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Effects of Changes in Brain Metabolism on the Levels of Citric Acid Cycle Intermediates*

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SUMMARY

The levels of all the free intermediates of the Krebs cycle except succinyl coenzyme A were measured in cerebral tissue of mice after periods of ischemia; anesthesia by Amytal, phenobarbital, and ether; hyperthermia; fluoroacetate poisoning; and insulin hypoglycemia. The levels of glucose, glucose 6-phosphate, pyruvate, phosphocreatine, adenosine triphosphate, adenosine diphosphate, and adenylic acid were also determined.

During 5 sec of complete ischemia (decapitation) the levels of citrate, α-ketoglutarate, and oxalacetate fell 15 to 60% whereas the concentrations of succinate and fumarate rose 40% over a 30-sec interval. These changes presumably represent the conversion of a steady state system into an equilibrium system. Succinate accumulation is postulated to result from pyruvate carboxylation and fumarate reduction rather than from α-ketoglutarate oxidation.

Decreasing the metabolic flux with anesthetic agents resulted in each case in marked decreases in α-ketoglutarate, fumarate, and malate, whereas increasing the flux by hyperthermia resulted in increases in these same three substrates. Neither anesthesia nor hyperthemia caused significant change in the levels of citrate, isocitrate, succinate, or oxalacetate. Fluoroacetate at first increased citrate levels without much effect on other substrates. Subsequently, when citrate had increased more than 3-fold, changes occurred in levels of the other Krebs cycle members. The accompanying rise in glucose 6-phosphate and fall in pyruvate suggested that inhibition of glycolysis at the phosphofructokinase step had taken place. Insulin hypoglycemia resulted in a general lowering of the levels of all Krebs cycle intermediates, as well as of glucose 6-phosphate and pyruvate.

The changes associated with increasing, decreasing, or blocking flux in the Krebs cycle by the various procedures are used to assess the kinetic behavior of individual steps and to attempt to identify control points.

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This is a report of the changes in tissue levels of citric acid cycle intermediates when metabolic activity of the brain is altered by anesthesia, hyperthermia, fluoroacetate poisoning, ischemia, and insulin hypoglycemia. The results are believed to aid in locating control points in the Krebs cycle and in establishing regulatory interrelationships between this oxidative cycle and the Embden-Meyerhof glycolytic pathway.

All recognized free intermediates in the Krebs cycle except succinyl coenzyme A were measured, and the analytical methods are described in detail.

EXPERIMENTAL PROCEDURE

Preparation of Animals

Adult, male, Swiss-Webster mice weighing 17 to 22 g were used after fasting overnight. All of the experiments were carried out during a single 10-hour experimental period with animals selected at random from one homogeneous group. After the desired treatment the whole animal, or the severed head in the case of the ischemia study, was rapidly frozen in Freon 12 (CH_2F_2) cooled to −150° with liquid nitrogen. This material was stored at −80° until it could be prepared for analysis.

Anesthetics—Amytal (135 mg per kg) and phenobarbital (225 mg per kg), as their sodium salts, were administered by intraperitoneal injection in a volume of 0.9% NaCl that did not exceed 0.4 ml. Ether anesthesia was produced by maintaining an ether concentration of 4.5% (by volume) in a closed 4-liter container. Anesthesia with each agent was maintained for exactly 60 min after loss of the righting reflex before freezing the animal.

Insulin—A dose of 125 units per kg was administered intraperitoneally in 0.02% bovine serum albumin solution. The animals were maintained in a cage warmed by a 60-watt bulb overhead and sacrificed at the first sign of convulsions, which ensued approximately 30 min after injection.

Hyperthermia—Animals were individually restrained in wire holders and placed in a chamber warmed to 48–50° by a heating pad. When body temperature reached 43–44°, measured rectally with a thermocouple, the animals were plunged into the freezing medium.

Fluoroacetate—Animals were frozen 10, 20, or 40 min after intraperitoneal injection of 100 mg per kg of sodium monofluoroacetate. These times after injection corresponded roughly to preconvulsive, early convulsive, and convulsive periods.

Ischemia—Mice were decapitated, and after intervals of 5, 10, and 30 sec the heads were frozen.

Preparation of Brain Tissue Extracts

The frozen forebrains were dissected, powdered, and weighed in a room maintained at −20°. The frozen powder, 200 to 250 mg, was placed on top of 600 μl of 3.0 M $HClO_4$ frozen on Dry Ice in an 8-ml test tube. The tube was placed in an ethanol-Dry Ice bath maintained at −12° and agitated to thaw the $HClO_4$ and extract the water. After the temperature was raised to 0–5°, each sample was diluted with 2 ml of cold H_2O and centrifuged. One portion of the supernatant fluid was brought to pH 6 with a predetermined volume of 2 N $KHCO_3$. A second portion was treated with Florisil (Floridin Company) and then neutralized in the same manner (see below). After centrifugation, the supernatant fluids were stored at −80° and thawed for intervals of only 1 or 2 min when aliquots of the extracts were removed for analysis. Extracts prepared in this manner represented an approximate 1:15 dilution of brain tissue.

