enzymatic reactions utilized to measure these substrates are the following.

$$\text{Xylulose-5-P} + \text{erythrose-4-P} \rightarrow \text{glyceraldehyde-P} + \text{fructose-6-P} \quad (1)$$

$$\text{Glyceraldehyde-P} \rightarrow \text{dihydroxyacetone-P} \quad (2)$$

$$\text{Dihydroxyacetone-P} + \text{DPNH} \rightarrow \text{glycero-P} + \text{DPN}^+ \quad (3)$$

Net reaction:

$$\text{Xylulose-5-P} + \text{erythrose-4-P} + \text{DPNH} \rightarrow \text{fructose-6-P} + \text{glycero-P} + \text{DPN}^+ \quad (4)$$

$$\text{Ribulose-5-P} \rightarrow \text{xylulose-5-P} \quad (5)$$

$$\text{Ribose-5-P} \rightarrow \text{ribulose-5-P} \quad (6)$$

The use of transketolase as an analytical reagent in the above

series of reactions makes this assay very specific, as well as sensitive, since the only keto sugar which gives rise to glyceralde-P in the presence of transketolase is xylulose-5-P (17). The assay also has the advantage of having an equilibrium in favor of the formation of fructose-6-P and glyceraldehyde-P (18).

The analytical sequence was initiated after endogenous glyceraldehyde-P and dihydroxyacetone-P present in the tissue extract had reacted with DPNH via glycero-P dehydrogenase and triose-P isomerase. Xylulose-5-P was then measured by the DPNH decrease upon addition of transketolase. It is essential that the enzymes used in this system be free of pentose-P isomerase and pentose-P epimerase. After the reaction with xylulose-5-P was completed, ribulose-5-P was measured by the addition of ribulose-5-P epimerase in a volume of 1 μl. Finally ribose-5-P (plus sedoheptulose-7-P) were measured by the addition of pentose-P isomerase. This last step does not distinguish between ribose-5-P and sedoheptulose-7-P since the latter is known to react with erythrose-4-P in the presence of transketolase to form ribose-5-P and fructose-6-P (17, 18). The erythrose-4-P concentration used in the present study, 0.015 mM, did not inhibit transketolase. At higher concentrations (0.05 mM) erythrose-4-P has been reported to inhibit red blood cell transketolase (19).

Erythrose-4-P—The assay of this substrate was attempted by adaptation of a spectrophotometric assay reported earlier by Srere *et al.* (9). In the presence of transaldolase a three-carbon moiety is transferred from fructose-6-P to erythrose-4-P, generating sedoheptulose-7-P and glyceraldehyde-P (12). The assay is not specific for erythrose-4-P in that other aldoses may serve as acceptors for the ketol unit (12). The concentration of such substances along with erythrose-4-P must be extremely low in cerebral tissue since the total amount of glyceraldehyde generated was less than 2 μmoles per kg, wet tissue.

Glucose-6-P and Fructose-6-P—These were measured in the same sample. The method for glucose-6-P is essentially that described earlier (15) except for the addition of dithiothreitol. This was added to reduce the oxidized glutathione present in brain extracts and thus prevent reoxidation of TPNH by glutathione reductase, a common contaminant of preparations of glucose-6-P dehydrogenase and P-glucoisomerase (20–22). After the glucose-6-P reaction was complete, P-glucoisomerase was added to the reaction mixture in a volume of 1 μl and fructose-6-P was measured by the second increment in fluorescence. Measurement of the two substrates in the same sample was facilitated by reduction of the tissue blank with charcoal as described.

