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PROPORTIONAL INHIBITION OF ACETYLCHOLINE SYNTHESIS ACCOMPANYING IMPAIRMENT OF 3-HYDROXYBUTYRATE OXIDATION IN RAT BRAIN SLICES

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Abstract—The synthesis of acetylcholine from 3-hydroxy[3-^{14}C]butyrate has been studied in brain slices from 19-day-old and adult rats. In the brains of 19-day-old rats, the synthesis of acetylcholine from 3-hydroxybutyrate was stimulated by the addition of physiological concentrations of glucose but CO_2 production was not. Conversely, physiological concentrations of unlabeled 3-hydroxybutyrate reduced the production of acetylcholine and of $^{14}CO_2$ from [U-^{14}C]glucose. Results with adult brains were qualitatively similar, except that glucose stimulated the production of CO_2 from 3-hydroxy[3-^{14}C]butyrate. Methylmalonic acid inhibited the oxidation of 3-hydroxybutyrate but did not affect the oxidation of either pyruvate or glucose. Addition of methylmalonic acid to slices incubated with both 3-hydroxybutyrate and glucose decreased the synthesis of acetylcholine from 3-hydroxybutyrate with a compensatory increase in synthesis from glucose. These studies demonstrate that impairing the oxidation of 3-hydroxybutyrate to CO_2 is accompanied by a proportional decrease in the incorporation of 3-hydroxybutyrate into acetylcholine, even though the flux through oxidative pathways is over 100 times that to acetylcholine.

Acetylcholine is known to be synthesized from acetyl-CoA and choline in cholinergic neurones by the action of choline acetyltransferase (EC 2.3.1.6), but the control of this reaction remains a matter of controversy [1–4]. It has been suggested that the ratio of reactants to products of the acetyltransferase reaction is close to equilibrium and that the reaction is governed by the principles of mass action. There have been extensive studies of the metabolism of choline with respect to acetylcholine synthesis, and there is a large body of evidence indicating that the supply of choline can limit acetylcholine synthesis [2–4]. There have been relatively few studies of acetyl-group metabolism with respect to acetylcholine synthesis. It is established that pyruvate derived from glucose is the usual physiological precursor of the acetyl-CoA used for acetylcholine synthesis in adult rat brain [5, 6]. Impairing the oxidation of carbohydrates leads to a proportional inhibition of the synthesis of acetylcholine even though the flux of carbohydrates through oxidative pathways is about 100-fold that to acetylcholine [7–10]. This proportionality reflects compartmentation of glucose and pyruvate metabolism with respect to acetylcholine synthesis [11]. These observations suggest that under some circumstances the supply of acetyl-CoA can limit acetylcholine synthesis.

The ketone bodies 3-hydroxybutyrate and acetoacetate can act as precursors of acetyl-CoA in brain more effectively in young than in adult animals. There have been extensive studies of the interactions between ketone bodies and carbohydrates in cerebral production of CO_2 [12–14], ATP [12], amino acids [12, 15], and lipids [14]. Itoh and Quastel [12] reported that acetoacetate can support the synthesis of acetylcholine by brain slices more effectively in the brains of 1- to 3-day-old rats than in adult rats, although acetoacetate did not support acetylcholine synthesis by cell-free preparations of brain [16].

In order to study further the role of acetyl-group metabolism in the control of acetylcholine synthesis, we have studied the interactions between 3-hydroxybutyrate oxidation and acetylcholine synthesis in rat brain slices. Impairing oxidation of 3-hydroxybutyrate leads to a proportional decrease in its incorporation into acetylcholine, even though its flux through oxidative pathways is over 100-fold its incorporation into acetylcholine. The results are consistent with compartmentation of 3-hydroxybutyrate metabolism with respect to acetylcholine synthesis. Details of these studies are presented below [17].