Analytical Methods

Fluorometry—All of the substrate analyses were made fluorometrically by measuring the appearance of TPNH or 3-acetylpyridine-DPNH, or the disappearance of DPNH after the addition of appropriate enzymes. Fluorescence of reduced pyridine nucleotides was measured in test tubes (8 × 100 mm) with a Farrand model A fluorometer modified with a tungsten lamp (1) with Corning No. 5840 as a primary filter, and Corning No. 4303 and No. 3387 as secondary filters. The turntable of the instrument was fixed as described previously (1). The measurements were made in carefully selected, unscratched tubes. The fluorescent blank of Tris and imidazole was reduced by treating solutions of the bases with charcoal.

In certain analyses of substrates present at very low concentrations the fluorescent blank contributed by the tissue extract was reduced by treating the acid extract with Florisil. The adsorbent was prepared by washing in several changes of 0.6 M $HClO_4$, rinsed acid-free with water, and dried at 50°. The fines were removed in the process. An aliquot of the extract was passed through a 2-mm internal diameter column containing 50 to 60 mg of Florisil per ml of extract. This removed over 90% of the tissue fluorescence whereas dicarboxylic and tricarboxylic acids were quantitatively recovered. Phosphorylated sugars and nucleotides, however, were partially adsorbed.

Enzymes—Beef heart lactate dehydrogenase was obtained from Worthington. All other enzymes except aconitase and succinate thiokinase were purchased from Boehringer.

The aconitase was prepared from pig heart according to Morrison (2) with the following modifications:[1] (*a*) the enzyme was extracted at pH 7.5 with 0.02 M Tris-HCl buffer; (*b*) tricarballylic acid was substituted for citrate in the solution used for the initial extraction, and in subsequent steps to dissolve enzyme precipitates; (*c*) the enzyme was finally precipitated from a 30 mM tricarballylate solution with $(NH_4)_2SO_4$ and stored either as the precipitate or in solution in 0.1 M Tris·HCl buffer, pH 7.5, containing 30 mM tricarballylate. The 20-fold purified aconitase (100 μmoles per mg of protein per hour) lost less than 50% of its activity after storage for 3 years at −80° in either case. The tricarballylate ("deoxycitrate") stabilizes aconitase and does not interfere with the determination of citrate under assay conditions.

Succinate thiokinase was purified by the procedure of Cha and Parks (3) to an activity of 50 μmoles per mg per hour.

General Procedure—Unless noted otherwise, 1 ml of a reagent having the composition indicated in Table I was placed in each fluorometer tube, the brain samples were added, and the fluorescence was read at an appropriate sensitivity. The necessary enzymes were then added in small volumes (1 to 10 μl). The tubes were read again when the reaction was complete (usually 5 to 10 half-times). Standards in volumes of 20 μl or less were added directly to reagent in the fluorometer tubes to which was also added blank solution equal to the tissue samples in volume and in $HClO_4$ and $KHCO_3$ composition.

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. This determined (*a*) whether the reaction was proceeding at the expected rate and (*b*) the proper time at which final readings should be made for the bulk of the samples. This test is critical both to ensure complete reaction and to avoid the equal danger of side reactions if the time is longer than necessary. No enzyme preparation can be regarded as absolutely free of other enzymes, consequently it is as hazardous to use too much enzyme or allow it to work too long, as it is to use too little. The time course of the reaction provides an extra measure of the specificity of the assay.

The methods employed for the analyses of glucose, glucose-6-P, pyruvate, P-creatine, ATP, ADP, and AMP have been described (1). An estimation was made of nucleoside diphosphates other than ADP, based on the differential rates of reaction of pyruvate kinase with these compounds (5). From the relative velocities it appears that GDP comprises most of the nucleoside diphosphate in brain other than ADP. The fluorometric methods described for citrate, isocitrate, α-ketoglutarate, and oxalacetate are modifications of the spectrophotometric methods of Ochoa (6), Strecker (7), and Mehler *et al.* (8), respectively. The determination of malate and fumarate is based on a method described by Fleming and LaCourt (9). Analytical details not covered in Table I are as follows.