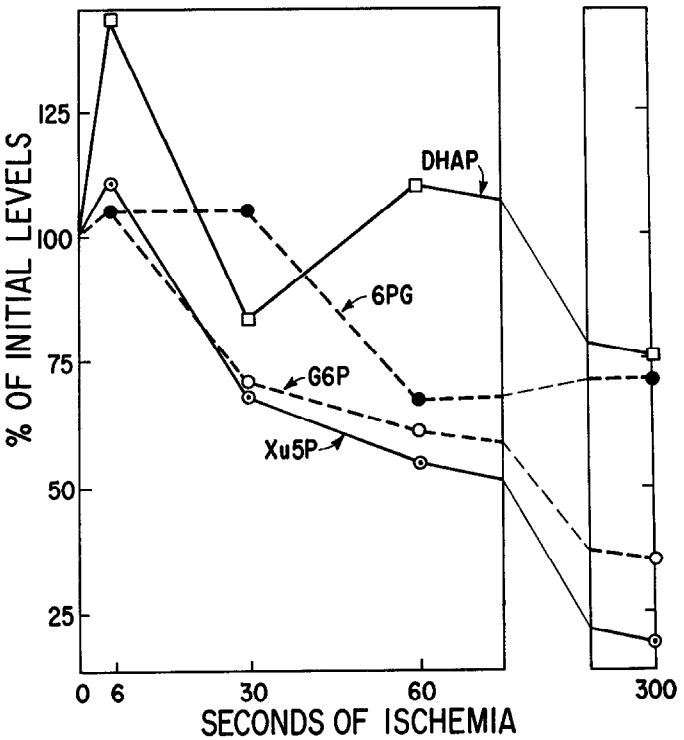

FIG. 1. Changes in glucose-6-P (*G6P*), 6-P-gluconate (*6PG*), xylulose-5-P (*Xu5P*), and dihydroxyacetone-P (*DHAP*) in mouse brain after decapitation. The zero time values are the average for 6 mice, of which 3 were frozen whole and 3 were decapitated directly into the freezing mixture. Each of the other values is based on the average for 3 to 4 mice, a total of 20 animals. Standard errors averaged about 20%. Ribulose-5-P levels were about half of those of xylulose-5-P up to 60 sec. At 300 sec the level was too low for accuracy.

TABLE II

Levels of pentose phosphate pathway metabolites in cerebral tissue of normal mice

Values were obtained from animals frozen whole or decapitated directly into the freezing medium. The standard errors are shown for the number of animals given in parentheses.

Metabolite	
	μmoles/kg
Glucose-6-P (12)	60.4 ± 9.2
6-Phosphogluconate (14)	9.7 ± 0.9
Ribulose-5-P (15)	7.2 ± 0.6
Xylulose-5-P (15)	14.2 ± 1.0
Ribose-5-P plus sedoheptulose-7-P (6)	64.3 ± 6.0
Erythrose-4-P (4)	<2.0
Fructose-6-P (8)	9.9 ± 0.6
Glyceraldehyde-P (12)	4.7 ± 0.7
Dihydroxyacetone-P (15)	38.0 ± 3.4

Stability of Substrates in Extracts—Test were made in solutions which simulated media to which tissue substrates were exposed. Xylulose-5-P, ribulose-5-P, ribose-5-P, and 6-P-gluconate were stable for at least 2 hours at 60° in 0.6 N $HClO_4$. In neutralized extracts, pH 6.8, xylulose-5-P and ribulose-5-P were measurably less stable, both undergoing a loss of approximately 50% in 2 hours at 60°. No loss of ribose-5-P or 6-P-gluconate was detected in the neutralized extract after 2 hours at 60°.

RESULTS

Normal Brain Levels—In general, the levels of pentose phosphate pathway metabolites in normal mouse brain are extremely low (Table II). The ratio of ribulose-5-P to xylulose-5-P (1:2.0) agrees with equilibrium measurements made *in vitro*. Incubation of pentose-P with purified P-riboisomerase and ribulose-5-P epimerase, until equilibrium was attained, resulted in a mixture containing 21.5 μM ribose-5-P, 11.0 μM ribulose-5-P and 25.8 μM xylulose-5-P (2.0:1:2.4). Various equilibrium values have been reported for the pentose phosphates (13, 23–26). The values we observed agree most closely with those reported by Tabachnick, Srere, Cooper, and Racker (2.83:1:2.90)