EXPERIMENTAL PROCEDURES

Materials. Dr. S. Zamenhof kindly provided 19-day-old rats from his own colony. Adult male Sprague–Dawley rats were from Mission Supply, Los Angeles, CA. Potassium D,L-3-hydroxy[3-^{14}C]butyrate (1–5 Ci/mole), D[3,4-^{14}C]glucose (10–15 Ci/mole), sodium [1-^{14}C]pyruvate (2–10 Ci/mole), and sodium [2-^{14}C]pyruvate (2–10 Ci/mole) were from New England Nuclear Corp., Boston, MA. D[U-^{14}C]glucose was from Amersham-Searle, Arlington Heights, IL. Methylmalonic acid was from ICN Pharmaceuticals, Plainview, NY. Nonradioactive potassium D,L-3-hydroxybutyrate, acetylcholinesterase (EC 3.1.1.7; from electric eel;

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200 units/mg) and lecithin were from Sigma Chemical Co., St. Louis, MO. Other reagents were as described previously [7].

Experiments with brain slices. All experiments except that shown in Tables 1 and 3 were with brains from 19-day-old rats. Whole brains were sliced in two dimensions with a McIlwain chopper and incubated, as described previously [8], in a modified Krebs–Ringer phosphate buffer [12], pH 7.4, containing 141 mM NaCl, 31 mM KCl, 2.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 10.3 mM Na_2HPO_4, 50 μM choline chloride, and 40 μM paraoxon to inhibit cholinesterase. The substrates were those specified in the legends to the figures and tables and included 1 μCi of radioactive substrate. The α- and β-anomers of glucose were allowed to equilibrate overnight before the incubations. Brain slices containing 5–10 mg protein were incubated in 3 ml medium in 25 ml siliconized Erlenmeyer flasks for 60 min unless otherwise specified. Incubations were terminated by the addition of 0.5 ml of 1.4 M $HClO_4$, and the incorporation of radioactivity into CO_2, acetylcholine, lipids, proteins and nucleic acids was determined as described previously [8]. Blanks were determined by adding perchloric acid to the media before adding the tissue slices. The nmoles of [U-^{14}C]glucose or of 3-hydroxy[3-^{14}C]butyrate incorporated into each fraction were calculated by dividing the dis./min in the fraction by the specific activity of the radioactive substrate. To determine the nmoles of acetylcholine produced from [U-^{14}C]glucose, this value was multiplied by 3 [18], since the labeled glucose contains three times as many C atoms as the acetyl moiety. It was multiplied by 2 to calculate the nmoles of acetylcholine produced from 3-hydroxy[3-^{14}C]butyrate [6], since each mole of 3-hydroxybutyrate gives rise to 2 moles of acetyl moieties.

The identity of the radioactive material in the acetylcholine fraction after incubation with 3-hydroxy[3-^{14}C]butyrate was demonstrated by hydrolysis with acetylcholinesterase exactly as described elsewhere for the identification of the radioactive products after incubations with pyruvate or glucose [7–9]. The values were comparable to the proportion of radioactive material hydrolyzed by acetylcholinesterase in the acetylcholine fraction of LeFresne *et al.* [19].

In vivo studies. The 250 g male rats used in the study of incorporation of [$^2H_4$]choline into acetylcholine were sacrificed by microwave irradiation at 2.5 kW for 1.2 sec, 1 min after the injection of [$^2H_4$]choline (20 μmoles/kg). Choline, acetylcholine, [$^2H_4$]choline and [$^2H_4$]acetylcholine were synthesized from [$^2H_4$]1,2-dibromoethane [20] and determined by gas chromatography–mass spectrometry [21].

Enzyme studies. A mitochondrial pellet was prepared as described by Clark and Nicklas [22] but omitting the centrifugation on a Ficoll gradient. 3-Hydroxybutyrate dehydrogenase (D-3-hydroxybutyrate: NAD^+ oxidoreductase, EC 1.1.1.30) was solubilized by freezing, thawing, and sonicating the mitochondrial pellet three times for 20 sec at 4° in an ice bath. Activity was assayed by the method of Tan *et al.* [23], except that NADH oxidases were inhibited with 80 μg/ml of rotenone instead of with antimycin A and that lecithin, 80 μg/ml, was added. Apparent K_i values were calculated using Biomedical Program BMDP-3R, which computes a best-fit curve for velocity vs substrate concentration data.