Glucose-6-P and Citrate—These were measured in the same sample because the aconitase preparation was contaminated with glucose-6-P dehydrogenase. The procedure is essentially that described earlier (1) except for a change in pH from 8.0 to 8.5 to favor measurement of citrate. After the glucose-6-P reaction was complete, isocitrate dehydrogenase was added, and a second reading was taken after allowing time for isocitrate oxidation. Aconitase was then introduced. The aconitase, as a 12.5 mg per ml solution in 30 mM tricarballylate, was activated with 10 mM cysteine and 0.5 mM $Fe(NH_4)_2SO_4$ 30 min prior to use as described by Dickman and Cloutier (10). Mn^{++} ion in excess of 0.1 mM inactivates aconitase, apparently by displacing the Fe^{++} from the enzyme. Florisil-treated extracts also interfered with aconitase activity, and such extracts are not recommended for this assay. The pH optimum of 8.5 determined in the isocitrate dehydrogenase-coupled system is higher than that of 7.4 previously reported for aconitase (11). The apparent K_m for citrate under these conditions is 1.3×10^{-5} M, which is 100 times smaller than that reported by Racker (12). The method used for the determination of citrate would include the small (5%) equilibrium proportion of *cis*-aconitate.

d-Isocitrate—It is possible to determine isocitrate in the pre-

[1] A detailed procedure of the purification, stabilization, and kinetics of aconitase will be published separately.

TABLE I

Analytical conditions

Analyses were conducted in most cases with 1 ml of reagent of the composition shown with the addition of neutralized $HClO_4$ extract equivalent to the amount of brain indicated (wet weight). In the case of oxalacetate, 600 μl of the given reagent were used with the addition of 400 μl of sample. α-Ketoglutarate and pyruvate were measured in this same sample. For the case of succinate see the text.

Substance	Reagent		Brain	Enzymes[b]	Half-time	Incubation
	Buffer	Other additions[a]				
			mg		*min*	
Glucose-6-P	Tris, 50 mM, pH 8.5	TPN^+, 0.05 mM	1–2	Yeast glucose-6-P dehydrogenase, 0.25 μg/ml	2	20
Citrate	Same	$MnSO_4$, 0.05 mM[c]	1–2	Same plus heart aconitase, 25 μg/ml and heart isocitrate dehydrogenase, 2.0 μg/ml	1½	20
Isocitrate	Tris, 50 mM, pH 8.1	$MnCl_2$, 0.1 mM; TPN^+, 0.05 mM	7[d]	Heart isocitrate dehydrogenase, 1 μg/ml	2	20
Oxalacetate	P_i, 200 mM, pH 6.8	NH_4 acetate, 400 mM; EDTA, 0.1 mM; DPNH, 0.0005 mM[e]	25[d]	Heart malate dehydrogenase, 0.3 μg/ml	½	10
α-Ketoglutarate	Same	Same plus ADP, 0.1 mM; DPNH, 0.01 mM	Same sample	Same plus liver glutamate dehydrogenase, 120 μg/ml	1	10
Pyruvate	Same	Same	Same sample	Same plus heart lactate dehydrogenase, 0.5 μg/ml	1	6
Succinate						
Step 1	Imidazole, 250 mM, pH 7.5	P-pyruvate, 15 mM; $MgCl_2$, 15 mM; KCl, 75 mM; DPNH, 0.6 mM; GTP, 2.0 mM	8	Heart lactate dehydrogenase, 15 μg/ml; muscle pyruvate kinase, 0.5 μg/ml[f]	1	15
Step 2	Imidazole, 150 mM, pH 7.5	CoA, 0.1 mM		Muscle pyruvate kinase, 10 μg/ml; succinate thiokinase, 240 μg/ml[f]	6	45
Step 3	Imidazole, 200 mM, pH 7.5	DPNH, 0.002 mM		Heart lactate dehydrogenase, 0.25 μg/ml	2	20
Malate	Hydrazine, 100 mM, pH 9.1	EDTA, 0.2 mM; acetyl-DPN^+, 0.06 mM	3.5[d]	Heart malate dehydrogenase, 4.0 μg/ml	1½	20
Fumarate	Same	Same	Same sample	Heart fumarase, 2.0 μg/ml	3	20

[a] Concentrations in the reagent are given. Except in the cases of oxalacetate and succinate, these are approximately the final concentrations. Unless noted, additions were made to the bulk reagent before transfer to fluorometer tube.

[b] Except for the two cases noted, the final enzyme concentration in the fluorometer tube is given.

[c] The $MnSO_4$ can equally well be incorporated in the glucose-6-P reagent. $MnCl_2$ and $MnSO_4$ are equally satisfactory for this and the isocitrate reagent.

[d] Florisil-treated extract.

[e] Added from stock DPNH (2 to 5 mM) prepared in carbonate buffer (0.15 M Na_2CO_3, 0.05 M $NaHCO_3$) and heated 20 min at 60° before use to destroy any DPN^+ present (4).

[f] These enzyme concentrations refer to the levels in the small volumes of reagent added (30 μl for Step 1, 60 μl for Step 2).

ceding analysis, from the TPNH increment after adding isocitric dehydrogenase. However, since tissue levels of *d*-isocitrate are very low, a separate sample was used, and the extract was treated with Florisil. The isocitrate dehydrogenase preparation that was used was free of interfering enzymes, but other lots were found to contain glucose-6-P dehydrogenase in substantial amounts.

