THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 239, No. 1, January 1964
Printed in U.S.A.

Effect of Ischemia on Known Substrates and Cofactors of the Glycolytic Pathway in Brain*

OLIVER H. LOWRY, JANET V. PASSONNEAU, FRANCIS X. HASSELBERGER, AND DEMOY W. SCHULZ

From the Department of Pharmacology and the Beaumont-May Institute of Neurology, Washington University, St. Louis 10, Missouri

(Received for publication, June 17, 1963)

This is a record of the concentrations of the nonenzyme components of the Embden-Meyerhof system in mouse brain measured at brief intervals after the production of complete ischemia by decapitation. All of the 18 recognized active components were looked for. Of these, 1,3-diphosphoglycerate did not reach levels measurable by the procedures used. Additional substances determined were glycogen, phosphocreatine, creatine, adenosine 5′-phosphate, α-glycerophosphate, and triphosphopyridinenucleotide and its reduced form. The pyridine nucleotide values are only provisional. Considerable attention is devoted to methodology, since there are numerous possibilities for serious errors in preparation of samples as well as in the analyses for individual substrates.

The substances measured account for all the known significant sources of energy available to the brain after its blood supply is cut off. Therefore it is possible to calculate the metabolic rate during the brief period of survival, as well as the time sequence according to which the brain taps its reserve sources of energy.

Ischemia resulted in increases in glycolytic rates of at least 4- to 7-fold in different experimental groups of mice. The coincident changes in substrate concentrations show which steps were facilitated to make this increase in flux take place, *i.e.* which steps control glycolysis in brain. These steps are the phosphorylations of glucose and fructose 6-phosphate, and the phosphorolysis of glycogen. There is no evidence that facilitation occurs at any other step in the glycolytic pathway.

EXPERIMENTAL PROCEDURE

Preparation of Animals

The white mice used were either young adult males (18 to 22 g) or 10-day-old animals of both sexes from litters of six to eight each (5 to 7.5 g). In the case of the young animals, litter mates were distributed as widely as possible among the groups exposed to different lengths of ischemia. Hair from the heads of the adult mice was trimmed on the previous day, under light ether anesthesia, in an effort to accelerate freezing. The series of anesthetized animals received phenobarbital intraperitoneally. The dosage averaged 150 mg per kg, but was adjusted individually with disappearance of the righting reflex (on stimulation) as the end point. Anesthesia was maintained for nearly 1 hour before decapitation. Four 10-day-old animals were lightly anesthetized with ether and then chilled in ice water until body temperature reached 18°, measured rectally with a thermocouple.

* Supported in part by grants from the American Cancer Society (P-78) and the United States Public Health Service (5T1-GM-96, NB 01352, and NB 00434).

Preparation of Tissue Extracts

The mice were decapitated, and at measured intervals the heads were frozen in Freon 12 (CCl_2F_2) at its melting point, −150°, with vigorous stirring. Until frozen, the heads were maintained at 38°. Zero time animals were either decapitated directly into Freon or the whole mouse was frozen.

In a room maintained at −15°, the forebrain was dissected and powdered in a mortar chilled in liquid nitrogen. Except for the few minutes required to dissect the brain and weigh the powder, the material was kept at −80°. Preliminary trials showed serious losses in labile constituents overnight at −10°, but not at −20°.

In the case of the 10-day-old mice, the brain powder (125 mg) was placed on top of 0.3 ml of 3 M $HClO_4$ previously frozen in the bottom of an 8-ml test tube and kept on Dry-Ice. The tubes with the powder were transferred to an alcohol bath at −10°, and the samples were stirred or agitated in the bath until the acid had completely penetrated the powder and removed the water (3 to 5 minutes). To each were then added 1.25 ml of 1 mM EDTA in H_2O, and, after repeated mixing at 4° for 5 or 10 minutes, the tubes were centrifuged and 1 ml of supernatant fluid was mixed with 0.33 ml of 2 M $KHCO_3$. EDTA was not used in all of the analyses, but its use is recommended. If the samples should contain even small amounts of bone, as accidentally occurs with small brains, calcium phosphate will precipitate on neutralization and remove part of the ATP, fructose diphosphate, and probably other phosphorylated compounds. The EDTA, by sequestering Ca^{++}, prevents this completely. After time had been allowed for CO_2 to come off, 0.025 ml of 2 M Tris base was added to bring the pH to 7.5 to 8.0. The addition of buffer is probably unnecessary and was omitted in one series of analyses. If pyruvate is to be determined, Tris should definitely be avoided (see below). A second sample of 0.4 ml was similarly treated with 0.11 ml of 2 M $KHCO_3$, followed by 0.012 ml of a buffer that was 1 M in sodium formate and 1 M in formic acid (final pH, about 3.5). The supernatant fluids were decanted from the $KClO_4$ precipitates for storage. The original $HClO_4$ precipitates, with as much supernatant fluid as possible removed, were saved for glycogen determination.

The method of extraction was designed to prevent thawing of

the tissue before acidification. When frozen powder is treated with weak acid solutions at 0°, the tissue must in principle thaw, however briefly, before the precipitation. Minard and David (1) have stressed this danger, and they observed much lower values for AMP as well as higher values for ATP when CCl_3COOH was used in acetone below 0° rather than in water at 0°. Seraydarian *et al.* (2) found lower P_i values in muscle when samples were extracted with alcohol below 0° before acidification. The procedure recommended here has the advantage that it is unnecessary to remove an organic solvent before the analyses. In comparison with the use of 0.3 M $HClO_4$ at 0°, the procedure adopted gave much lower AMP values and somewhat lower P_i values, but the same values for lactate and glucose. Clearly the magnitude of artifact with extraction at 0° will depend on the particular technique for bringing frozen powder and acid solution together.

The samples at pH 8 were kept at −80° except for very brief intervals when they were thawed to remove aliquots for analysis. These samples were used for most of the analyses. Triose phosphate and fructose diphosphate analyses were made with the pH 3.5 samples except in the 10-day-old Group B samples. Pyridine nucleotides were measured in separate samples of brain powder which were dried at −40°. The powders were brought to room temperature under vacuum, 0.5-mg samples were homogenized with either dilute H_2SO_4-Na_2SO_4(DPN^+, TPN^+) or dilute NaOH-cysteine (DPNH, TPNH), and the individual nucleotides were measured as previously described (3).

Analytical Methods

The majority of the analyses were performed by measuring fluorometrically the appearance of TPNH or the disappearance of DPNH upon addition of appropriate enzymes. Greengard (4) and more recently Seraydarian, Mommaerts, and Wallner (5) have pointed out the advantages of fluorometry with pyridine nucleotides for measurement of tissue metabolites. The recent availability in crystalline form of most of the enzymes of the glycolytic pathway has simplified the analyses, and permitted rather exact specifications for amounts of enzymes and incubation times. The procedures used therefore are given in explicit form.

In general, the procedures are based on the spectrophotometric studies of many investigators starting with the pioneer work of Warburg and collaborators. Specifically, the methods for ATP, ADP, glucose-6-P, pyruvate, and P-pyruvate are basically the same as those of Greengard (4), which were derived from spectrophotometric procedures of a number of authors (6–9). Those for fructose diphosphate and dihydroxyacetone-P are similar to the spectrophotometric methods used by Thorn *et al.* (10), which are based on the principles employed by Slater (11). Glycero-P was measured by a fluorometric adaptation of the method of Bublitz and Kennedy (12). The ATP and glucose-6-P methods are comparable to the respective spectroscopic methods of Slein, Cori, and Cori (13, 14). The method for 2,3-diphosphoglycerate is new.

Fluorometry—All fluorometric measurements were made at a volume of about 1 ml in 3-ml tubes in the Farrand model A fluorometer. To increase stability, the mercury arc light source was replaced with a GE No. 2331 lamp with tungsten filament run at 8 volts from batteries. Since much less ultraviolet light is produced by the tungsten light, the filters were changed from those ordinarily employed. The primary filter was Corning No. 5840, and the secondary was a combination of Corning No. 4303 and 3387 with the latter facing the phototube. When highest sensitivity was used for substrates present at very low concentrations, the turntable of the instrument, which ordinarily carries three tubes at once, was fixed rigidly in one position, and tubes were read one at a time. Care was taken to use only unscratched tubes. With these changes, readings were reproducible to 0.2% of the scale reading. This resulted in reproducibility to 3×10^{-9} M (concentration in the fluorometer tube) at the highest useful sensitivity. This corresponds to 0.5×10^{-6} mole per kg of brain at the lowest tissue dilution used (1:150); *i.e.* analyses for substrates present at levels of 5 or 10 μmoles per kg were reproducible to about 10%, whereas substrates present at much higher levels were reproducible to 2 to 5% depending on the particular procedure. Practical limits are finally set by the fluorescence contribution from the brain extracts themselves, which was equivalent on the average to 5×10^{-5} mole of DPNH per kg of brain. Blank fluorescence of reagents varied from 2 to 5×10^{-7} M, expressed as equivalent DPNH contribution.

Enzymes and Reagents—All enzymes, unless noted, were obtained from Boehringer and Sons through the California Corporation for Biochemical Research. Imidazole, as a 2 M solution of the free base, was treated with charcoal to reduce an otherwise highly fluorescent blank. Significant details in regard to reagent components are given with the individual procedures. The stock reagents, unless noted, were stable when frozen for weeks or months. DPNH, when required, was added shortly before use.

General Procedure—Most of the reactions were carried out with substrate concentrations at levels which resulted in first order rates (Table I). The approximate half-times for the reactions under the conditions of analysis with the stated enzyme concentrations have been given as a guide. The incubation periods recommended are in general longer than the theoretical five or six half-times, since sometimes the rates fall off because of depletion of a cosubstrate or product inhibition. All enzymes were diluted for use in 0.02 M buffer, pH 7 to 7.5, containing 0.02% bovine plasma albumin. The dilutions were such as to provide the required amounts in 2 to 20 μl. When more than one enzyme was required, these were added as a mixture. Standards in 5 to 20 μl volumes of appropriate strength were added directly to reagent in the fluorometer tubes, which also contained blank solution equal to the tissue samples in volume and in $HClO_4$ and $KHCO_3$ composition.

Incubations were at room temperature (25–28°). For each set of analyses, the course of the reaction was followed for one standard, blank, and tissue sample, to make sure that the reaction concerned was complete at the time selected. This was particularly important, since excessive ClO_4 from $HClO_4$ extracts can slow some of the enzyme reactions. Also in some cases there may be slow side reactions which could disturb if samples were incubated too long.