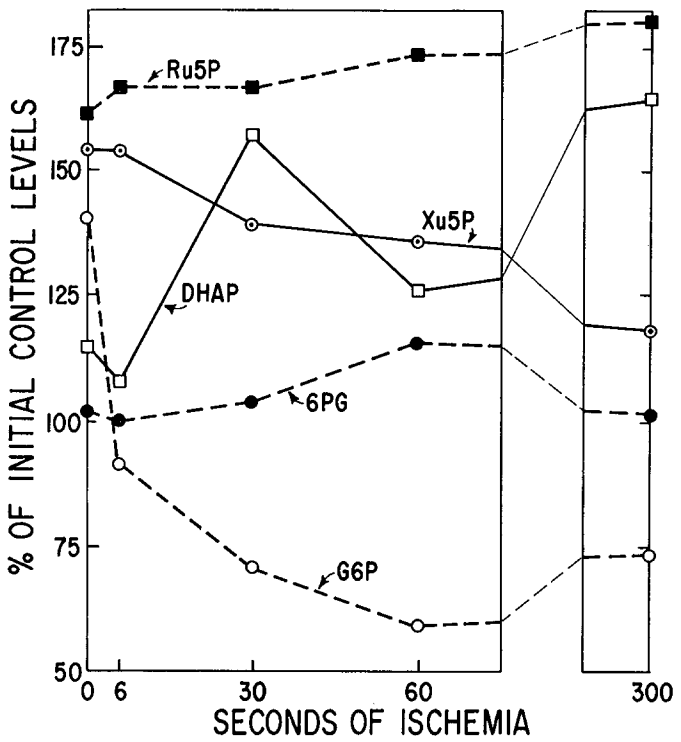

FIG. 2. Changes in metabolites of mouse brain as the result of phenobarbital anesthesia and of anesthesia plus ischemia (decapitation). The levels are expressed as the percentage of those for the initial animals of Fig. 1. The abbreviations are as given in Fig. 1 except for *Ru5P*, for ribulose-5-P. The zero time values are the average for 6 mice; each of the rest represents 4 mice, a total of 22 animals.

(26). The observed values for glyceraldehyde-P and dihydroxyacetone-P are in the proportion of 1:8.1 rather than in the equilibrium ratio value of 1:24 (27). A similar discrepancy in the ratio of these trioses was reported by Williamson (28) for perfused rat heart (1:5.6 to 1:9.1).

The analytical method for ribose-5-P does not distinguish between it and sedoheptulose-7-P. The sum of the two is much larger than either ribulose-5-P or xylulose-5-P (Table II). An attempt was made to differentiate between sedoheptulose-7-P and ribose-5-P in brain extracts by adding epimerase and isomerase, to allow equilibration of pentose phosphates, stopping the reaction by adjusting the pH to 4.0, heating 3 min at 100°, then measuring xylulose-5-P. This permitted calculation of the total pentose-P by means of the equilibrium ratios given above. From this the preformed xylulose-5-P and ribulose-5-P values were deducted to give preformed ribose-5-P. The results of this preliminary experiment indicated that ribose-5-P levels in normal brain were about 25 μM, suggesting that ribose-5-P is essentially in equilibrium with ribulose-5-P. This also suggests that sedoheptulose-7-P levels may be as high as 40 μmoles per kg.

Ischemia—After the blood supply to the brain is cut off, there is a 6- to 8-fold increase in glycolysis which is associated with a prompt fall in glucose-6-P levels and large increases in levels of fructose-1,6-di-P and the triose phosphates (15, 16). Since O_2 is cut off, it would seem probable that flux through the oxidative limb of the pentose pathway would be reduced or halted. In the present experiments, 6-P-gluconate fell by one-third during the 1st min of ischemia but did not change further (Fig. 1). Since oxidation is required for both formation and removal of this intermediate, greater change might not be expected. In contrast, xylulose-5-P fell continuously, somewhat in parallel to glucose-6-P, reaching 18% of the initial value at the end of 5 min. Within analytical limitations, ribulose-5-P was found to fall similarly. This suggests that, with pentose-P formation interrupted, metabolites below 6-P-gluconate were drained off into the Embden-Meyerhof stream.