Oxalacetate, α-Ketoglutarate, and Pyruvate—These three substrates were measured on the same sample. The acid extracts were treated with Florisil, neutralized, and assayed within 30 min of neutralization. (There is a 5% loss of oxalacetate after 30 min at pH 7.0 at 25°.) Because of the very low levels of this substance, 400 μl of extract were used with 600 μl of reagent. It was a convenience to use the same sample for α-ketoglutarate and pyruvate determination, but to keep the blank fluorescence as low as possible the ADP and the full amount of DPNH were not added until after the oxalacetate reaction was complete. α-Ketoglutarate was measured second with glutamic dehydrogenase. In spite of the fact that glutamate was present at levels 100-fold higher than those of α-ketoglutarate, the reaction went at least 95% to completion because of the high concentration of added NH_4^+, and because DPN^+ had been removed from the tissue extract with Florisil and from the DPNH stock solutions by alkaline treatment (4).

After the glutamic dehydrogenase reaction was complete, pyruvate was measured with lactic dehydrogenase. The time and enzyme were kept to a minimum to avoid erroneously high results (1).

Succinate—The analytical enzyme sequence is:

$$\text{Succinate} + \text{GTP} + \text{CoA} \rightarrow \text{succinyl-CoA} + \text{GDP} + P_i \quad (1)$$

$$\text{GDP} + \text{P-pyruvate} \rightarrow \text{GTP} + \text{pyruvate} \quad (2)$$

$$\text{Pyruvate} + \text{DPNH} \rightarrow \text{lactate} + DPN^+ \quad (3)$$

Special procedures were necessary because of various interfering substances present: (*a*) nucleoside diphosphates in the tissue and in commercial preparations of GTP react with the pyruvate kinase; (*b*) there are endogenous lactic dehydrogenase-reacting

TABLE II

P-creatine and free nucleotide levels in mouse brain following various treatments

Values are recorded as micromoles per kg, wet weight, ± the standard errors.

Treatment	No. of animals	P-creatine	ATP	ADP	"N"DP[a]	AMP	Total adenylate	Total high energy phosphate
Controls	10	3,630 ± 90	2,950 ± 40	331 ± 10	212 ± 5	50 ± 6	3,330	10,070
Decapitation, 5 sec	6	2,210 ± 40	2,660 ± 40	469 ± 45	216 ± 15			8,220
Decapitation, 10 sec	4	1,830 ± 40	2,640 ± 30	491 ± 15	230 ± 24			7,830
Decapitation, 30 sec	5	640 ± 40	1,670 ± 130	653 ± 19	301 ± 9			4,930
Phenobarbital	6	5,080 ± 180	3,120 ± 70	260 ± 53	189 ± 14	32 ± 4	3,500	11,950
Amytal	4	4,870 ± 70	3,200 ± 80	203 ± 13	191 ± 6	31 ± 3	3,430	11,660
Ether	6	4,420 ± 90	3,190 ± 60	218 ± 4	174 ± 5	33 ± 2	3,440	11,190
Hyperthermia	5	3,220 ± 140	2,770 ± 60	375 ± 13	182 ± 11	129 ± 11	3,280	9,320
Insulin	6	3,370 ± 70	2,610 ± 80	401 ± 17	194 ± 5	97 ± 9	3,110	9,180
Fluoroacetate, 10 min	5	3,480 ± 120	2,860 ± 10	416 ± 11	161 ± 10	76 ± 8	3,350	9,780
Fluoroacetate, 20 min	6	3,620 ± 210	2,910 ± 100	396 ± 29	184 ± 16	57 ± 5	3,370	10,020
Fluoroacetate, 40 min	4	4,040 ± 180	3,040 ± 180	390 ± 20	177 ± 14	67 ± 12	3,500	10,690

[a] "N"DP indicates nucleotide diphosphate other than ADP capable of reacting with P-pyruvate in the presence of pyruvate kinase (see "Analytical Methods").

materials in tissue extracts; and (*c*) the succinic thiokinase used was contaminated with DPNH-oxidizing activity. To circumvent these problems, the assay was conducted in three steps. First, the pyruvate kinase and lactic dehydrogenase-reacting materials in the tissue extracts were removed. In test tubes (6 × 75 mm), 120 μl of extract were incubated for 15 min at room temperature with 30 μl of the reagent specified in Step 1 of Table I (including the two enzymes). The enzymes and unreacted DPNH were destroyed by adding 5 μl of 5 N HCl. To each sample, 12 mg of acid-washed Florisil were added, and the contents of the tubes were mixed and centrifuged. Although the Florisil treatment was carried out at 0°, the temperature is presumably not critical.