The majority of the samples were read only twice: initially after the sample had been added but before the reaction was started, and a second time after adding the required enzyme or enzymes and waiting the predetermined time interval. Groups of 10 to 20 or more samples were carried through together.

Notes and further analytical details not covered in Table I are as follows.

Glucose—In analyzing serum for glucose, the same method

TABLE I

Analytical conditions

All reagents contained 0.005% or 0.01% bovine serum albumin. Analyses were conducted, unless noted, with 1 ml of reagent in fluorometer tubes (8 × 100 mm) plus neutralized $HClO_4$ extract equivalent to the amount of brain indicated (wet weight).

Substance	Buffer	Enzymes	Other additions	Brain used	Half-time	Incubation time
				mg	*min*	*min*
Glucose	Tris, 100 mM, pH 7.5	Yeast hexokinase, 2.5 μg/ml; yeast glucose-6-P dehydrogenase, 0.5 μg/ml	ATP, 0.3 mM; TPN^+, 0.03 mM; $MgCl_2$, 5 mM	1–2	0.5	20
ATP	Tris, 100 mM, pH 7.5	Yeast hexokinase, 5 μg/ml; yeast glucose-6-P dehydrogenase, 2 μg/ml	Glucose, 1 mM; TPN^+, 0.03 mM; $MgCl_2$, 5 mM	1–2	<0.5	10
P-Creatine	Tris, 100 mM, pH 7.5	Same plus muscle creatine kinase, 80 μg/ml	Same plus ADP, 0.03 mM	Same sample	2	20
Dihydroxyacetone-P	Imidazole, 20 mM, pH 7	Muscle glycerol-P dehydrogenase, 7 μg/ml	DPNH, 0.0005 mM	5–10	0.4	5
Fructose diphosphate	Imidazole, 20 mM, pH 7	Same plus muscle triose-P isomerase, 1 μg/ml, and muscle, aldolase, 1 μg/ml	Extra DPNH as needed	Same sample	1	10
3-P-Glycerate	Imidazole, 20 mM, pH 7.6	Muscle glyceraldehyde-P dehydrogenase, 25 μg/ml; muscle P-glycerate kinase, 1 μg/ml	DPNH, 0.001 or 0.002 mM; $MgCl_2$, 5 mM; ATP, 0.3 mM; mercaptoethanol, 5 mM	6	0.8	10
2,3-Diphosphoglycerate	Same	Same plus muscle P-glycerate mutase, 15 μg/ml	Same	Same sample	See text	25
Pyruvate	P_i, 100 mM,[a] pH 7.0	Heart lactic dehydrogenase,[b] 0.5 μg/ml	DPNH, 0.0003 mM to 0.001 mM	4	0.5	6
P-Pyruvate	P_i, 50 mM, pH 7.0	Heart lactic dehydrogenase,[b] 5 μg/ml; muscle pyruvate kinase, 3 μg/ml	DPNH, 0.00025 mM; ADP, 0.02 mM; $MgCl_2$, 5 mM; hydrazine·HCl, 10 mM	7	1	5
2-P-Glycerate	Same	Same plus muscle enolase, 25 μg/ml	Same	Same sample	1	6
ADP	P_i, 50 mM[c] pH 7.0	Heart lactic dehydrogenase,[b] 8 μg/ml; muscle pyruvate kinase, 0.5 μg/ml	DPNH, 0.003 mM; ATP, 0.02 mM; P-pyruvate, 0.02 mM; $MgCl_2$, 2 mM	1	1	5
AMP	Same	Same plus muscle adenylate kinase, 1 μg/ml	Same	Same sample	1	10
Creatine	Glycylglycine, 50 mM, pH 8.2	Heart lactic dehydrogenase,[b] 10 μg/ml; muscle pyruvate kinase, 5 μg/ml; muscle creatine kinase, 600 μg/ml	DPNH, 0.01 mM; ATP, 0.05 mM; $MgCl_2$, 5 mM; P-pyruvate, 0.025 mM; KCl, 30 mM	0.7	6	40
α-Glycerol-P	See 4th column	Muscle glycerol-P dehydrogenase, 10 μg/ml	DPN^+, 0.2 mM; EDTA, 1 mM; hydrazine, 350 mM; hydrazine·HCl, 50 mM	3	0.8	10
Lactate[d]	Carbonate,[e] 200 mM, pH 9.7	Heart lactic dehydrogenase, 75 μg/ml	Acetyl-DPN^+, 1 mM	3–6	1	10
Glucose-6-P[f]	Tris, 100 mM, pH 8.0	Yeast glucose-6-P dehydrogenase, 0.25 μg/ml	TPN^+, 0.01 mM	3	0.4	5

[a] This level is recommended although 200 mM was used for the present analyses.

[b] If skeletal muscle enzyme is used, the amount should be increased 4-fold. Heart lactic dehydrogenase is essential in the method for lactate determination. It was obtained from Worthington Biochemical Corporation.

[c] Potassium salt.

[d] Conducted in spectrophotometer with 400 μl of reagent.

[e] $NaHCO_3$, 0.133 M; Na_2CO_3, 0.067 M.

[f] Direct method at 1-ml volume. For indirect procedures for glucose-6-P, fructose-6-P, and glyceraldehyde-P, see the text.

was used as for brain except that since no preliminary extraction was required 0.5 to 1 μl of whole serum was added directly to the reagent.

Commercially available preparations of hexokinase contain TPNH-oxidizing activity. This need not cause difficulty if the amount of hexokinase used is kept to a minimum, but for any of the procedures with the hexokinase-glucose-6-P dehydrogenase system, it is desirable to check the stability of TPNH under the conditions chosen and with all enzymes present. Glutathione reductase may also be present in some preparations of glucose-6-P dehydrogenase, and with tissue extracts this could cause trouble not detectable with simple standards because of the likelihood that oxidized glutathione may be present in tissue extracts. This problem can usually be avoided by using the minimal amount of the dehydrogenase.

Glycogen—This was measured with the reagents used for glucose. To the $HClO_4$-insoluble residue (from 100 mg of brain) was added 1 ml of 1 N HCl. The tube was sealed and heated for 2 hours at 100°. Breckenridge[1] has shown that such treatment releases all the glucose from glycogen plus

[1] B. M. Breckenridge, personal communication.

about one-third of that present in gangliosides. Glycogen was calculated by deducting the amount of glucose released by hydrolysis of those brain samples that had undergone the longest periods of ischemia (10 or 20 minutes). It has been shown that by this time glycogen has practically disappeared from brain, and that the contribution from nonglycogen sources is unchanged.[2] The values are also corrected for preformed glucose calculated to be present in the precipitate. No glycogen was found in the original $HClO_4$ extracts; *i.e.* there was no increase in glucose on hydrolysis. (It is not safe to assume from this that $HClO_4$ extracts of brain will never contain glycogen.[2])

ATP and P-Creatine—These were both measured on the same sample. After the reaction with ATP was complete, and the second reading made, 20 μl containing the creatine kinase and 1.5 mM ADP were added. This mixture of ADP and enzyme was prepared (at 0°) within 1 hour of use. Available ADP contained about 1% ATP, and therefore gave a substantial blank. More ADP and less creatine kinase could be used if the ATP contamination were less, although this would increase the danger from possible adenylate kinase contamination. An increase in ADP accelerates adenylate kinase more than it does creatine kinase in this concentration range (15). Because of the stability of adenylate kinase to acid, $HClO_4$ extracts had to be separated cleanly from precipitates, and samples were checked to make sure that the reaction stopped after P-creatine was gone. It is because of the danger of adenylate kinase contamination that ADP was not added until needed.

Under the analytical conditions used, CTP, GTP, and UTP did not react significantly.

Dihydroxyacetone-P and Fructose Diphosphate—After the reaction with dihydroxyacetone-P was complete, and the second reading made, extra DPNH was added according to the expected magnitude of fructose diphosphate. The samples were read once more but at a lower instrumental sensitivity before adding the mixture of aldolase and triose-P isomerase.

Tris buffer, although used instead of imidazole for part of the analyses, is not ideal for the reaction since it can combine slowly with glyceraldehyde-P (16), presumably as a Schiff base. High P_i concentrations markedly inhibit triose-P isomerase, and imidazole is probably the buffer of choice.[3]

3-Phosphoglycerate and 2,3-Diphosphoglycerate—These were measured by disappearance of DPNH upon enzymatic conversion to glyceraldehyde-P. Measurement of 2,3-diphosphoglycerate was made possible by the fact that the P-glycerate mutase (Boehringer) contained phosphatase activity capable of converting diphosphoglycerate to 3-P-glycerate. This phosphatase activity may have been due either to a contaminant or to a secondary activity of mutase, as suggested by Joyce and Grisolia (17).

Originally 3-P-glycerate was assessed by enzymatic conversion to lactate and measurement of the amount of DPNH consumed. This required the use of four enzymes and the prior determination of pyruvate, P-pyruvate, and 2-P-glycerate. The above direct procedure gave more reproducible results with standard mixtures, and in addition gave decidedly lower results with brain samples. The discrepancy was traced to the P-glycerate mutase used when the assay was made via pyruvate. Evidence that 2,3-diphosphoglycerate was responsible for the extra DPNH consumption by brain samples analyzed with the pyruvate system is as follows: (*a*) in the complete glyceraldehyde-P dehydrogenase system, addition of mutase caused the expected extra consumption of DPNH, but if either P-glycerate kinase, glyceraldehyde-P dehydrogenase, or ATP was omitted, addition of mutase did not cause DPNH consumption; (*b*) in the pyruvate system, the contributions after mutase addition from both 3-P-glycerate and from suspected 2,3-diphosphoglycerate were abolished if either lactic dehydrogenase or enolase was omitted; (*c*) the sum of 3-P-glycerate plus suspected diphosphoglycerate is the same through both analytical systems; (*d*) known 2,3-diphosphoglycerate was attacked at the same rate as the substrate present in brain samples.

Sutherland, Posternak, and Cori (18) first reported 2,3-diphosphoglycerate phosphatase activity associated with mutase activity but found that much of the phosphatase activity disappeared on purification. The activity found here in the crystalline muscle mutase is very low in absolute magnitude, 0.4 mole per kg of protein per hour at 28°. This is about one-fourth of the activity reported by Pizer for mutase from muscle (19) and twice that found by Rodwell, Towne, and Grisolia (20) for yeast mutase.