Anesthesia—Deep anesthesia produces a marked fall in the metabolic rate of brain (29–31). As shown earlier (15), this is associated with an increase in glucose-6-P. Ribulose-5-P and xylulose-5-P were also found to increase during anesthesia, and in about the same proportion as glucose-6-P, but 6-P-gluconate was not significantly changed (Fig. 2). In addition, anesthesia prevented large decreases in pentose pathway metabolites during ischemia (Fig. 2). Although glucose-6-P decreased as expected, 6-P-gluconate and ribulose-5-P did not change for at least 5 min and xylulose-5-P was still 18% above the control level at that time. It will be observed that during ischemia dihydroxyacetone-P levels were distinctly higher in anesthetized than in nonanesthetized mice and that the same is true of the 5-min value for glucose-6-P.

Hypothermia and Hyperthermia—Hyperthermia and hypothermia were employed as additional means of altering metabolic rate. Hyperthermia is known to increase cerebral oxygen consumption (31) whereas hypothermia quite certainly reduces cerebral metabolic rate. A recent study indicated that entry of glucose-6-P into the pentose phosphate pathway is favored at elevated temperatures (32). Hypothermia resulted in marked decreases in ribulose-5-P and xylulose-5-P, whereas hyperthermia resulted in increases in the same metabolites (Fig. 3). The triose phosphates, especially dihydroxyacetone-P, also decreased in hypothermia but did not change significantly in hyperthermia.

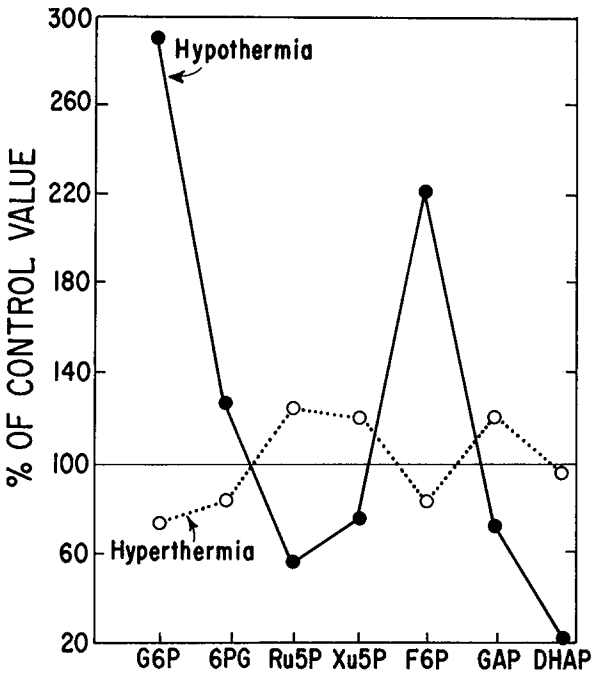

FIG. 3. Changes in metabolites during hypothermia and hyperthermia. The results are compared to those in Table II. Each *set of points* represents six animals. Abbreviations in addition to those of Fig. 1 are *Ru5P*, ribulose-5-P; *F6P*, fructose-6-P; and *GAP*, glyceraldehyde-P.

