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Effect of Various Substrates on Oxygen Uptake of Rat Cerebral Cortex Slices.* (29078)

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The well-known association of liver disease and coma implies that adequate liver function is required for normal brain metabolism. The usual explanation for this association has been that the liver is necessary to inactivate some substance which is probably absorbed from the gastrointestinal tract and is toxic to brain. Indeed, the demonstration that administration of ammonium salts or a precursor of ammonia induced a syndrome indistinguishable from spontaneous impending hepatic coma in certain patients with liver disease(1) lent support to this theory. The role of ammonia in development of spontaneous hepatic coma, however, has not been clarified.

Normal brain metabolism may depend on liver function in another way, by requiring some nutrient which is synthesized only or predominantly in the liver. In support of this theory, Geiger *et al*(2) reported that glucose uptake of perfused cat brain could be restored for a short time by adding fresh cat liver extract or cat blood to the perfusate and for a longer time by including the liver in the perfusion system. Geiger and Yamasaki(3) subsequently reported that this effect on glucose uptake could be produced by adding cytidine and uridine to the perfusate. Similarly, Lang, Goldstein, and Levine(4) showed that hepatectomy in dogs markedly decreased utilization of glucose by peripheral tissues, an effect which was independent of insulin, and postulated that the liver released a "humoral" agent necessary for normal peripheral glucose consumption.

In the present study an attempt was made to find a substance synthesized by the liver which might be required for normal brain metabolism. To do this, the effect of various known substrates which are made in the liver and might fulfill this role on oxygen uptake of surviving rat cerebral cortex slices was studied.

Materials and methods. Sherman rats weighing about 100 g each were used. The animals were stunned by a blow on the back of the head and exsanguinated; the brains were removed within a few minutes and placed in cold buffer of composition described below. Hemorrhagic brains were not used. Slices from each brain were cut free-hand from the cerebral cortex grey matter and placed into 3 manometric flasks, the first serving as a control and the other 2 containing different concentrations of the substrate to be tested. The buffer volume in each flask was 3.0 ml. The standard "direct" Warburg technique(5) was employed at 37.0°C using 9 test flasks (3 brains) and 2 thermo-barometer flasks for each run. The time elapsed between sacrifice of the rat and first manometric reading was about 60-75 minutes, and manometric readings were taken at 30-minute intervals for 5 hours. The brain slices were then removed, rinsed in distilled water, dried at 110°C for about 18 hours, and

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TABLE I. Effect of Various Substrates on Oxygen Consumption of Rat Brain Slices.

Substrate		$-Q_{O_2}^{O_2}$ (μl/mg/hr) Control	1mM	3mM	% change to 3mM
Krebs-Ringer-phosphate buffer (modified)					
Na acetate	(6)*	10.18	11.01	11.68	+15[A]
DL-Na β-hydroxybutyrate	(5)	9.23	9.71	11.23	+22[A]
Ethyl acetoacetate	(6)	10.95	11.80	11.18	+ 2
Na acetoacetate	(6)	10.08	10.09	12.52	+24[A]
NaOh	(6)	9.67	11.15	11.56	+20[B]
Acetic acid	(6)	11.17	10.41	11.26	+ 1
Na lactate	(6)	10.62	11.55	11.68	+10[B]
NaCl	(6)	10.25	10.05	10.29	0
Oxaloacetic acid	(6)	9.71	9.72	9.81	+ 1
Fumaric acid	(5)	9.95	10.03	9.55	− 4
Krebs-Ringer-phosphate buffer without calcium					
Na acetate	(6)	11.33	12.00	11.49	+ 1
" "	(5)	11.29	10.95†	10.99‡	− 3
DL-Na β-hydroxybutyrate	(5)	11.46	11.70	12.07	+ 5[B]
" "	(6)	9.55	9.81†	11.02‡	+15[A]
Na acetoacetate	(6)	10.49	11.18	12.08	+15[A]
NaOh	(6)	10.64	10.71	11.38	+ 7
Cytidine + uridine	(6)	10.97	11.37§	11.35§	+ 3
Creatine	(6)	10.13	10.40	9.99	− 1

* Numbers in parentheses refer to No. of rats.
† 2mM concentration used.
‡ 6mM " "
§ 1mM and 3mM of each, respectively.
[A] Significant at 1% level as judged by t test on average difference.
[B] Significant at 5% level as judged by t test on average difference.

weighed. Average total dry weight of the slices in each flask was about 9 mg. The pH of the buffer was measured before and usually after each run.

Krebs-Ringer phosphate buffer was found to produce a precipitate, as noted by Krebs (6). Since this precipitate was probably calcium phosphate, the buffer as used in the present experiments was modified to contain one-half the calcium concentration described by Krebs; at this concentration no precipitate was evident. The calcium concentration used, moreover, corresponds more closely to the ionized calcium concentration of rat serum(7). The calcium-free buffer used was made up in the same way except for omission of the calcium chloride.