Although the absolute activity is low, the Michaelis constant is exceedingly low (in the order of 2×10^{-7} M). The specific 2,3-diphosphoglycerate mutase reported by others as a possibly separate enzyme has a Michaelis constant 1000 times larger (17, 21). Because of the low Michaelis constant of the phosphatase activity in crystalline mutase, this enzyme, whether contaminant or side activity, is satisfactory for measuring the low concentrations of 2,3-diphosphoglycerate of the present fluorometric assays. If concentrations higher than 3×10^{-7} M were to be measured, the enzyme would need to be increased in direct proportion.

The method used for 2,3-diphosphoglycerate would be expected to include 2-P-glycerate. However, no correction has been applied for the following reasons: (*a*) the P-glycerate kinase used was contaminated with traces of P-glycerate mutase and an enzyme, probably a phosphatase, capable of destroying 2-P-glycerate, and (*b*) under the conditions of the assay, the low amount of 2-P-glycerate present would increase 3-P-glycerate results by not more than 3 to 5% and 2,3-diphosphoglycerate results by a maximum of 10%.

Pyruvate—Upon the addition of lactic dehydrogenase to reagent containing brain extract, there is a rapid reduction of DPNH at the rate expected from pyruvate standards. However, the reaction does not stop when expected, but proceeds slowly thereafter until the total DPNH consumption may have at least doubled. This is presumably due to other keto acids in brain, and was anticipated by Meister (22) after a careful study of the specificity of lactic dehydrogenases. Heart and skeletal muscle lactic dehydrogenase give the same results when used at comparable activities; this is in keeping with Meister's findings with a series of keto acids. The nonpyruvate DPNH oxidation can be controlled by using a reagent at pH 6.8 to 7.0 provided that the time and dehydrogenase concentration are carefully chosen on the basis of pyruvate standards. (At pH 7.5 to 8.0, the rate of nonpyruvate oxidation of DPNH increases relative to the rate with pyruvate.)

Phosphate is at present the buffer of choice. Tris buffer is unsuitable for measuring low levels of pyruvate because of the

[2] D. Gatfield, O. H. Lowry, and J. V. Passonneau, to be published.

[3] D. B. McDougal, Jr., personal communication.

presence of lactic dehydrogenase-reacting materials. In imidazole buffer the rate of reaction with the nonpyruvate substances of brain is increased. P_i accelerates the rate of destruction of DPNH, and therefore blank readings are carefully made in parallel.

P-Pyruvate and 2-P-Glycerate—After the sample was added, 20 or 30 minutes were allowed for reaction between hydrazine and keto acids present, at which time lactic dehydrogenase was added. Five minutes later, the fluorescence was measured and pyruvate kinase added. Subsequent steps were performed as indicated in Table I. Enolase was used in relatively high concentration since it was inhibited 80 and 92%, respectively, by addition to 1 ml of reagent of 100 and 200 µl of solution saturated with $KClO_4$ at 0°. The higher of the two $KClO_4$ levels inhibited the other enzymes 65% or less.

Earlier, an attempt was made to assay P-pyruvate and 2-P-glycerate in the same sample used for pyruvate. There were two difficulties. First, since pyruvate is usually present in much greater amount than the other two, it is hard to gauge the DPNH excess to provide just the right amount for a reliable assay. More serious is the fact that nonpyruvate tissue components react slowly with either muscle or heart lactic dehydrogenase (see "Pyruvate" above). By the use of hydrazine, both pyruvate and these nonpyruvate-reacting materials are tied up. It is now only necessary to add DPNH in modest excess, and there is little or no drift in readings in the presence of lactic dehydrogenase after a slight drop in the first few minutes.

Hydrazine reacts with pyruvate and with other lactic dehydrogenase-reacting tissue components at comparable speeds. With 10 mM hydrazine, the half-time for reaction with pyruvate at 28° is about 3 minutes; therefore, to avoid a negative error of more than 5%, sufficient lactic dehydrogenase must be added to react with pyruvate with a half-time of 10 seconds or less. The reaction rate with hydrazine was the same at pH 6.8 and 7.6. The pyruvate hydrazide can slowly dissociate and allow reaction with DPNH on long exposure to lactic dehydrogenase; therefore, the assay is completed promptly. The concentration of hydrazine used had no effect in 1 hour on enolase or pyruvate kinase; there was a small loss of lactic dehydrogenase activity.

It is necessary to test lactic dehydrogenase carefully for contamination with pyruvate kinase. Some ADP preparations contain P-pyruvate, part of which may be converted to pyruvate on storage. Consequently there may be a substantial blank after pyruvate kinase addition, particularly if more ADP than necessary is used.

ADP and AMP—Currently available DPNH preparations contain 5 to 15% AMP on a molar basis. Since for each mole of AMP 2 moles of DPNH are consumed, this may result in a very troublesome blank after the addition of adenylate kinase. The AMP may be removed by treating a 5 mM DPNH solution at pH 8 to 9 with 70 µg of alkaline phosphatase (Sigma, type III, "bacterial" (*E. coli*)) per ml for 20 minutes at 38°, adding NaOH in 0.02 N excess (*pH must be at least 12*), heating for 2 minutes at 100° to destroy the phosphatase, and then restoring the solution to the original pH.[4]

Current preparations of ATP (added to the reagent for the sake of the second step) contain about 1% ADP, but negligible AMP. The adenylate kinase is specific for 5′-AMP, but the pyruvate kinase reaction is not specific for ADP (23). Under the given conditions, the relative rates with other nucleoside diphosphates known to be present in brain are close to those measured with phosphate buffer by Strominger (24), who found the relative rates with ADP, GDP, IDP, UDP, and CDP to be 100, 19, 12, 3, and 2, respectively. Since substantial amounts of GDP may be present in brain, the results reported for ADP are greater than the true values.

ADP values were corrected for pyruvate concentrations measured independently. However, if pyruvate is not to be measured, it would be preferable to add lactic dehydrogenase separately with a reading before the addition of pyruvate kinase. For reasons discussed in the section on P-pyruvate and 2-P-glycerate determination, a further improvement in the present procedure would be to put hydrazine into the reagent and permit the samples to incubate for 20 or 30 minutes before adding the enzymes. This would tie up pyruvate and any other preformed lactic dehydrogenase-reacting compounds in sample or reagent.

Creatine—This was measured in a glycylglycine buffer rather than Tris for the reasons mentioned in the section on pyruvate. Tris was particularly troublesome in this case, because the creatine reaction is rather slow and has to be conducted at a slightly alkaline pH.

α-Glycero-P—The hydrazine buffer was made freshly each day since it may develop a fluorescent blank on standing.

Lactate—The reagent was prepared within 1 or 2 hours of use because of the instability of acetyl-DPN at alkaline pH. The molar extinction coefficient of the reduced nucleotide is 9300 at 363 mµ. Blanks and standards were provided with the use of equal volumes of neutralized perchlorate solutions prepared in the same way as the tissue samples, since any decrease in pH would cause a decrease in the blank absorption (of the acetyl-DPN^+) as well as a decrease in the completeness of lactate oxidation. $(NH_4)_2SO_4$ (from the enzyme preparation) was kept to a minimum for the same reasons. If the pH does not fall below 9.7, the reaction is essentially complete with lactate up to 0.02 mM; higher concentrations were measured with the aid of a standard curve. The reaction time increases with increasing levels of lactate. Heart lactic dehydrogenase is much to be preferred to the muscle enzyme, because at high pH it is much more stable and the Michaelis constant is much lower (1.5 mM as opposed to 13).

Inorganic Phosphate—This was measured by adding 50 µl of extract, equivalent to 3 mg of brain, to 400 µl of 0.1% ascorbic acid in 0.125 M acetate buffer, pH 4. To each sample were added 20 µl of 2.5% ammonium molybdate containing 0.004% $CuSO_4 \cdot 5H_2O$. Optical density was read 10 minutes later at 700 mµ. This is a minor modification of a published procedure (25).

Glucose-6-P and Fructose-6-P—With care, glucose-6-P can be measured with sufficient accuracy directly in the fluorometer (Table I). However, fructose-6-P measurement was not considered to be accurate enough. A satisfactory method was provided by a "cycling" procedure (3) as follows. The stock solution consisted of 0.005 mM TPN^+ and 0.01% bovine plasma albumin in 0.02 M Tris, pH 8.0. To this was added glucose-6-P dehydrogenase to a concentration of 0.25 µg of protein per ml. A volume of 5 µl of extract, equivalent to 0.3 mg of brain, was added to 15 µl of reagent in a tube of 4-mm bore and 60-mm length. After 15 minutes at room temperature, a 5-µl aliquot

[4] According to personal communication from Dr. Mark Stewart, some DPNH preparations contain a substance which yields 5′-AMP in hot alkali; such preparations must be treated with alkali at 100° both before and after phosphatase treatment.

was mixed with 5 μl of 0.1 N NaOH in a similar tube of 2.5-mm bore, and heated for 10 minutes at 60° to destroy excess TPN^+. This left TPNH equivalent to the original glucose-6-P. To the remaining 15 μl of each sample in the 4-mm tube was added 1 μl of 1 N HCl to destroy the TPNH formed from glucose-6-P. After 5 minutes at 38°, this sample was treated with 2 μl of a mixture of glucose-6-P dehydrogenase (1.5 μg of protein per ml) and 0.1% bovine plasma albumin in 1 M Tris (20:1 ratio of Tris base to Tris hydrochloride), together with sufficient P-glucoisomerase to give a maximal velocity of 15 mM per hour at 25° (this is approximately equal to 1 μg of crystalline isomerase per ml). After 15 minutes at room temperature, 2.5 μl of 1 N NaOH were added followed by 10 minutes of heating at 60° to destroy excess TPN^+, leaving TPNH equivalent to the original fructose-6-P.

The TPNH was measured with 3- and 10-μl aliquots, respectively, from the first and second set of samples, representing glucose-6-P and fructose-6-P. The aliquots were added to 100-μl volumes of cycling reagent (3) containing 0.01% crystalline glutamic dehydrogenase and 0.005% of the glucose-6-P dehydrogenase preparation. Cycling was carried out for 1 hour at 38°. The danger from possible glutathione reductase contamination, mentioned above in the section on glucose, is particularly relevant for measurement of fructose-6-P and glucose-6-P. As pointed out by Hohorst *et al.* (7), commercially available isomerase may be seriously contaminated with glutathione reductase.