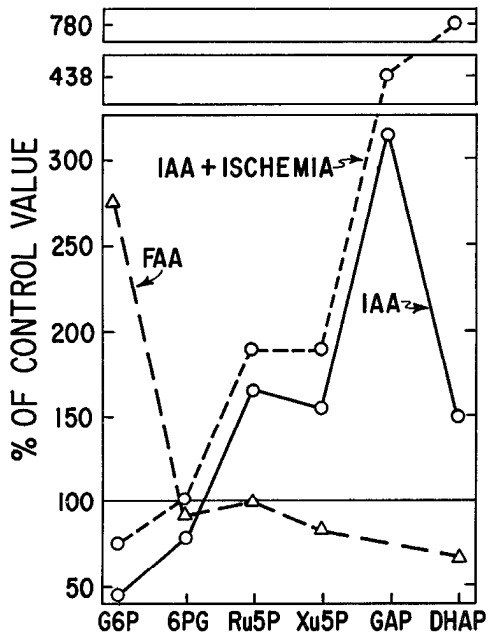

FIG. 4. Changes in metabolites in mouse brain after iodoacetate (*IAA*) administration, after the combination of iodoacetate and 30 sec of ischemia (decapitation), and after fluoroacetate (*FAA*) poisoning. The controls are the animals of Table II. Each *line* represents the averages for three mice. The abbreviations and symbols are the same as for Fig. 3.

(Small percentage changes in glyceraldehyde-P are not significant because of the low absolute levels.) In contrast to both pentose and triose phosphates, glucose-6-P and, to a lesser extent, 6-P-gluconate increased in hypothermia and decreased in hyperthermia.

If flux through the pentose pathway changes in the same direction as metabolic rate, these results point to control at both dehydrogenase steps. Control between 6-P-gluconate and ribulose-5-P is suggested by the crossover point. Control between glucose-6-P and 6-P-gluconate is suggested by the much smaller changes in 6-P-gluconate levels than in the levels of its precursor. It will be noted that hypothermia and anesthesia, both associated with decreased metabolic rate, have opposite effects on xylulose-5-P and ribulose-5-P. Consequently, factors other than flux, such as the triose-P concentration, may affect the tissue levels.

Iodoacetate—In the above experiments it is uncertain to what extent the levels of the pentose phosphates might be influenced by the levels of glyceraldehyde-P. Therefore, the levels of this intermediate were raised by blocking its utilization with iodoacetate. An hour after administration, major increases were observed in ribulose-5-P and xylulose-5-P as well as in both triose phosphates (Fig. 4). As with hyperthermia increases in pentose phosphates were associated with decreases in 6-P-gluconate and glucose-6-P. Although glyceraldehyde-P and the pentose phosphates moved in the same direction, it does not seem certain that one caused the other. Thus the superimposition of ischemia to accelerate glycolysis caused even greater changes in triose phosphates but no significant further increases in pentose phosphates (Fig. 4). Nor was there any correlation between levels of triose phosphates and pentose phosphates among individual animals. For example, in one case glyceraldehyde-P and dihydroxyacetone-P rose 7-fold and 18-fold, respectively, but xylulose-5-P and ribulose-5-P only doubled. Therefore, it is possible that the increased pentose-P levels after iodoacetate are due to some cause other than blockade by the rise in glyceraldehyde, perhaps an increase in flux through the pentose pathway.

Fluoroacetate—Poisoning with fluoroacetate causes not only an increase in citrate levels but also an increase in glucose-6-P (16), presumably through inhibition by citrate of P-fructokinase. In spite of 3-fold increases in glucose-6-P and fructose-6-P (not shown), there were no demonstrable changes in levels of 6-P-gluconate, ribulose-5-P, or xylulose-5-P (Fig. 4).

DISCUSSION

An understanding of the place of the pentose pathway in brain economy would require knowledge of (*a*) the flux through the pathway under various circumstances and (*b*) the mechanism by which that flux is controlled. Clearly the present study does not settle either issue. While it was possible to vary the metabolic rate and to make large changes in the flux from glucose-6-P to triose phosphate, it is not certain what fraction of the flux went by the alternate routes. Nevertheless, certain necessary relevant information is presented and the possibilities are narrowed.

The most logical regulatory step, as others have pointed out (33, 34), is the first one. The present results suggest that both of the two dehydrogenase steps may participate in control. That glucose-6-P dehydrogenase is a control step is suggested by the relatively small changes in 6-P-gluconate levels when glucose-6-P is caused to vary widely by ischemia, anesthesia, hypothermia, or fluoroacetate poisoning. Evidence that 6-P-gluconate dehydrogenase may also be involved is the fact that ribulose-5-P and xylulose-5-P levels changed much more than 6-P-gluconate levels under the several experimental manipulations and even changed in a direction opposite to that of 6-P-gluconate. In particular, there appeared to be a crossover point between 6-P-gluconate and ribulose-5-P when body temperature was raised and lowered, and again when the brain was poisoned with iodoacetate.