RESULTS

Effective utilization of 3-hydroxybutyrate for the synthesis of acetylcholine required the simulta-

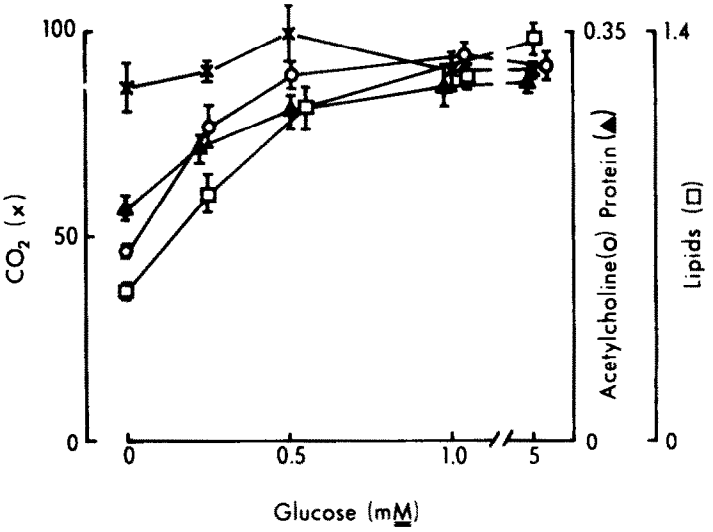

Fig. 1. Utilization of 3-hydroxy[3-^{14}C]butyrate by rat brain slices incubated with different concentrations of glucose. Slices from 19-day-old rats were incubated with 2 mM 3-hydroxybutyrate and the indicated concentrations of nonradioactive glucose, as described in detail in the text and in Ref. 8. Three different vertical axes are used for graphical clarity, because of differences in the magnitudes of the fluxes to CO_2, to proteins and acetylcholine, and to lipids. Values are expressed in nmoles of 3-hydroxy[3-^{14}C]butyrate incorporated/mg of tissue protein/hr and are the means + S.E.M. of six incubations.

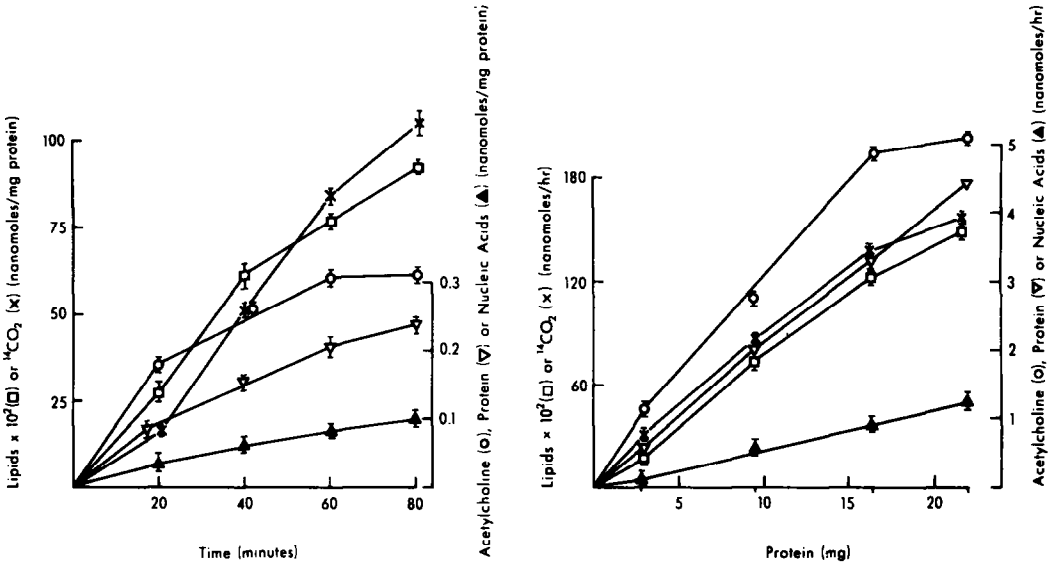

Fig. 2. Utilization of 3-hydroxy[3-^{14}C]butyrate by rat brain slices. Slices from 19-day-old rats were incubated with 5 mM glucose and 2 mM 3-hydroxy[3-^{14}C]butyrate and the incorporation of radioactivity into various fractions was determined, as described in detail in the text and in Ref. 8. In the lefthand panel, the flasks contained 12 mg of brain protein and the lengths of the incubations were varied. In the righthand panel, incubations were for 60 min and the amount of tissue protein was varied. Values are expressed in nmoles of 3-hydroxy[3-^{14}C]butyrate incorporated and are the means ± S.E.M. of six incubations; where error bars are not shown they were smaller than the symbols. Note the difference in magnitude for CO_2 compared to the other products.