Glyceraldehyde-P—This substrate was also too low to measure directly, and again resort was had to cycling. The stock reagent consisted of 0.05 M K_2HPO_4, 0.1 M Na_2CO_3, 0.05 $NaHCO_3$, 1 mM EDTA, and 0.02% bovine plasma albumin. To this was added, just before use, DPN^+ (5×10^{-6} M), mercaptoethanol (5 mM), and glyceraldehyde-P dehydrogenase (0.002%), at the final concentrations indicated. This reagent (10 μl) was added to 5 μl of tissue extract equivalent to 0.3 mg of brain. After 30 minutes at 25°, excess DPN^+ was destroyed by adding 3 μl of 1 N NaOH and heating for 10 minutes at 60°. This left DPNH equivalent to the original glyceraldehyde-P, which was measured by incubating a 10-μl aliquot for 1 hour at 25° with 100 μl of DPN-cycling reagent (3) containing 0.02% glutamic dehydrogenase and 0.005% heart lactic dehydrogenase.

Stability of Substrates in Extracts—The less stable substrates were tested to determine the best conditions for storage. Tests were made in 0.3 M $HClO_4$ or in solutions made from this acid by neutralization with $KHCO_3$ with adjustment of pH to 3, 5, or 8, to simulate the media to which the tissue substrates might be exposed. Dihydroxyacetone-P and glyceraldehyde-P were stable for at least 2 hours at 60° in either 0.3 M $HClO_4$ or at pH 3, and for at least 1 week at 4° at pH 3. Stability was measurably less at pH 5, particularly in the case of glyceraldehyde-P (50% loss in 2 hours at 60°). Fructose diphosphate and pyruvate were stable for 2 hours at 60° at pH 3, 5, and 8, for at least 5 days at 4° in 0.3 M $HClO_4$ or at pH 3, and for 5 days at −80°, pH 8. The reported instability of fructose diphosphate on storage at neutral pH (25) was thus not observed.

Pyruvate may be lost on storage either through polymerization or by reaction with other constituents present. Storage of 0.01 mM pyruvate in the presence of 0.05 M Tris buffer, pH 8, resulted in 40% loss in 1 month at 7°, and 30 to 100% loss in 3 days at −18°. In the absence of Tris at pH 9, 40% loss occurred in 28 days at −18°. No loss was observed under the same conditions at pH 4. If polymerization occurs, this can be reversed by heating for 1 to 2 minutes at 100° in 0.1 N NaOH. The loss in Tris is not due to polymerization, since reversal was not observed in this case.

P-Creatine was not detectably cleaved in 60 minutes either at 0° in 0.6 M $HClO_4$ or at −10° in 3 M $HClO_4$. At −80°, pH 8, there was no loss in either P-creatine or ATP in 1 month.

General Comment—Considerable effort was made in the above procedures to control sources of error. Nevertheless, the history of substrate measurement in tissues makes caution imperative. There are at least five kinds of possible error: (*a*) freezing may not be fast enough to stop enzymatic processes (1, 26) or change may continue in the frozen state; (*b*) change may occur during extraction or preparation of the tissue for extraction (1, 2); (*c*) acidic extracting agents may affect a metabolite differently in the form in which it exists in the tissue than when it is in the free state, as seems to be the case for TPN^+ (27); (*d*) loss may occur before the analysis is made, as a result of incomplete extraction, adsorption onto the $KClO_4$ precipitate (see above), or instability during storage of extracts; (*e*) the enzymatic assay may fail through nonspecificity of the enzymes used; this can be particularly troublesome and difficult to detect, and the error from this source can either be positive or negative.

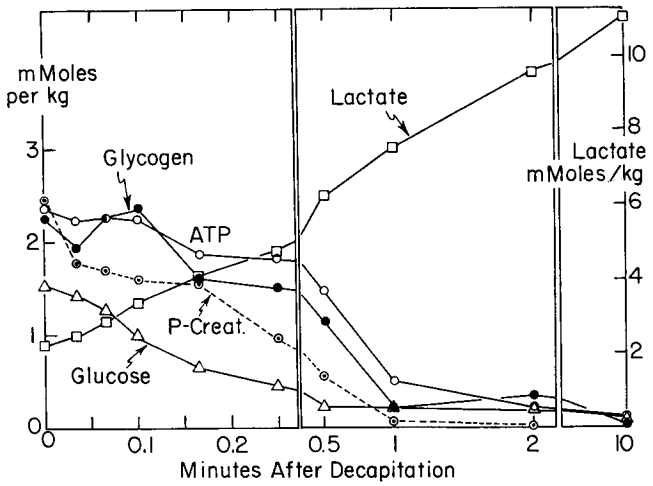
RESULTS

After the blood supply has been cut off, the brain is forced to use its emergency sources of energy as long as they are available. Therefore, the analytical results can be examined with two questions in mind. (*a*) How *fast* are the energy reserves used and in what order? (*b*) What are the *mechanisms* by which these energy supplies are mobilized; or perhaps more to the point, what are the mechanisms for keeping the energy supplies from being used until needed?

Use of Energy Reserves—Without a supply of oxygen the major sources of energy are ATP, P-creatine, glucose, and glycogen. The rate of energy use, *i.e.* the metabolic rate, can be calculated in terms of ~P use from the changes in these four substances, since for each mole of lactate formed from free glucose 1 mole of ~P is generated, and for each mole of lactate formed from glycogen there are about 1.45 moles of ~P generated (corrected for an assumed 10% branching).

The rates of use of these compounds differed markedly among the adult, 10-day-old, and anesthetized 10-day-old groups. In general, the order of depletion of the compounds of the energy reserve was P-creatine, glucose, ATP, and glycogen, with the rates for the last two being nearly equal (Figs. 1 to 3; Table II). That glucose is used more rapidly than glycogen under ischemia was observed in the dog by Kerr and Ghantus (26). Many authors have observed that P-creatine is more rapidly depleted than ATP by anoxia or ischemia (10, 28, 29).

The initial rates of fall of P-creatine were nearly the same for all three groups. Glucose fell initially as rapidly as P-creatine in the adult mice, but only one-third to one-fourth as rapidly in the younger animals. The very low initial glucose levels in the nonanesthetized 10-day-old animals might have been a factor causing the low initial rate for that group. The effects of age and anesthesia on the rates of glycogen and ATP use are striking. Glycogen was not used at all for at least 2 minutes in the anesthetized young animals. The peak rates of use of glycogen were 3, 0.6, and 0.75 mmole per kg per minute for the adult, 10-day, and 10-day anesthetized groups; *i.e.* once glycogen was required by the anesthetized brains, it was used as rapidly as in



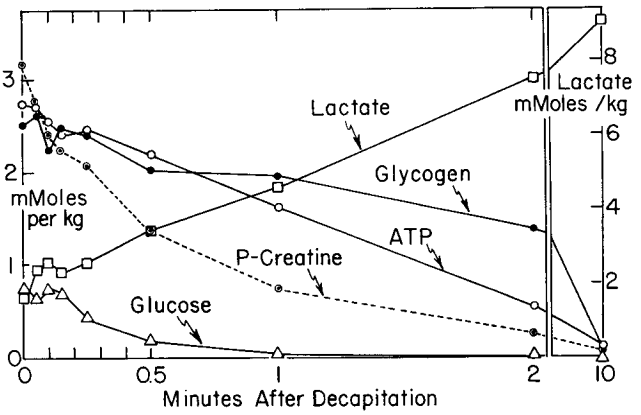


FIG. 1 (*left*). Concentrations of P-creatine, ATP, glucose, glycogen, and lactate in the brains of unanesthetized adult mice frozen quickly at different intervals after decapitation. Each point is the average of values from 2 to 4 mice, a total of 28 animals.

FIG. 2 (*right*). This is similar to Fig. 1 except that the data are from 25 unanesthetized, 10-day-old animals.

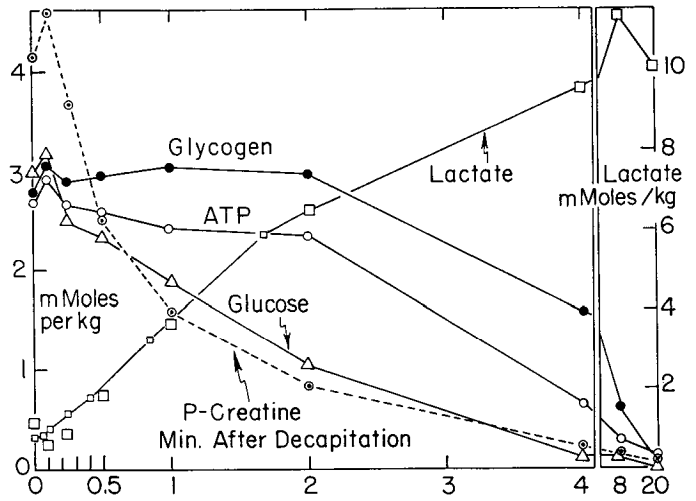


FIG. 3. This is similar to Fig. 2 except that the 10-day-old mice had been anesthetized with phenobarbital for about 1 hour before decapitation. All of the values refer to a single lot of 25 mice (Group A) except in the case of the lactate values represented by the *smaller squares* (0 to 100 seconds only), which come from an additional lot of 32 mice (Group B).

the controls. In none of the groups did the peak rate of glycogenolysis equal the peak (initial) rate of glucose utilization. Apparently glycogen and ATP are not depleted until the rate of delivery of ~P from P-creatine and from conversion of glucose to lactate is less than the rate of ~P use. It is clear, however, that it is not the *concentration* of P-creatine which triggers the use of glycogen and ATP, since in the anesthetized group P-creatine fell to 0.8 mmole per kg before glycogen began to fall, whereas in the adult group glycogen had fallen 40% when P-creatine had reached the same level.

The initial rates of use of ~P were maintained for only about 15 seconds in the unanesthetized animals and for 30 seconds in the anesthetized. Possibly by these times the more active cells had exhausted their P-creatine and glucose. The rates in all cases fell to about half after the initial burst, and were maintained roughly constant for 45 seconds in the adult group, almost 2 minutes in the 10-day-old group, and 3.5 minutes in the young anesthetized group. The anesthetized animals started with about 20% more total reserve energy than the controls. This was chiefly due to higher glucose levels plus slightly higher levels of P-creatine (Table III).

Changes in lactate reflected changes in glucose and glycogen within experimental limits. Other things being equal, initial

TABLE II

Initial fluxes for major energy sources after decapitation

The values have been estimated from initial slopes of the curves in Figs. 1 to 4. The glycogen fluxes are calculated as glucose equivalents.