In the second step, 40 μl of the supernatant fluid from Step 1 were incubated with 60 μl of Step 2 reagent of Table I (enzymes included) in a fluorometer tube. The aliquot from the first step contained sufficient GTP, P-pyruvate, and K^+ to give desired concentrations. The reaction was complete after 45 min at 25°, and 7.5 μl of 5 N HCl were then added to destroy DPNH-oxidizing activity in the succinic thiokinase preparation. Within 10 min 1 ml of Step 3 reagent (Table I) was added (*lactic dehydrogenase omitted*). After an initial reading lactic dehydrogenase was added, and the pyruvate formed in the second step was measured.

The procedure was assessed for the presence of interfering substances or reactions. There was no detectable hydrolysis of succinyl-CoA either during the second step or during the brief subsequent exposure to acid. Since ATP was present in the tissue extracts, either adenylate kinase or nucleoside diphosphokinase, if present at Step 2, would be expected to interfere. Difficulty on this score was not encountered.

The levels of succinyl-CoA in brain must be very low (less than 2 or 3% of the succinate levels) since alkaline pretreatment of tissues (which completely hydrolyzed added succinyl-CoA) produced no measurable increment in tissue succinate.

Malate and Fumarate—These two intermediates were measured sequentially in the same sample. Malate was first oxidized to oxalacetate with acetyl-DPN and malic dehydrogenase after which fumarate was converted to malate with fumarase. Florisil-treated extracts were used. The conditions of the reaction were chosen to circumvent the unfavorable equilibrium of the malic dehydrogenase system. The pH was made alkaline to shift the equilibrium in the favorable direction; acetyl-DPN^+, because of its higher oxidation potential, was substituted for DPN^+; and hydrazine was used to trap the oxalacetate. The acetyl-DPN^+ was added to the reagent just before use since the fluorescent blank increases with time and 3-acetylpyridine-DPN^+ is unstable in the reagent.

RESULTS

High Energy Phosphate—Before presenting the findings in regard to carbohydrate intermediates, it is desirable to describe the effects of the experimental procedures on the high energy phosphate stores in the brain (Table II). Except in the case of complete ischemia, none of the procedures caused serious depletion of P-creatine or ATP. Small increases in total high energy phosphate were observed as expected in anesthesia, and small significant decreases were found in hyperthermia and during insulin hypoglycemia. The most sensitive index of these changes is the AMP level (Table II). Since even a mouse brain does not freeze rapidly enough to prevent some anoxic effects, the observed differences in high energy phosphate with anesthesia and hyperthermia could be at least partially due to the differences in metabolic rates (*cf.* Minard and Davis (13)).

Control Tissue Levels—The control levels of Krebs cycle intermediates were almost identical for mouse (Fig. 1) and rat (not shown). These levels differ markedly from the published values of Frohman, Orten, and Smith for rat brain (14) except in the cases of citrate and isocitrate. The values shown here are about twice as high for succinate and malate, $\frac{1}{10}$ as high for fumarate and α-ketoglutarate, and less than $\frac{1}{100}$ as high for oxalacetate. The minimal turnover time for a metabolite in a system in steady state must be its concentration divided by the flux. With the assumption that the brain normally oxidizes about 250 μmoles of glucose per min (500 μmoles of triose) through the Krebs cycle, the turnover time for the intermediates would vary from 1.3 min for succinate to 0.5 sec for oxalacetate.

Ischemia—Within 5 sec after the blood supply is cut off by decapitation, 20% of the brain glucose has disappeared, and by 30 sec only 9% remains (Fig. 1). This rapid use of glucose is associated with a prompt fall in glucose-6-P and rise in pyruvate. Concomitantly with the appearance of these signs of increased

glycolysis there occur changes in the intermediates of the Krebs cycle which are presumably the result of complete block of this oxidative system. The changes consist of rapid decreases in citrate, α-ketoglutarate, and oxalacetate and delayed increases in succinate, fumarate, and probably malate. Isocitrate did not change. Since decreased oxidation must have preceded increased glycolysis, it is of interest that α-ketoglutarate and oxalacetate both fell maximally before the maximal changes in glucose-6-P and pyruvate had taken place.

The changes may be interpreted as due to transition from a steady state of flux to a static state with equilibration wherever possible. There is a shift in the ratio of citrate to isocitrate from 20:1 at "zero time" to the equilibrium ratio of 15:1 (15). α-Ketoglutarate fell with remarkable speed during ischemia (75 μmoles per kg in 5 sec). This rate may be compared to an estimated normal oxidation rate of 40 μmoles per kg per 5 sec (see above). The large fall may be due in part to equilibration by means of glutamic dehydrogenase with ammonia and DPNH, which are known to increase with anoxia (16).