neous availability of glucose in slices from the brains of 19-day-old rats (Fig. 1). The flux of 3-hydroxybutyrate to CO_2 was more than 100 times that to acetylcholine (Figs. 1 and 2). Glucose also stimulated the utilization of 3-hydroxybutyrate for the synthesis of proteins and lipids, in agreement with the results of Patel and Owen [13]. Oxidation of 3-hydroxybutyrate to CO_2 in young rat brain slices did not require glucose, in agreement with the observations of Itoh and Quastel [12].

In the presence of 5 mM glucose, the utilization of 3-hydroxybutyrate for the synthesis of acetylcholine, lipids, proteins and nucleic acids and for its oxidation to CO_2 was proportional to the amount of tissue present and to the length of the incubation, under the conditions used (Fig. 2).

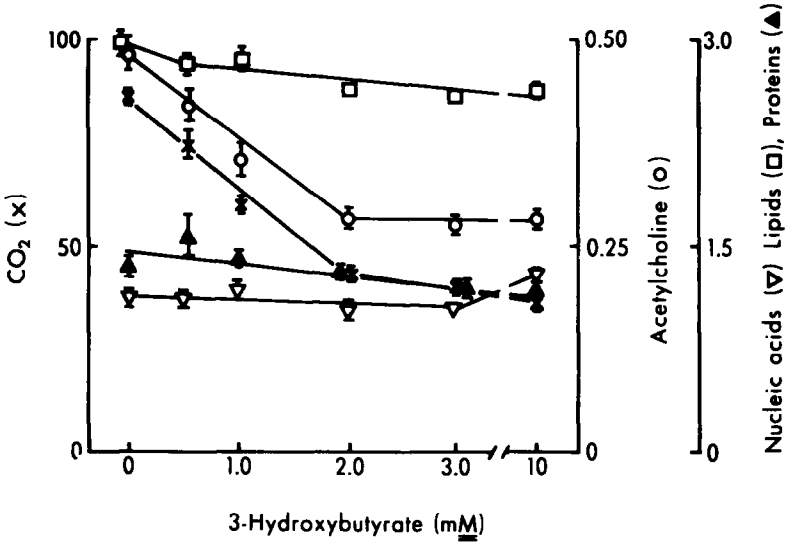

Fig. 3. Utilization of [U-^{14}C]glucose by rat brain slices incubated with different concentrations of 3-hydroxybutyrate. Slices from brains of 19-day-old rats were incubated with 5 mM [U-^{14}C]glucose and the indicated concentrations of nonradioactive 3-hydroxybutyrate. The conditions are described in detail under Experimental Procedures and in Ref. 8. Values are expressed in nmoles of [U-^{14}C]glucose incorporated/mg of tissue protein/hr and are the means ± S.E.M. of six incubations. Note the different magnitudes of the three vertical axes.

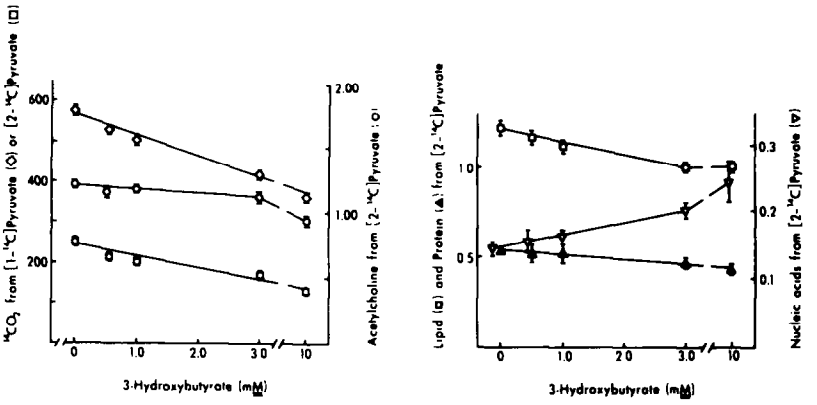

Fig. 4. Effect of 3-hydroxybutyrate on the utilization of [1-^{14}C]- or [2-^{14}C]pyruvate by rat brain slices. Slices from 19-day-old rats were incubated with 5 mM sodium [1-^{14}C]-or [2-^{14}C]pyruvate and with the indicated concentrations of 3-hydroxybutyrate, as described in detail in Experimental Procedures and in Ref. 8. Values are expressed in nmoles of pyruvate incorporated/mg of protein/hr and are the means ± S.E.M. of six incubations.