Group	P-Creatine	ATP	Glucose	Glycogen	Lactate	~P[a]	Maximal predecapitation glucose flux[b]
	mmoles/kg/min						
Adult	11.	2.2	6.5	2.2	11.3	25	0.76
10-Day	7.8	1.1	1.9	0.6	3.6	13	0.40
10-Day anesthetized							
A	4.8	0.26	1.6	0.0	—[c]	8	0.24
B	4.2	0.36	1.5		0.6	7.5	0.22

[a] This is the rate of decrease in available sources of ~P. It is the sum of the initial fluxes in P-creatine, ATP (multiplied by 2), and lactate (ignoring the initial lag) with due allowance for accumulation of ADP and for the fact that more ~P is generated by lactate formation from glycogen than from glucose (see Fig. 4 and the text).

[b] This is the glucose flux that would be required *aerobically* to provide the ~P flux of the preceding column. It is based on assumptions given in the text, and it is probably higher than the actual predecapitation flux, since decapitation undoubtedly increases the metabolic rate.

[c] The peak lactate fluxes were 3.4 and 3.2 mmoles per kg per minute in anesthetized Groups A and B, respectively (Figs. 2 and 3). There appeared to be a lag in both groups, but only in Group B do the data permit an estimate of the initial rate. The lag period in Group B was between 2 and 3 seconds (Fig. 3).

brain glucose levels are proportional to plasma glucose levels;[5] these were measured only in the case of anesthetized Group B. This introduces discrepancies in the carbohydrate balance at some of the time intervals. The initial rate of lactate formation was very low in the anesthetized animals, but the peak rate was about the same as in the controls (Table II). It would therefore appear that over-all glycolytic capacity was not reduced by anesthesia.

It is possible to calculate the rates of glycolysis before decapitation if it is assumed (*a*) that the rates of ~P use were unchanged during the first few seconds after decapitation, (*b*) that glucose had been the only source of energy, (*c*) that 15% of the glucose used was converted to lactate (28), and (*d*) that the remaining 85% of the glucose used was oxidized to CO_2 with a yield of 38 equivalents of ~P per mole oxidized (30).

According to these assumptions, for each mole of glucose utilized there would be 33 equivalents of ~P generated. For example, the adult mice with 25 mmoles of ~P used per kg per minute would require 25/33 = 0.76 mmole of glucose per kg per minute (1.52 mmoles of triose). Based on such calculations, glycolysis increased not less than 7.4-, 4.6-, and 7.1-fold in the adult, 10-day, and 10-day anesthetized groups, respectively (Table II). These figures are lower limits, since the stimulation caused by decapitation and anoxia undoubtedly increased ATP expenditure above predecapitation rates. From data summarized by McIlwain (28), it would be calculated that normally brain uses only 0.3 to 0.5 mmole of glucose per kg per minute.

The indirect method of calculating metabolic rate would give erroneous results during a transition stage if changes in intermediates were significant compared to total flux. Thus, when glucose is added to ascites tumor cells, there is transient fall in ATP as the glycolytic intermediates accumulate (31–34). Correction for such transients could be made if the intermediates are measured. In the present instances, the balance sheet indicates that errors from this source are not large.

Intermediate Substrates—The first few seconds of ischemia were characterized by a fall in glucose-6-P and fructose-6-P and a concomitant rise in fructose diphosphate (Figs. 5, 6, 8, and 9). The peak values for fructose diphosphate were found at 3 to 5 seconds in the adult and 5 to 8 seconds in the younger animals, but not for 25 to 50 seconds in the young anesthetized animals. These changes, occurring at a time when glycolysis has increased at least 4- to 7-fold, clearly show that phosphorylation of fructose-6-P had been greatly facilitated. The fact that hexose monophosphates remained much higher in the anesthetized animals than in the controls indicates that the P-fructokinase step had not been facilitated as much as in the unanesthetized groups. Dihydroxyacetone-P (Figs. 6 to 9) underwent changes which parallel those of fructose diphosphate. Both compounds showed a second rise at 2 to 4 minutes. This could be due to a second flood of glucose-6-P from glycogenolysis; however, the rates of lactate formation do not support this explanation. At no time were the ratios of dihydroxyacetone-P to fructose diphosphate those expected from the equilibrium constant (see "Discussion").

Fructose-6-P and glucose-6-P paralleled each other at least roughly. The fact that the ratio of glucose-6-P to fructose-6-P exceeded the expected value of 3:1 (35) might indicate that equilibrium is not quite maintained, but there is a possibility that the true fructose-6-P values were somewhat higher than the observed levels (see "Experimental Procedure").

[5] C. Mayman, D. Gatfield, and B. M. Breckenridge, to be published.

TABLE III

Substrate levels in mouse before and after periods of ischemia

The periods of ischemia (time after decapitation) were chosen at the approximate time of maximal glycolytic rate. The anesthetized animals had received 125 to 150 mg of phenobarbital per kg 1 hour before the experiment. All values are recorded as micromoles per kg, wet weight.

Substrate	Adult		10-day-old				
			Control		Anesthesia		Refrigerated[a]
	Initial	4 sec	Initial	6 sec	Initial	25 sec	Initial
Glycogen	2250	2260	2510	2240	2780	2930	2640
Glucose	1540	1270	750	770	2560	1930	1870
Glucose-6-P	80	66	91	40	224	91	240
Fructose-6-P	16	11	23	12	50	27	56
Fructose diphosphate	120	216	109	170	27	153	42
Dihydroxyacetone-P	46	53	14	39	13	39	13
Glyceraldehyde-P					0.9	3.3[b]	
1,3-Diphosphoglycerate					<1	<1	
3-Phosphoglycerate					25	85	
2,3-Diphosglycerate					29	29	
2-Phosphoglycerate			(8)	(12)	2.8	8.8	(4)
P-Pyruvate			(10)	(10)	3.5	8.5	(1)
Pyruvate			(21)	(35)	39	72	(36)
Lactate	2260	2830	1650	2600	770	1820	1790
α-Glycero-P			61	106	48	76	57
ATP	2360	2260	2740	2530	2580	2410	2660
ADP	910	1080	670	850	690	730	650
AMP	210	330	140	180	130	130	130
P-Creatine	2430	1670	3180	2400	3740	2000	4560
P_i			4370	5070	3350	4410	2210

[a] These mice, four in number, were lightly anesthetized with ether and chilled to 18° before decapitation.

[b] Sixty seconds after decapitation.

Glyceraldehyde-P (Table III), 3-P-glycerate, 2-P-glycerate, P-pyruvate, and pyruvate (Figs. 8 and 9) all increased with the increase in glycolytic rate, although none increased as much as fructose diphosphate and dihydroxyacetone-P. Pyruvate lagged behind the rest and then continued to rise when its precursors were falling. Satisfactory values for the above four compounds are available only for young anesthetized animals from 0 to 100 seconds post-decapitation, although less complete and less precise data were obtained for unanesthetized young animals which agree with those presented. After 10 minutes of ischemia, pyruvate fell to very low levels, 5 to 8 μmoles per kg (not shown), *i.e.* much lower than dihydroxyacetone-P levels at the same time.

The concentration of 1,3-diphosphoglycerate was below the

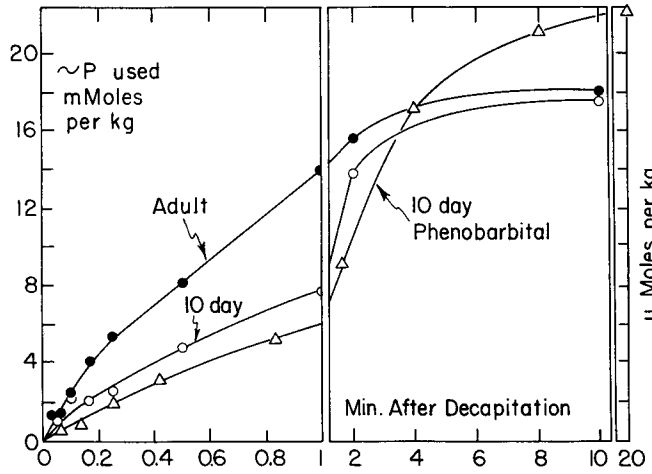


FIG. 4 (*left*). Calculated use of ~P in brain after decapitation. Each point represents the sum of decreases in the major anaerobic sources of ~P, both actual and potential, *viz.* P-creatine, ATP, ADP, glycogen, and glucose (see text).

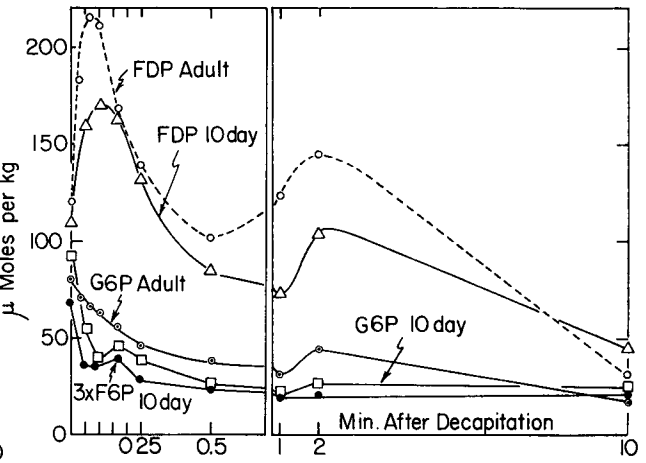


FIG. 5 (*right*). Concentrations of fructose diphosphate (*FDP*) and glucose-6-P (*G6P*) in brains of adult and 10-day-old nonanesthetized mice. Fructose-6-P (*F6P*) is also recorded for the younger mice. The animals are those of Figs. 1 and 2.