Since in preliminary studies increasing the concentration of glucose in the buffer over 6 mM did not increase oxygen uptake by brain slices over a period of 5 hours, all of the buffer used contained a 6 mM concentration of glucose which was added to the buffer as an isotonic solution. All of the substrates used were also added as isotonic solutions so that the isotonicity of the suspending medium was preserved.

The following substrates were tested: sodium acetate, ethyl acetoacetate (Fisher, Reagent), sodium acetoacetate (kindly supplied by Dr. D. Tapley, College of Physicians and Surgeons, Columbia University), sodium lactate, acetic acid, DL-sodium betahydroxybutyrate (Mann, C. P.), oxalacetic acid (Mann, M. A.), fumaric acid (Mann, C. P.), creatine (Eastman), cytidine (Schwarz Labs.), and uridine (Nutritional Biochem. Corp.).

Each substrate was tested on 6 rat brains except where otherwise stated (Table I).

Results. The effects of at least 2 concentrations of each of the various substrates on the oxygen uptake of rat cerebral cortex slices are shown in Table I. Q_{O_2} values were similar to those found for rat brain by other investigators(8-10); these figures represent the average Q_{O_2} over a 5-hour period during which time the rate of oxygen uptake gradually decreased, while most previous investigators have measured Q_{O_2} over shorter pe-

riods. The variation in control values between runs was probably due largely to technical factors, since the 3 brains used in any one run showed close agreement. An important technical factor may have been differences in the time between sacrifice of the rats and start of manometric readings.

When the calcium-containing buffer was used, the largest percentage increases in oxygen uptake were seen with sodium acetoacetate and DL-sodium betahydroxybutyrate. Sodium acetate, however, also stimulated oxygen uptake, while acetic acid or sodium chloride in the same molar concentrations had no such effect, indicating that the effect of sodium acetate might have been related to its alkalinity. Indeed, sodium hydroxide similarly appeared to augment oxygen uptake. In 2 experiments in which oxygen uptake was measured over a wide range of buffer pH's, a higher oxygen uptake was seen over a pH range of 7.6 to 7.9 than at the pH of the buffer used in these studies, which was 7.30. A rising Qo_2 with an increasing pH of the medium from 7.0 to 9.0 has been noted before(11). Acetic acid has a higher $_pK_A$ at 25°C than both acetoacetic and betahydroxybutyric acids, and lactic acid has a higher $_pK_A$ at 25°C than acetoacetic acid, yet sodium acetoacetate and DL-sodium betahydroxybutyrate had a greater stimulating effect on Qo_2 than sodium acetate or sodium lactate, suggesting that these substances had a stimulating action on Qo_2 independent of their alkalinity.

Since the elimination of calcium from the buffer has been shown to result in an increase in oxygen uptake of rat brain(8,12), the possibility arose that these alkaline substrates raised oxygen uptake by decreasing the ionization of calcium phosphate. The effect of several of these substrates on the Qo_2 was studied, therefore, in the identical buffer except for the exclusion of calcium chloride. Indeed, in the absence of calcium, sodium acetate had no effect and sodium hydroxide little, if any, effect on Qo_2, while sodium acetoacetate and DL-sodium betahydroxybutyrate both caused an increase in the Qo_2. However, a 6 mM concentration of DL-sodium betahydroxybutyrate produced a similar response as a 3 mM concentration of sodium acetoacetate, as would be expected if only one optical isomer of this racemate were effective. Sodium acetate in 6 mM concentration was without effect. Cytidine and uridine or creatine, likewise appeared to have no effect at the concentrations used.

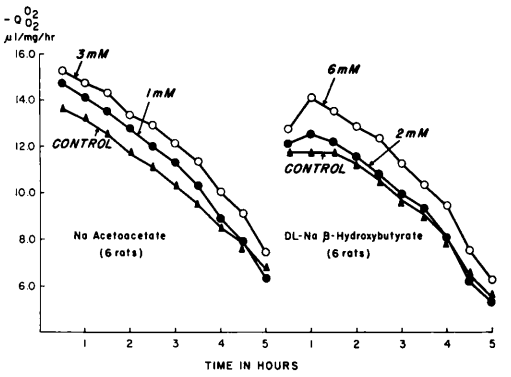

FIG. 1. Effect of sodium acetoacetate and DL-sodium betahydroxybutyrate on oxygen uptake of rat cerebral cortex slices in Krebs-Ringer phosphate buffer without calcium at 37.0°C.

The pH of the buffer was significantly reduced, usually to a pH of about 7.0, at the end of the run both in the control flasks and with all of the substrates used except ethyl acetoacetate, with which it was not measured.

Fig. 1 shows that the rate of decline of Qo_2 with sodium acetoacetate or DL-sodium betahydroxybutyrate was approximately parallel to the control curve over the entire 5-hour period; these curves appeared to approach the control curves, however, toward the end of this period. One can not conclude, therefore, that these substances prolonged the survival of the slices. If these substances were limiting, they must have been limiting from the start of the measurements.