Interpretation of the changes in pentose phosphate levels is complicated by the possibility that these levels may be affected not only by the flux, but also by approach to equilibrium with fructose-6-P and glyceraldehyde-P. The major sequence below 6-P-gluconate is presumably

$$\text{Ribose-5-P} + \text{xylulose-5-P} \rightleftharpoons \text{sedoheptulose-7-P} + \text{glyceraldehyde-P} \quad (7)$$

$$\text{Sedoheptulose-7-P} + \text{glyceraldehyde-P} \rightleftharpoons \text{erythrose-4-P} + \text{fructose-6-P} \quad (8)$$

$$\text{Erythrose-4-P} + \text{xylulose-5-P} \rightleftharpoons \text{fructose-6-P} + \text{glyceraldehyde-P} \quad (9)$$

The respective equilibrium equations can be combined, together with the equilibrium ratio between ribose-5-P and xylulose-5-P (approximately 1:1), to give

$$(\text{Xylulose-5-P})^3 = \frac{(\text{fructose-6-P})^2\ \text{glyceraldehyde-P}}{K_7K_8K_9} \quad (10)$$

where K_7 etc. are the respective equilibrium constants in Equations 7 to 9. These are reported to be, respectively, 1.11 (21), 1.05 (12), and 11.9 (21). Application of Equation 10 to the experimental data yields equilibrium levels of xylulose-5-P

which are in every case lower by factors of 3 to 5 than the observed levels. Although this suggests that equilibrium is not maintained, the possibility of compartmentation or fractional binding of some of the components prevents assurance on this point.

An upper limit for flux through the pentose pathway can be estimated from the activity of the enzymes concerned. The activity of 6-P-gluconate dehydrogenase was measured in mouse brain homogenates at 37° in isotonic potassium acetate at pH 7.2. The Michaelis constant for 6-P-gluconate was 7 μM. With 10 μM 6-P-gluconate, *i.e.* the normal brain concentration, and a saturating level of TPN^+, the velocity was 2.4 mmoles per kg per min. This would correspond to triose consumption of 0.8 mmole per kg per min, or nearly 100% of the total rate of triose oxidation (15). A similar measurement of transketolase under the same conditions gave a Michaelis constant for xylulose-5-P of 14 μM. At this level (which happens to be the normal brain concentration) the velocity was 0.4 mmole per kg per min. Since there are two transketolase steps for each complete cycle this would set an upper limit for triose oxidation of 0.2 mmole per kg per min. This is 20 to 30% of the normal rate of triose consumption.

A recent estimate of the contribution of the pentose pathway to the metabolism of glucose-2-^{14}C in mammalian brain *in vivo* indicated that only 3 to 5% of glucose is catabolized via this route (35). The randomization of ^{14}C of glucose-2-^{14}C into carbon 1 relative to carbon 2 in rat cerebral glycogen was used to estimate net carbon flow through the pentose pathway. The value obtained represents a maximal value since ^{14}C introduced by any mechanism into carbon 1 of glucose-6-P is ascribed to the pentose pathway. The difference between results obtained in this isotope study and the estimate of the upper limit for flux based on kinetic constants of transketolase may easily be ascribed to less than optimal levels of the cosubstrates and to back reactions.