Since oxalacetate fell as promptly as α-ketoglutarate and by a comparable percentage, it may be that these keto acids equilibrate (or remain in equilibrium) with aspartate and glutamate through the mediation of transaminase. Observed initial levels of oxalacetate and α-ketoglutarate and reported levels of aspartate and glutamate (17) are approximately those demanded for equilibrium at pH 7.5 (18); aspartate and glutamate are known not to change substantially during ischemia (17). In contrast, the levels of pyruvate are such as to make it improbable that equilibrium exists between pyruvate and α-ketoglutarate via the glutamate-pyruvate transaminase system, either before or after brief ischemia.

The late increase in succinate may not be the result of α-ketoglutarate oxidation, since the brain is anoxic. It seems more likely, in accordance with the recent observations of Hoberman, Prosky, and Arfin for liver (19), that succinate arises during anaerobiosis from lactate by way of a pathway involving fumarate and the reversal of succinic dehydrogenase.

The initial ratio of fumarate to malate is 1:6, which may be compared to the equilibrium figure of 1:4 calculated for physiological conditions (20). The observed ratio may in fact represent equilibrium, since it is not affected by brief ischemia. Considering the direction of flow, if fumarate were not in equilibrium with malate, one would expect the ratio of fumarate to malate to be too high rather than too low. The ratio is known to be affected by both pH and ionic strength (20). Equilibration between fumarate and malate appears to be the usual situation in brain since in none of the experiments (Figs. 2 and 3) were fumarate to malate ratios found which differed much from 1:6.

Anesthesia and Hyperthermia—In order to uncover control points in the Krebs cycle, it seemed necessary to change the flux without blocking it completely. Changes in the metabolic rate of the brain should bring about similar changes in citrate cycle flux. Hyperthermia increases cerebral oxygen consumption (21) whereas anesthesia produces a distinct fall in brain respiration (22, 23). (Part of the decrease in metabolism may be the result of decreased body temperature, which is known to accompany anesthesia (24)).

All three anesthetic agents were found to produce similar changes in substrate levels (Fig. 2). As observed by Mayman, Gatfield, and Breckenridge (25), the anesthetics increase brain glucose beyond the level expected from the diminished glycolytic

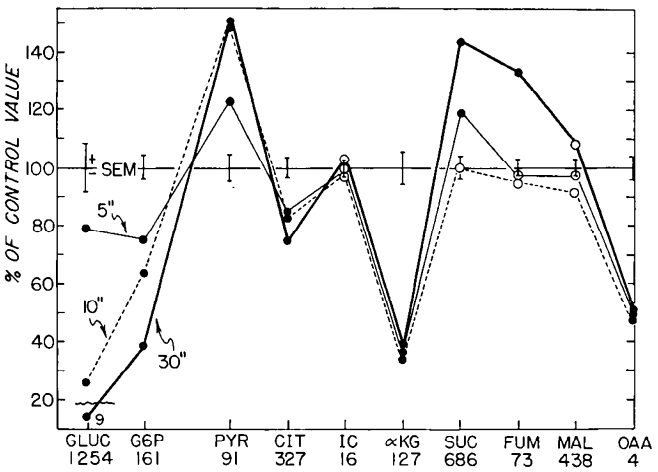

FIG. 1. Percentage changes of 10 constituents in mouse brain at 5, 10, and 30 sec after decapitation. The abbreviations are: *Gluc*, glucose; *G6P*, glucose-6-P; *Pyr*, pyruvate; *Cit*, citrate; *IC*, isocitrate; *αKG*, α-ketoglutarate; *Suc*, succinate; *Fum*, fumarate; *Mal*, malate; *OAA*, oxalacetate. The control levels are given below each substrate in micromoles per kg, wet weight. The standard error of the mean (*SEM*) is given for control values. Each value is based on averages of 10 mice for controls and 4 to 6 mice after decapitation, a total of 25 animals. The *solid symbols* indicate values significantly different from control values ($P < 0.05$).

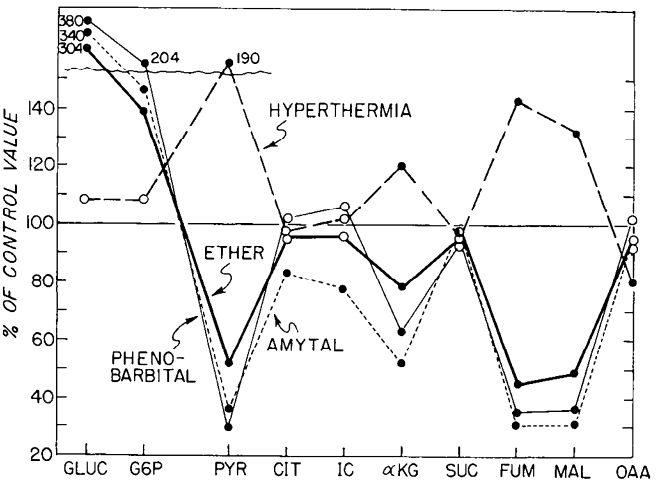

FIG. 2. Percentage changes in substrates of mouse brain following hyperthermia, or anesthesia with ether, Amytal, or phenobarbital. The conditions are given in the text. The controls are the same animals as for Fig. 1. The experimental values are based on the averages for 5 or 6 mice, a total of 32 animals. The abbreviations and symbols are the same as for Fig. 1.

flux or any possible small increases in blood glucose concentration. The decreased glycolytic flux is associated with increases in glucose-6-P and decreases in pyruvate concentration, as shown earlier in the case of phenobarbital (1).