Discussion. Of the various substances made in liver which were tested for an effect on oxygen uptake of surviving rat cerebral cortex slices, only acetoacetate and DL-betahydroxybutyrate had a stimulating effect in the calcium-containing and calcium-free media. The lack of such an effect by the other substrates tried, however, could have resulted

if a non-limiting concentration of the substrate already had been present in the slice, possibly as a consequence of synthesis *in situ,* or if inadequate substrate concentrations had been tested. But the failure of a substance to stimulate oxygen uptake in brain slices does not rule out its necessity for proper brain function in a less disorganized preparation. Such may be the case for uridine and cytidine, which had no apparent effect on oxygen uptake in the *in vitro* system used in this study but were reported to restore brain function in the perfused brain preparation(3), even though much higher concentrations were used in the present study.

Very few natural substances have been shown to increase the oxygen uptake of rat cerebral cortex slices in the presence of an adequate concentration of glucose. Potassium chloride in 0.1 M concentration(8,10) and succinate(13) have been shown to have this effect. According to Krebs(12), a combination of pyruvate, fumarate, and glutamate added to the glucose-containing medium increases Qo_2, but Elliott *et al*(13) have been unable to confirm this finding. The effectiveness of acetoacetate and DL-betahydroxybutyrate in this system thus far appears to be rather unique.

DL-betahydroxybutyrate was less effective than acetoacetate in the calcium-free medium, although both betahydroxybutyrate isomers have been reported to be oxidized aerobically by mitochondrial preparations (14). Both isomers, in addition, are found naturally, but the initial pathway of oxidation of each is different(14). The intermediate formed on the oxidation of fatty acids is L (+)-betahydroxybutyryl-Coenzyme A, while the free betahydroxybutyrate of body fluids appears to be of the D(—) configuration(14). Since only the D(—) isomer has been reported to be active as a transmitochondrial electron carrier (see below), appears to be the naturally occurring free form, and has been shown to be utilized by rat heart as readily as acetoacetate(15), the difference in activity of DL-betahydroxybutyrate as compared to acetoacetate in the present study may have been a result of the inactivity of the L(+) isomer.

Acetoacetate and betahydroxybutyrate are well qualified to fill the role of substances produced by the liver which may be required by the brain and perhaps other tissues. These substances are made almost exclusively in the liver(16,17); no production has been detected in brain(16). Even though their concentration in blood may be greatly increased when utilization of carbohydrate is reduced, they are present in the blood of healthy subjects and should be regarded as normal blood constituents. These substances can be utilized in quantity and serve as a major substrate of respiration for rat brain(18,19); in fact, they appear to be utilized by all tissues tested so far(16-19), with the single exception of liver(18-20). In studies on the perfused rat heart, acetoacetate appeared to be used preferentially to glucose(15,21) or endogenous substrates(15). DL-betahydroxybutyrate has been shown to be readily converted to acetoacetate by a number of tissues, including brain(16,18,22), a reaction which appears to be reversible(22).

A vital role for these substances in brain or other tissue metabolism might not be merely as a fuel of respiration. Lehninger has shown that intact rat liver mitochondria do not oxidize reduced diphosphopyridine nucleotide (DPNH)(23) and concluded that DPNH does not penetrate the intact mitochondrion(24). Since DPN is necessary for the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglyceric acid in the process of glycolysis, which takes place in the cytoplasm, a carrier substance may be required to carry electrons into the mitochondrion to the electron transport chain. Devlin and Bedell(25) have shown that acetoacetate or D(—) betahydroxybutyrate may function in this capacity, since in catalytic amounts they stimulated markedly the oxidation of DPNH by intact rat liver mitochondria, while succinate, α-ketoglutarate, malate, citrate, glutamate, and pyruvate had no effect on DPNH oxidation; DL-betahydroxybutyrate appeared to be one-half as active as D(—) betahydroxybutyrate. This mechanism may explain,

at least in part, the effects of acetoacetate and DL-betahydroxybutyrate noted in the present study, although much larger concentrations were necessary to produce an effect on Qo_2. A possible reason for this is that the mitochondria of the brain, unlike those of liver, may have metabolized the acetoacetate further so that less was transported to the extramitochondrial environment.

Even if acetoacetate and betahydroxybutyrate are required by brain, their production in patients with liver disease may not be decreased enough to interfere with brain function. Whether a deficiency af acetoacetate and betahydroxybutyrate does play any role in the development of hepatic coma in man will have to await clinical studies.

Summary. The effect of various substrates on oxygen uptake of rat cerebral cortex slices was studied in a modified Krebs-Ringer phosphate medium containing glucose. A stimulating effect on oxygen uptake by the salts of several of these substrates appeared to be related to their alkalinity. Since an alkaline substance might produce this effect by depressing the ionization of calcium phosphate, several of these substrates were tested in the same buffer except for exclusion of calcium chloride. Of the various substances tested, only acetoacetate and DL-betahydroxybutyrate consistently produced an elevation in oxygen uptake. The possibility is raised that acetoacetate and betahydroxybutyrate may play an important role in normal brain function.

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