REFERENCES

1. Bloom, R., *Proc. Soc. Exp. Biol. Med.*, **88**, 317 (1955).
2. Di Pietro, D., and Weinhouse, A., *Arch. Biochem. Biophys.*, **80**, 268 (1959).
3. Sacks, W., *J. Appl. Physiol.*, **16**, 175 (1961).
4. O'Neill, J. J., Simon, S. H., and Shreeve, W. W., *J. Neurochem.*, **12**, 797 (1965).
5. Buell, M. V., Lowry, O. H., Roberts, N. R., Chang, M. W., and Kapphahn, J. I., *J. Biol. Chem.*, **232**, 979 (1958).
6. Friede, R. L., Fleming, L. M., and Knoller, M., *J. Neurochem.*, **10**, 263 (1963).
7. Dreyfus, P. M., and Moniz, R., *Biochim. Biophys. Acta*, **65**, 181 (1962).
8. Burt, A. M., *Develop. Biol.*, **12**, 213 (1965).
9. Srere, P., Cooper, J. R., Tabachnick, M., and Racker, E., *Arch. Biochem. Biophys.*, **74**, 295 (1958).
10. De Lahaba, G., Leder, I. G., and Racker, E., *J. Biol. Chem.*, **214**, 409 (1955).
11. Pontremoli, S., Prandini, B. D., Bonsignore, A., and Horecker, B. L., *Proc. Nat. Acad. Sci. U. S. A.*, **47**, 1942 (1961).
12. Venkataraman, R., and Racker, E., *J. Biol. Chem.*, **236**, 1876 (1961).
13. Ashwell, G., and Hickman, J., *J. Biol. Chem.*, **226**, 65 (1957).
14. Racker, E., in H. Bergmeyer (Editor), *Methods of enzymatic analysis*, Academic Press, New York, 1963, p. 201.
15. Lowry, O. H., Passonneau, J. V., Hasselberger, F. X., and Schulz, D. W., *J. Biol. Chem.*, **239**, 18 (1964).
16. Goldberg, N. D., Passonneau, J. V., and Lowry, O. H., *J. Biol. Chem.*, **241**, 3997 (1966).
17. Racker, E., in P. D. Boyer, H. Lardy, and K. Myrback (Editors), *The enzymes, Vol. 5*, Academic Press, New York and London, 1960, p. 196.
18. Datta, A. G., and Racker, E., *J. Biol. Chem.*, **236**, 617 (1961).
19. Dische, Z., and Igals, D., *Arch. Biochem. Biophys.*, **93**, 201 (1961).
20. Hohorst, B. Z., *Biochem. Z.*, **332**, 18 (1952).
21. Needleman, P., Passonneau, J. V., and Lowry, O. H., *Fed. Proc.*, **26**, 507 (1967).
22. Schulz, D. W., Passonneau, J. V., and Lowry, O. H., *Anal. Biochem.*, **19**, 300 (1967).
23. Stumpf, P. K., and Horecker, B. L., *J. Biol. Chem.*, **218**, 753 (1956).
24. Dickens, F., and Williamson, D. H., *Biochem. J.*, **64**, 567 (1956).
25. Dische, Z., and Shigeura, H., *Biochim. Biophys. Acta*, **24**, 87 (1957).
26. Tabachnick, M., Srere, P. A., Cooper, J., and Racker, E., *Arch. Biochem. Biophys.*, **74**, 315 (1958).
27. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **149**, 71 (1943).
28. Williamson, J. R., *J. Biol. Chem.*, **242**, 4476 (1967).
29. Kety, S. S., and Schmidt, C. F., *Fed. Proc.*, **5**, 264 (1946).
30. Homburger, E., Himwich, W. A., Etsten, B., York, G., Maresca, R., and Himwich, H. E., *Amer. J. Physiol.*, **147**, 343 (1946).
31. Himwich, H. E., Bowman, K. M., Goldfarb, W., and Fazekas, J. F., *Science*, **90**, 398 (1939).
32. Hochachka, P. W., *Comp. Biochem. Physiol.*, **25**, 107 (1968).
33. Tepperman, J., and Tepperman, H. M., *Amer. J. Physiol.*, **193**, 55 (1958).
34. Tsutsui, E. A., Marks, P. A., and Reich, P., *J. Biol. Chem.*, **237**, 3009 (1962).
35. Hostetler, K. Y., and Landau, R., *Biochemistry*, **6**, 2961 (1967).