The levels of citrate, isocitrate, succinate, and oxalacetate were in general not affected by the anesthetic agents. (Citrate and isocitrate were lowered somewhat by Amytal.) In marked contrast, there were consistent moderate decreases in α-ketoglutarate and marked decreases in fumarate and malate.

The data offer little evidence of inhibition of electron transport by the barbiturates. If this had occurred, one would expect a

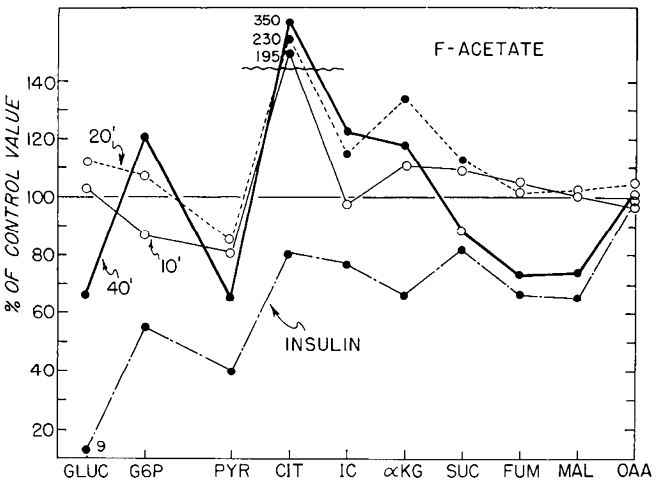

FIG. 3. Percentage changes in substrates in mouse brain at 10, 20, or 40 min after fluoroacetate administration or insulin injection as described in the text. The controls are the same animals as in Fig. 1. The experimental values are based on 4 to 6 mice, a total of 31 animals. The abbreviations and symbols are the same as for Fig. 1.

decrease in the succinate to malate ratio, whereas just the opposite took place. The ratio increased whether the anesthetic used was Amytal (the most effective inhibitor *in vitro*) or ether (no reported inhibition *in vitro* of electron transport).

In general, the substrate changes produced by hyperthermia were mirror images of those seen during anesthesia. This applies to pyruvate as well as the Krebs cycle intermediates but not to glucose and glucose-6-P. Plasma glucose levels were lowered from 6.2 mmoles per kg to 3.2 mmoles per kg by hyperthermia. Consequently the ratio of brain glucose to plasma glucose was doubled by hyperthermia in spite of the presumed greater glucose use. This is surprising in view of the fact that anesthesia also increases the brain to plasma glucose ratio (24). The failure of the glucose-6-P level to change in hyperthermia suggests that the increased intake of glucose is just balanced by increased P-fructokinase activity.

While deferring discussion of control implications of these results, it may be pointed out that between the anesthesia and hyperthermia experiments there is a 4- to 5-fold difference in malate, fumarate, and pyruvate levels. In contrast, citrate and succinate levels are relatively unaffected. Malate levels may represent a valid and convenient index of Krebs cycle flux in the oxidative state.

Fluoroacetate—The results of administration of fluoroacetate seem straightforward (Fig. 3). Although a block at the aconitase step is clearly shown by the large increases in citrate levels, it seems probable that actual flux along the citrate pathway is maintained near normal by these increases for at least 20 min. This is indicated by the fact that at 10 and 20 min citrate cycle substrates do not fall below control levels. In addition, the high energy phosphate data presented above show that oxidative phosphorylation has not been seriously compromised. By 40 min succinate, fumarate, and malate have fallen below normal, suggesting that the block has now become too great to permit normal flux.

At the end of 40 min, in spite of the presumed need for increased glycolysis, the rise in glucose-6-P and fall in pyruvate indicate diminished glycolytic flux. This is reasonably explained by inhibition of P-fructokinase by the high citrate concentration (26–28).

It is puzzling that in spite of normal ATP and P-creatine levels the poisoned mice showed evidence of neurological difficulties. There were early convulsive signs at 20 min, and at 40 min the mice appeared somewhat sedated and convulsed intermittently. Possibly certain small regions of the brain or parts of neurons are more severely affected than the whole.