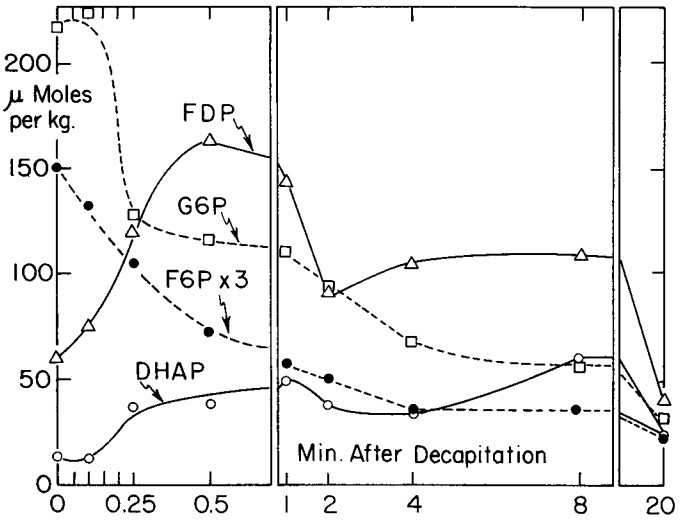


FIG. 6. The same as Fig. 5 except that the mice were anesthetized (10-day-old Group A of Fig. 3). Also shown are the dihydroxyacetone-P (*DHAP*) values for the same mice.

limit of the method used (1 μmole per kg), both initially and at times of peak glycolysis.

Glycero-P was found to maintain, during ischemia, an almost strict parallelism with lactate (Fig. 10). Even when there occurred considerable variations between animals of a given time interval, the ratios of lactate to glycero-P were nearly constant. The average ratios were 23.8 ± 0.7 (standard error) and 25.0 ± 0.7 for the control and anesthetized groups, respectively. There were, however, some variations in the relative rates of accumulation of the two products. During the first 30 seconds, glycero-P accumulated about in proportion to lactate. In the second phase, particularly seen in the anesthetized group, glycero-P accumulation lagged behind lactate. In the final stage, when glucose and glycogen were largely depleted, glycero-P accumulated at a greater proportionate rate than did lactate (Fig. 10).

Thorn *et al.* observed changes for fructose diphosphate, dihydroxyacetone-P, and pyruvate in ischemic and anoxic rabbit brain (36) which were similar to those reported here, although the peak pyruvate levels were considerably lower than in the mouse brains.

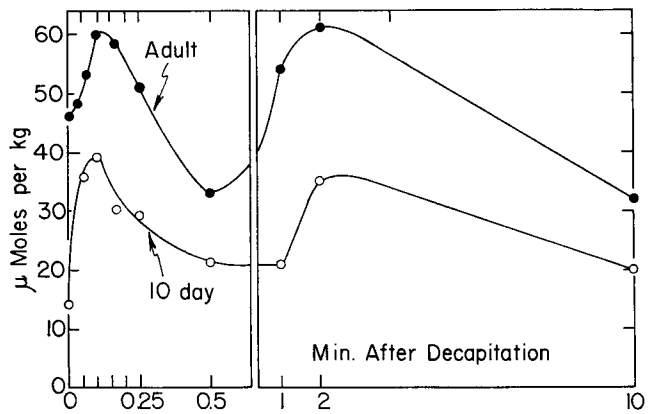


FIG. 7 (*left*). Dihydroxyacetone-P concentrations in brains of adult and 10-day-old nonanesthetized mice, the same mice as in Figs. 1 and 2.

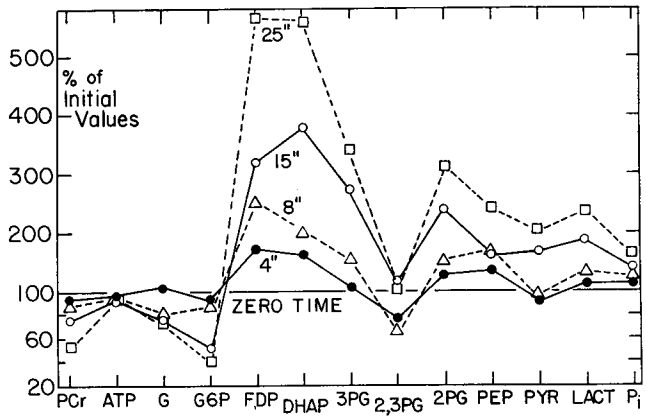


FIG. 8 (*right*). Percentage changes in 13 constituents of mouse brain during the first 25 seconds after decapitation. The mice were 10 days old and anesthetized with phenobarbital (Group B of Fig. 3). The figure is based on averages for eight mice at zero time and four mice at each subsequent time interval. The abbreviations used are: *PCr*, P-creatine; *G*, glucose; *G6P*, glucose-6-P; *FDP*, fructose diphosphate; *DHAP*, dihydroxyacetone-P; *3PG*, 3-P-glycerate; *2,3PG*, 2,3-diphosphoglycerate; *2PG*, 2-P-glycerate; *PEP*, P-pyruvate; *LACT*, lactate. Actual initial values are given in Table III.

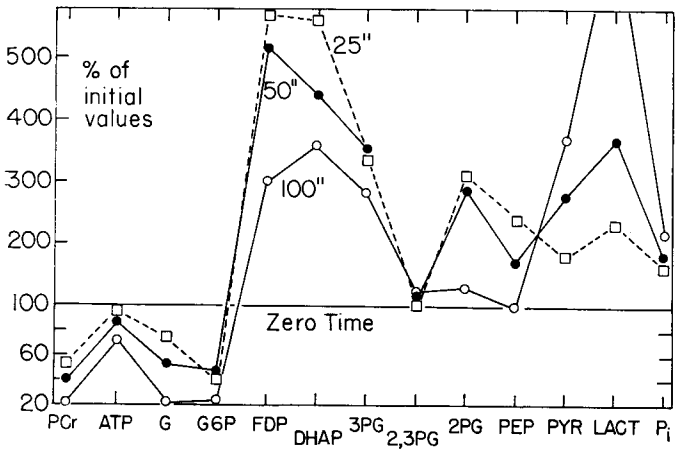


FIG. 9. Continuation of Fig. 8 covering the interval from 25 to 100 seconds after decapitation. Values at each time interval are averages for four mice. The abbreviations used are the same as in Fig. 8.

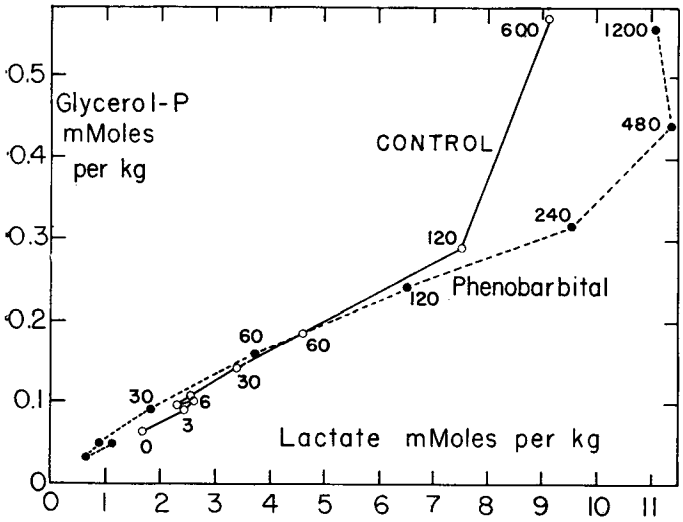


FIG. 10. Relationship between changes in glycero-P and lactate in brains of 10-day-old mice after decapitation. The nonanesthetized (control) mice are the same as those of Fig. 2; the anesthetized animals are Group A of Fig. 3. The *numbers* refer to time in seconds after decapitation.

The coenzyme 2,3-diphosphoglycerate did not increase after decapitation; in fact, there was a small but statistically significant decrease during the first few seconds (Fig. 8). It may be noted that at zero time (anesthetized animals, Table III) this coenzyme is higher than either the substrate or product of the reaction it serves. The results would seem to rule it out as a control factor under these circumstances.

The changes in ADP and AMP (Figs. 11 and 12) are consistent with those in ATP, but are presumably more accurate measures of ATP breakdown in the early stages when absolute changes are small. Initial values were almost the same in anesthetized and unanesthetized young animals. The initial figures for the adult group may be erroneously high, in part because of the less satisfactory manner of preparing original extracts (see "Experimental Procedure") and in part because of the longer time required to freeze the heads of adults. The expression (ATP)(AMP)/(ADP)^2 averaged about 0.6 in all groups throughout, except that it fell somewhat in the adult group after the first 6 seconds (Table IV). (The value would be raised slightly if correction were made for GDP, which is partially included in the ADP values.) The value 0.6 is within the range of values reported for the equilibrium constant (37).

The initial values for total adenylate were almost identical in all groups (Table IV). After 2 minutes, this total decreased in all groups, as has been previously described in brain (10), and as is well known to occcur in other tissues whenever AMP accumulates. The values for the expression (ATP) (Cr)/(ADP) (P-Cr) are much lower (Table IV) than would be predicted from the data of Nihei, Noda, and Morales (38) for equilibrium at a pH near 7. During ischemia there occurs in all groups a rise in the calculated equilibrium value as would be expected if the pH fell.

Changes in P_i values agree within reasonable limits with the changes calculated from decreases in P-creatine, ATP, and ADP except that after prolonged ischemia an excess of P_i was observed (Fig. 13). Too much significance should not be attached to this until the possibility of contamination with bone is ruled out. With these small mouse brains, great difficulty was encountered in avoiding minute fragments of bone.