The effects of 3-hydroxybutyrate on the utilization of [U-^{14}C]glucose by brain slices from 19-day-old rats fell into two patterns (Fig. 3): a dose-dependent reduction in the production of acetylcholine and of CO_2, but minimal effects on the production of lipids, proteins and nucleic acids. The reduction was proportional to the concentration of 3-hydroxybutyrate in the range of 0–2 mM, i.e. physiological concentrations, but then remained virtually constant even though the concentration of 3-hydroxybutyrate was increased to above 10 mM. Itoh and Quastel [12] attributed the effect of ketone bodies on CO_2 production from glucose to accumulation of acetyl-CoA derived from the ketones. Patel and Owen [13] have shown that the lipids derived from glucose under these conditions are derived primarily from acetate, not glycerol. It, therefore, appears that the acetyl moieties derived from 3-hydroxybutyrate dilute the pool of cytoplasmic acetyl groups going to acetylcholine without significantly diluting the pool of cytoplasmic acetyl groups converted into lipids.

Hydroxybutyrate had less effect when slices were incubated with 5 mM sodium pyruvate (Fig. 4). This concentration of pyruvate is between one and two orders of magnitude higher than that in brain slices incubated with glucose [24]. Hydroxybutyrate and pyruvate have been shown to compete for the monocarboxylate carrier system of brain mitochondria [25].

In agreement with the results of others [12–14], 3-hydroxybutyrate was a less effective substrate for adult than for young brain. Thus, the addition of 2 mM 3-hydroxybutyrate did not reduce the conversion of [U-^{14}C]glucose to acetylcholine by adult rat brain slices (Table 1), in contrast to the results with slices from 19-day-old animals (Fig. 3). On the other hand, the requirement for glucose for optimal utilization of hydroxybutyrate was even more marked in adult brain slices than in those from 19-day-old rats. Addition of 5 mM glucose doubled the conversion of 3-hydroxy[3-^{14}C]butyrate into CO_2 [12], proteins and lipids, but increased its conversion into acetylcholine 7-fold (Table 1).

The effects of using methylmalonate to inhibit 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) were also studied (Tables 2 and 3). In agreement with the observations of others [23, 26], methylmalonic acid inhibited the oxidation of 3-hydroxybutyrate by disrupted mitochondria more effectively than did a number of other 2-keto acids. The K_i values (mM) were determined on two different preparations of disrupted mitochondria and at a minimum of six different concentrations: methylmalonate, 0.2 ± 0.1; 2-oxobutyrate, 0.7 ± 0.2; phenyl-

Table 1. Utilization of 3-hydroxy[3-^{14}C]butyrate or of [U-^{14}C]glucose by brain slices from adult rats*

	3-Hydroxy[3-^{14}C]butyrate		Incubation with [U-^{14}C]glucose	
	3-Hydroxybutyrate (2 mM)	3-Hydroxybutyrate (2 mM) plus glucose (5 mM)	Glucose (5 mM)	Glucose (5 mM) plus 3-hydroxybutyrate (2 mM)
$^{14}CO_2$	19.6 ± 0.5	33.9 ± 1.9	95.2 ± 3.7	82.4 ± 3.5
Acetylcholine	0.020 ± 0.006	0.143 ± 0.009	0.549 ± 0.029	0.526 ± 0.019
Protein	0.032 ± 0.001	0.064 ± 0.006	1.013 ± 0.060	1.013 ± 0.044
Nucleic acid	0.021 ± 0.001	0.027 ± 0.002	0.654 ± 0.031	0.669 ± 0.038
Lipid	0.086 ± 0.012	0.199 ± 0.021	1.86 ± 0.10	1.84 ± 0.12

* Adult male rats were given water but no food for 48 hr before being sacrificed. Brain slices were prepared and incubated as described in detail in Experimental Procedures. Values are expressed in nmoles of ^{14}C-substrate incorporated/mg of protein/hr and are the means ± S.E.M. of six incubations.