Insulin Hypoglycemia—In the experiments above the citrate cycle flux was reduced either by reducing the metabolic needs (anesthesia) or blocking oxidation (ischemia), or by blocking a single step (fluoroacetate). Still another way to decrease the flux is to cut off the glucose supply with insulin (Fig. 3). In this case, in contrast to all the other experiments, the level of every intermediate measured from glucose to malate is diminished. Only the oxalacetate level is normal.

The contrast between the results with insulin and those with anesthesia should be stressed. In both cases less pyruvate than usual is converted to citrate. With anesthesia the reduced flux in the Krebs cycle is just sufficient for the needs of the tissue; the cycle is kept filled with intermediates, and a signal is sent back to shut off the input (high level of glucose-6-P). In the case of hypoglycemia, the cycle is depleted of intermediates, the needs of the cell are not met, and although a signal may be sent back to increase the input (lowered glucose-6-P level) it cannot be successful.

DISCUSSION

In the following discussion it is assumed that most of the net oxidation in brain proceeds by way of the Krebs cycle. The slowest turnover time for any member of this cycle is about 1 min (see above). Therefore, except in the case of ischemia, it can be assumed that in the experiments reported steady states exist; *i.e.* in any given case all steps in the cycle are proceeding at the same rate.

When in a sequence of enzyme steps the steady state flux, v, is changed, the concentrations of the different intermediate substrates may change in different ways depending on the circumstances. Assuming a homogeneous system, four cases may be considered.

Case 1: The enzyme step proceeds in accordance with the Michaelis-Menten equation, $v = V(S)/((S) + K_m)$. This means that any back reaction is negligible. In this case S must change in the same direction as v, and by at least the same amount.

Case 2: The enzyme step is rapid compared to v in both forward and backward directions. In this case the changes in S will tend to parallel the changes in the next succeeding substrate, and the two substrate levels will approximate equilibrium ratios.

Case 3: The enzyme reaction involves more than one reactant, where the second reactant may be a second substrate, or a coenzyme, or a prosthetic group of the enzyme which becomes oxidized or reduced. In this case, what happens to S will depend both on v and on what happens to the other components. Case 3 becomes Case 1 or 2 if the other substrate or the coenzyme or the state of oxidation of the prosthetic group remains constant.

Case 4: The enzyme activity itself can be changed, either by

the presence of substances not involved directly in the enzyme reaction, as in feedback control, or by actual change in the enzyme molecule, as in the conversion of phosphorylase *b* to *a*. Case 3 and particularly Case 4 represent control possibilities; the others do not.

The steps fumarate to malate, citrate to isocitrate, and oxalacetate to citrate may represent Case 2. In the various experiments fumarate and malate varied together almost in parallel over a 5-fold range and were never far from equilibrium. Citrate and isocitrate were close to equilibrium until aconitase was poisoned following fluoroacetate administration. In this event the step became a Case 1 situation, or more exactly, a modified version of Case 1 in which competitive inhibition is overcome by increasing levels of substrate. The classification of the oxalacetate to citrate step is less certain since acetyl-CoA levels were not measured. Oxalacetate levels remained constant as long as oxygen was present. Since the equilibrium constant for the condensing reaction is about 150 (29), the ratio of citrate to oxalacetate (150:1) could well approach that anticipated at equilibrium. However, the fact that the normal oxalacetate to citrate ratio was not maintained after fluoroacetate or insulin argues against this being a Case 2 step.

When oxygen was present, pyruvate, α-ketoglutarate, and malate all varied in the same direction as the flux and probably by at least as large a degree. Therefore, a step between pyruvate and citrate, a step between α-ketoglutarate and succinate, and the step between malate and oxalacetate appear to be in the Case 1 category. Since these steps are all known to involve more than one reactant, they would be classified as Case 3 with all components constant except the one measured.

The steps between isocitrate and α-ketoglutarate and between succinate and fumarate are clearly Case 3 or 4, since isocitrate and succinate were but little affected by changes in flux when glucose and oxygen were present. The isocitrate dehydrogenase step may be Case 4 with ADP playing a regulatory role (30–32).[2] The changes in brain ADP levels, with anesthesia and hyperthermia, are in the right direction for this explanation. Some control at the succinate dehydrogenase step could also be exerted indirectly by ADP levels through influence on electron transport. Whatever factors there may be to stabilize the levels of isocitrate and succinate, when the Krebs cycle is in a steady state the flux must be controlled by the input into the cycle. This control may be imposed at a step upstream from pyruvate. As noted, the changes in pyruvate levels in anesthesia and hyperthermia are more than sufficient to explain the decreased or increased input into the cycle. Actual control may be exerted at the P-fructokinase step through a balance between inhibition by citrate and ATP and deinhibition by AMP, ADP, P_i, or NH_4^+ (33).

[2] J. Holowach and D. B. McDougal, Jr., to be published.

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