The pyridine nucleotides were measured in 28 brain samples selected from the different groups. The analyses were made on frozen-dried brain powder after a period of storage at −20°,

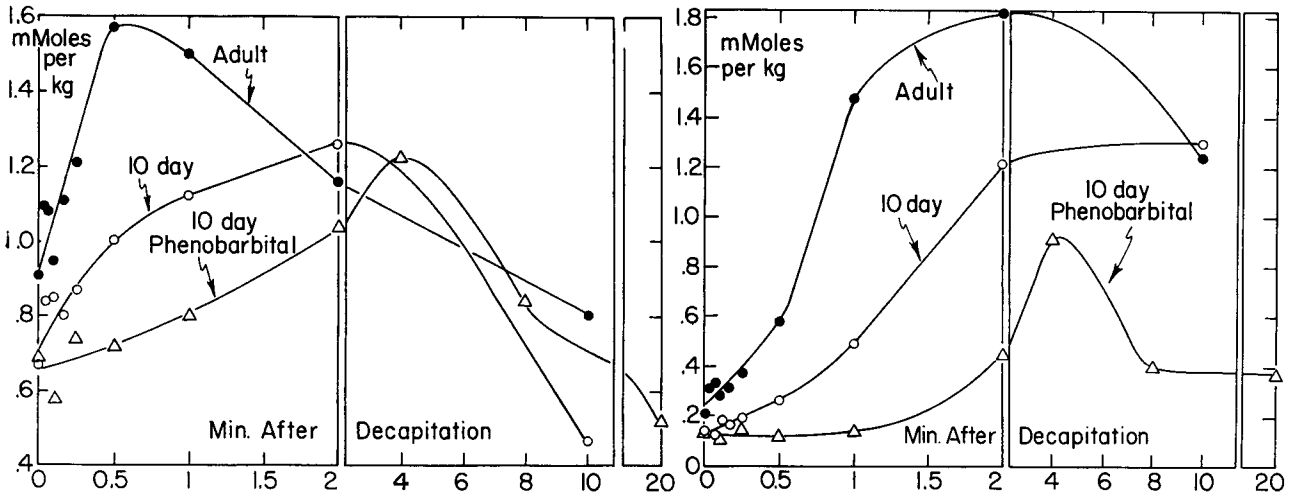


FIG. 11 (*left*). ADP concentrations in brain after decapitation. The mice are those of Figs. 1 and 2 and Group A of Fig. 3.
FIG. 12 (*right*). AMP concentrations in brain after decapitation. The same mice are represented as in Fig. 11.

and there is reason to suspect that there had been some oxidation of DPNH. Consequently the data are only provisional. The DPN^+:DPNH ratios fell progressively, and the rate of change was slower in the anesthetized animals. In the anesthetized Group A, the respective values at 0, 0.5 to 2, and 8 minutes post mortem were: for DPNH, 29, 45, and 100 μmoles per kg, and, for DPN^+, 332, 350, and 290 μmoles per kg. There was very little effect of ischemia on TPN^+:TPNH ratios, which averaged about 0.6, but total TPN fell 20% in 10 minutes.

Substrate Levels at Zero Time and during Peak Glycolysis (Table III)—Since freezing of even young mouse brains takes a number of seconds, values obtained at "zero time" are subject to possible error, and need to be evaluated in terms of the rapidity of change after decapitation. The anesthetized and refrigerated groups of animals seem least liable to this source of error. To judge from the rates of subsequent change, there are probably no serious errors in the anesthetized groups for glycogen, glucose, glucose-6-P, pyruvate, glycerol-P, the adenylates, P-creatine, or P_i. Since, however, most of the intermediates between glucose-6-P and pyruvate changed during the first 4 seconds, initial values for these intermediates may be in error.

TABLE IV

Total adenylate and ratios among adenylates, creatine, and P-creatine

The creatine values were not determined individually, but are based on a value of 8.9 ± 0.3 (standard error) mmoles per kg for the sum of creatine plus P-creatine obtained with a dozen brains from 10-day-old mice decapitated 0 to 20 minutes before freezing. There was no post-mortem change observed in total creatine. Adenylates are recorded as millimoles per kg. Gaps in the table indicate that ATP or P-creatine was too low for reliable calculation of ratios.

Group	Time	Total adenylate	$\frac{(ATP)(AMP)}{(ADP)^2}$	$\frac{(ATP)(Cr)}{(ADP)(P\text{-}Cr)}$
Adult	0.0	3.48	0.60	6.9
	0.03	3.62	0.58	8.3
	0.07	3.67	0.64	9.1
	0.10	3.46	0.69	10.8
	0.17	3.27	0.46	8.0
	0.25	3.40	0.46	13
	0.50	3.62	0.34	14
	1.0	3.49	0.33	
	2.0	3.20		
	10.0	2.16		
10-day controls	0	3.53	0.85	7.3
	0.05	3.66	0.46	7.2
	0.10	3.55	0.63	8.0
	0.17	3.38	0.60	9.0
	0.25	3.49	0.61	9.2
	0.50	3.45	0.57	12
	1.0	3.23	0.63	16
	2.0	3.50	0.41	15
	10.0	1.82		
10-day phenobarbital	0	3.48	0.73	4.3
	0.10	3.63	0.87	4.7
	0.25	3.52	0.68	5.0
	0.50	3.42	0.60	9.2
	1.0	3.69	0.53	14
	2.0	3.82	0.97	22
	4.0	2.76	0.39	20
	8.0	1.51		
	20.0	0.68		
Refrigerated	0	3.44	0.82	3.9

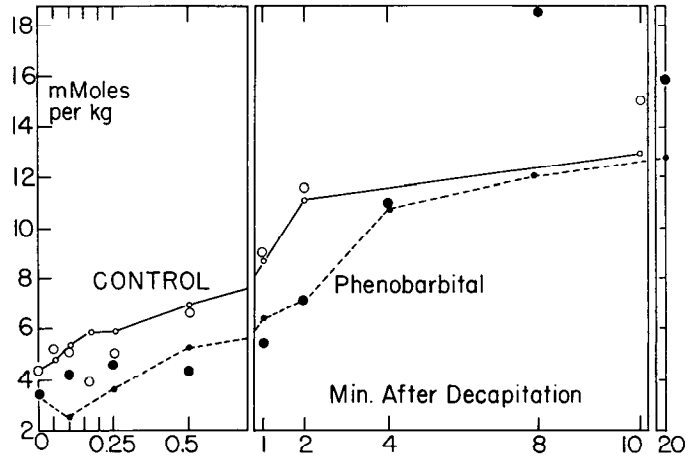


FIG. 13. P_i concentrations in brain after decapitation. The mice are those of Fig. 2 (control) and Fig. 3 (anesthetized Group A). The *large symbols* represent observed P_i values; the *small symbols (connected)* are calculated from the initial P_i values and the subsequent changes in P-creatine, ATP, and ADP.

In comparing the young anesthetized and control animals, there is probably no significant difference in true initial levels of glycogen or any of the adenylates. (The latter agrees with Minard and Davis (1), who have attributed earlier reports that anesthesia raises P-creatine and ATP to artifacts in preparing brain of nonanesthetized animals for analysis.) All of the other compounds except glucose could also conceivably have been the same in both groups *in vivo*. The difference in glucose levels must be real, since the sum of lactate (divided by 2) plus glucose is nearly double in the anesthetized group. This effect of phenobarbital is not the result of differences in plasma glucose levels. Brain glucose concentration is proportional to plasma glucose concentration with ratios of 0.14 and 0.37, respectively, in control and heavily anesthetized adult mice.[5] In the 10-day-old anesthetized mice (Group B), the ratio of initial brain glucose to plasma glucose was found to be 0.6.

DISCUSSION

Shutting off the blood supply converts the brain into a closed biological system, and at the same time limits the chemical events to those which can occur anaerobically. These advantages compensate for the rather cumbersome technical problems arising from the fact that each biological sample comes from a different animal.

By measuring nearly all components of the glycolytic system, three control points become evident: these are the respective phosphorylation steps for glucose, glycogen, and fructose-6-P. The control of glycogen breakdown is most clearly shown in the anesthetized young animal group in which no glycogen disappeared for 2 minutes even though glucose had fallen to one-third of the initial value. Presumably the control was exercised at the phosphorylase step; however, since glucose-1-P was not measured, this is only an assumption. Dr. Bruce Breckenridge kindly measured the state of phosphorylase in these anesthetized animals and found that within 1 minute after decapitation

most of the phosphorylase *b* had been converted to *a*. Consequently, this conversion is not sufficient in itself to cause glycogenolysis. Nor do changes in P_i levels (Fig. 11) seem to offer sufficient explanation, since in the control mice glycogen had fallen substantially with P_i in the range of 5 to 7 mM, but in the anesthetized animals glycogen did not fall until P_i rose above 7mM. Possibly the level of glucose-1-P is also a factor. It was probably higher in the anesthetized than in the control mice, since glucose-6-P levels were much higher in the latter.

That phosphorylation of glucose is a control mechanism cannot be established from the data presented, but can be inferred from the fact that glucose and ATP in brain at zero time are far from being in equilibrium with ADP and glucose-6-P and yet the rate of glucose phosphorylation can be increased 6- to 10-fold by ischemia (from a diminishing glucose store). These facts do not distinguish between hexokinase inhibition and inaccessibility of glucose to enzyme as the control mechanism. Both mechanisms appear to be available in skeletal muscle (39) and heart (40, 41). The initial levels of glucose-6-P do not appear to be high enough to produce the degree of hexokinase inhibition demanded (42).

A facilitation of the P-fructokinase step as the result of ischemia is clearly shown in all groups by the changes in fructose-6-P and fructose diphosphate levels. Cori in 1942 (43) concluded from earlier data on these two substrates that phosphorylation of fructose-6-P is a limiting step in glycolysis in muscle. It seems likewise to be a control point in liver fluke (44), ascites tumor cells (31, 45), yeast (46), heart (47), and diaphragm (48). Lynen and Koenigsberger (49) and Chance and Hess (50) suggested that control at this point may be exercised by inaccessibility of ATP to P-fructokinase. However, the kinetic properties of P-fructokinase are such as to permit almost complete inactivity of the enzyme in the presence of high local levels of ATP. Lardy and Parks found that ATP is inhibitory to P-fructokinase (51), and it has been found that inhibition may be overcome by low levels of either ADP, AMP, P_i, 3′,5′-cyclic adenylic acid, or fructose diphosphate (52), A combination of these substances is particularly effective. Consequently, even very slight simultaneous changes in concentration of these materials can cause a large increase in P-fructokinase activity. The only substances in this list of deinhibitors that increased significantly in the whole mouse brains, at the time of peak glycolysis (cyclic adenylate was not measured), are P_i and fructose diphosphate (Table III). However, because of the fall in P-creatine, there must also have been local increases in ADP at least as great as those demanded by the equilibrium constant for the reaction catalyzed by creatine kinase. Thus, the local ADP concentrations must have increased by the time of peak glycolysis at least 48, 33, and 56% in the adult, 10-day, and 10-day anesthetized groups, respectively. In addition, if equilibrium is maintained among the adenylates by adenylate-kinase, the local concentration of AMP must increase proportionately much more than the local ADP concentration (since $AMP = ADP^2/ATP$, and ATP is decreasing).

These considerations lead to the following theory of glycolytic control as far as the P-fructokinase step is concerned. Whenever ATP formation does not keep up with ATP use, then ADP and P_i must increase, however slightly. Through the activity of adenylate kinase this results in an even greater percentage increase in AMP. The combination of increased P_i, ADP, and particularly AMP increases P-fructokinase activity in a somewhat autocatalytic fashion, since the products fructose diphosphate and ADP are both deinhibitors. Creatine kinase tends to damp the reaction somewhat by removing part of the ADP, but not the P_i. The activity of P-fructokinase is not completely returned to initial activity until the P_i level (and therefore the P-creatine level) has returned to the initial value.

Since all of the substrates beyond fructose-6-P increased as the glycolytic flux increased, there is no obvious sign that any step beyond P-fructokinase had been a limiting factor in glycolysis. How closely the substrate levels agree with those predicted from enzyme activities and kinetics, and from equilibrium constants, will be discussed in the companion paper (42). It may be pointed out, however, that many steps are not maintained at or even near equilibrium, if the measured values represent free concentrations in a homogeneous system. In comparison with equilibrium concentrations, fructose diphosphate, at the aldolase step, is 100- to 1000-fold too high; 2-P-glycerate, at the enolase step, is 3- to 6-fold too high; ADP (or P-pyruvate), at the pyruvate kinase step, is 100- to 200-fold too high; and pyruvate (or DPNH), at the lactic dehydrogenase step, is 200-fold too high. A discussion of these points in relation to results of other investigators working with other biological systems will be made in the companion paper.

The changes in major sources of ~P which occurred after the blood supply was cut off have been used to calculate the metabolic rates. Subject to precautions mentioned above in regard to transient changes in intermediates, this principle could be extended to the histological and cytological level. The concentrations of ATP, P-creatine, glucose, lactate, and glycogen could be measured in single cells or groups of cells from a series of animals in which the organ concerned had been made ischemic for different lengths of time. Even at the level of the whole organ, this principle has advantages in comparison with measurements of metabolism in tissue slices. This is particularly true in an active organ such as brain, which does not perform in the same manner *in vitro* and *in vivo* (28). A case in point is that of the much lower rate of ~P use by the 10-day-old than by the adult brains. This may only be due in part to a lower basal metabolic rate measurable *in vitro* in terms of oxygen consumption, since there is probably a lower proportion of active neurons in the immature brain. Greengard and McIlwain (53) found that unstimulated cortex of 8-day-old rats *in vivo* has about half the metabolic rate of the adult, but that its metabolic rate only increases 25% on electrical stimulation compared to a 60% increase in the adult. Electrical stimulation is believed to mimic the status *in vivo*.

SUMMARY

Adult, 10-day-old, and anesthetized 10-day-old mice were decapitated and frozen after intervals of 0 to 20 minutes. The brains were analyzed for all known substrates and cofactors of the Embden-Meyerhof pathway from glucose to lactate. Values are presented for all of these except 1,3-diphosphoglycerate, which was present at a concentration of less than 1 μmole per kg, below the sensitivity of the method used. In addition, the concentrations of glycogen, phosphocreatine, creatine, adenosine 5′-phosphate, α-glycerophosphate, and triphosphopyridine nucleotide and its reduced form were measured.

From the initial rates of change of adenosine triphosphate, phosphocreatine, glucose, glycogen, and lactate, metabolic rates were calculated (in terms of high energy phosphate) to be 25,

13, and 7 mmoles per kg per minute, respectively, for the adult, 10-day, and anesthetized groups. Decapitation, *i.e.* ischemia, resulted in calculated increases in glycolytic rates of not less than 4- to 7-fold. The onset of these increased rates was characterized in all groups by decreases in glucose, glucose 6-phosphate, and fructose 6-phosphate, and by increases of all substrates from fructose diphosphate to lactate. The changes observed are interpreted as resulting from facilitation of the phosphorylation of glucose and fructose 6-phosphate. The facilitation of the phosphofructokinase step is attributed to increases in inorganic orthophosphate, adenosine 5′-phosphate, and adenosine diphosphate (and later fructose diphosphate), which are capable of overcoming adenosine triphosphate inhibition of this enzyme.

REFERENCES

1. Minard, F. N., and Davis, R. V., *J. Biol. Chem.*, **237**, 1283 (1962).
2. Seraydarian, K., Mommaerts, W. F. H. M., Wallner, A., and Guillory, R. J., *J. Biol. Chem.*, **236**, 2071 (1961).
3. Lowry, O. H., Passonneau, J. V., Schulz, D. W., and Rock, M. K., *J. Biol. Chem.*, **236**, 2746 (1961).
4. Greengard, P., *Nature*, **178**, 632 (1956).
5. Seraydarian, K., Mommaerts, W. F. H. M., and Wallner, A., *J. Biol. Chem.*, **235**, 2191 (1960).
6. Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **193**, **481** (1951).
7. H. V. Bergmeyer (Editor), *Methoden der enzymatische Analyze*, Verlag Chemie, GmbH, Weinheim, Bergstrasse, 1962.
8. Ochoa, S., Salles, J. B. V., and Ortiz, P. J., *J. Biol. Chem.*, **187**, 863 (1950).
9. Ochoa, S., Mehler, A. H., and Kornberg, A., *J. Biol. Chem.*, **174**, 979 (1948).
10. Thorn, W., Pfleiderer, G., Frowein, R. A., and Ross, I., *Arch. ges Physiol., Pflüger's*, **261**, 334 (1955).
11. Slater, E. C., *Biochem. J.*, **53**, 157 (1953).
12. Bublitz, C., and Kennedy, E. P., *J. Biol. Chem.*, **211**, 951 (1954).
13. Slein, M. W., Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **186**, 763 (1950).
14. Slein, M. W., *J. Biol. Chem.*, **186**, 753 (1950).
15. Greengard, P., *Bull. Photoelec. Spectrophotometry Group*, **11**, 292 (1958).
16. Segal, H. L., and Boyer, P. D., *J. Biol. Chem.*, **204**, 265 (1953).
17. Joyce, B. K., and Grisolia, S., *J. Biol. Chem.*, **233**, 350 (1958).
18. Sutherland, E. W., Posternak, T., and Cori, C. F., *J. Biol. Chem.*, **181**, 153 (1949).
19. Pizer, L. I., *J. Biol. Chem.*, **235**, 895 (1960).
20. Rodwell, V. W., Towne, J. C., and Grisolia, S., *J. Biol. Chem.*, **228**, 875 (1957).
21. Rapoport, S., and Luebering, J., *J. Biol. Chem.*, **189**, 683 (1951).
22. Meister, A., *J. Biol. Chem.*, **184**, 117 (1950).
23. Kornberg, A., in W. D. McElroy and B. Glass (Editors), *Symposium on phosphorus metabolism, Vol. I*, The John Hopkins Press, Baltimore, 1951, p. 392.
24. Strominger, J. L., *Biochim. et Biophys. Acta*, **16**, 616 (1955).
25. Hohorst, H. J., Kreutz, F. H., and Buecher, Th., *Biochem. Z.*, **332**, 18 (1959).
26. Kerr, S. E., and Ghantus, M., *J. Biol. Chem.*, **117**, 217 (1937).
27. Burch, H. B., Lowry, O. H., and Von Dippe, P., *J. Biol. Chem.*, **238**, 2838 (1963).
28. McIlwain, H., *Biochemistry and the central nervous system*, Little, Brown and Company, Boston, 1955.
29. Doring, H. J., Knoff, A., and Martin, T., *Arch. ges. Physiol., Pflüger's*, **269**, 375 (1959).
30. Hunter, F. E., in W. D. McElroy and B. Glass (Editors), *Symposium on phosphorus metabolism, Vol. I*, The Johns Hopkins Press, Baltimore, 1951, p. 297.
31. Lonberg-Holm, K., *Biochim. et Biophys. Acta*, **35**, 464 (1959).
32. Ibsen, K. H., Coe, E. L., and McKee, R. W., *Biochim. et Biophys. Acta*, **30**, 384 (1958).
33. Wu, R., and Racker, E., *J. Biol. Chem.*, **234**, 1036 (1959).
34. Hess, B., and Chance, B., *J. Biol. Chem.*, **236**, 239 (1961).
35. Kahana, S. E., Lowry, O. H., Schulz, D. W., Passonneau, J. V., and Crawford, E. J., *J. Biol. Chem.*, **235**, 2178 (1960).
36. Thorn, W., Scholl, H., Pfleiderer, G., and Mueldener, B., *J. Neurochem.*, **2**, 150 (1958).
37. Atkinson, M. R., Burton, R. M., and Morton, R. K., *Biochem. J.*, **78**, 813 (1961).
38. Nihei, T., Noda, L., and Morales, M. F., *J. Biol. Chem.*, **236**, 3203 (1961).
39. Kipnis, D. M., Helmreich, E., and Cori, C. F., *J. Biol. Chem.*, **234**, 165 (1959).
40. Randle, P. J., and Smith, G. H., *Biochem. J.*, **70**, 490, 501 (1958).
41. Morgan, H. E., Henderson, M. J., Regen, D. M., and Park, C. R., *J. Biol. Chem.*, **236**, 253 (1961).
42. Lowry, O. H., and Passonneau, J. V., *J. Biol. Chem.*, **239**, 31 (1964).
43. Cori, C. F., *A symposium on respiratory enzymes*, University of Wisconsin Press, Madison, 1942, p. 175.
44. Bueding, E., and Mansour, J. M., *Brit. J. Pharmacol.*, **12**, 159 (1957).
45. Hess, B., *Funktionelle und Morphologische Organisation der Zelle*, Springer-Verlag, Berlin, 1963, p. 163.
46. Lynen, F., Hartmann, G., Netter, K. F., and Schuegraf, A., in G. E. W. Wolstenholme and C. M. O'Connor (Editors), *Ciba Foundation symposium on the regulation of cell metabolism*, Little, Brown and Company, Boston, 1959, p. 256.
47. Park, C. R., Morgan, H. E., Henderson, M. J., Regen, D. M., Cadenas, E., and Post, R. L., in G. Pincus (Editor), *Recent progress in hormone research, Vol. 7*, Academic Press, Inc., New York, 1961, p. 493.
48. Newsholme, E. A., and Randle, P. J., *Biochem J.*, **80**, 655 (1961).
49. Lynen, F., and Koenigsberger, R., *Ann. Chem. Liebigs*, **573**, 60 (1951).
50. Chance, B., and Hess, B., *Science*, **129**, 700 (1959).
51. Lardy, H. A., and Parks, R. E. Jr., in O. H. Gaebler, (Editor) *Enzymes: units of biological structure and function*, Academic Press, Inc., New York, 1956, p. 584.
52. Passonneau, J. V. and Lowry, O H., *Biochem. and Biophys. Research Communs.*, **7**, 10 (1962).
53. Greengard, P., and McIlwain, H., in H. Waelsch (Editor), *Biochemistry of the developing nervous system*, Academic Press, Inc., New York, 1955, p. 251.