Table 2. Methylmalonic acid and the utilization of [U-^{14}C]glucose and of 3-hydroxy[3-^{14}C]butyrate by brain slices from 19-day-old rats*

	CO_2	ACh	Protein	Nucleic acids	Lipids
[U-^{14}C]glucose					
Control	78.8 ± 4.5	0.39 ± 0.02	1.00 ± 0.05	0.72 ± 0.06	2.75 ± 0.02
+MMA (10 mM)	72.9 ± 5.7	0.37 ± 0.01	0.90 ± 0.08	0.77 ± 0.09	2.69 ± 0.18
3-Hydroxy[3-^{14}C]butyrate					
Control	82.5 ± 1.3	0.12 ± 0.01	0.16 ± 0.01	0.025 ± 0.001	0.413 ± 0.047
+MMA (10 mM)	35.5 ± 1.4	0.06 ± 0.02	0.078 ± 0.005	0.023 ± 0.002	0.207 ± 0.01

* Slices from the brains of 19-day-old rats were incubated with either 5 mM [U-^{14}C]glucose or 2 mM 3-hydroxy[3-^{14}C]butyrate and the incorporation of radioactivity into various fractions was determined as described in detail in the text. Values are expressed in nmoles of substrate incorporated/hr/mg of tissue protein and are the means ± S.E.M. of six incubations. MMA = methylmalonic acid.

Table 3. Effect of methylmalonate on utilization of 3-hydroxybutyrate and glucose in brain slices of adult rats*

	Decrease in synthesis from 3-hydroxy-[3-^{14}C]-butyrate	Increase in synthesis from [U-^{14}C]glucose
	(nmoles/hr/mg tissue protein)	
$^{14}CO_2$	−19.9 ± 1.9	+8.09 ± 3.45
Acetylcholine	−0.079 ± 0.009	+ 0.006 ± 0.019
Protein	− 0.028 ± 0.006	+ 0.016 ± 0.044
Nucleic acid	− 0.013 ± 0.002	+ 0.062 ± 0.038
Lipid	− 0.076 ± 0.021	+ 0.17 ± 0.12

* Brain slices from adult rats were incubated with 2 mM 3-hydroxybutyrate and 5 mM glucose containing 1 μCi of either 3-hydroxy[3-^{14}C]butyrate or of [U-^{14}C]glucose, and the tissue was fractionated exactly as described in Experimental Procedures. Values are the means ± S.E.M. of the increase or decrease in synthesis of the specific fraction from the indicated precursor due to the addition of 20 mM methylmalonate to the incubation. The absolute values for synthesis in the absence of methylmalonate are given in the text.

pyruvate, 1.6 ± 0.3; pyruvate, 5.0 ± 0.1; 2-oxo-3-methylbutanoate, 26 ± 7; 2-oxo-methylpentanoate, 33 ± 7; and 2-oxo-4-methylpentanoate, 40 ± 16. Methylmalonate (2 mM) did not inhibit the oxidation of [1-^{14}C]pyruvate, [2-^{14}C]pyruvate or [3,4-^{14}C]glucose (data not shown) or of [U-^{14}C]glucose (Tables 2 and 3), but it did impair the utilization of 3-hydroxybutyrate in the brains of 19-day-old (Table 2) and adult (Table 3) rats. At lower concentrations of methylmalonic acid, the reduction in the synthesis of acetylcholine from 3-hydroxybutyrate was accompanied by a compensatory increase in the production of acetylcholine from glucose in young rat brain slices (Fig. 5). Higher concentrations of methylmalonate (10–20 mM), which are known to inhibit the tricarboxylate acid cycle [27, 28] caused a net decrease in the utilization of carbon from both substrates together, i.e. in the sum of biosynthesis from [U-^{14}C]glucose and from 3-hydroxy[3-^{14}C]-butyrate in both young (Fig. 5) and adult (Table 3) brain slices. Induction *in vivo* of ketonemia and hypoglycemia by 48 hr of fasting did not alter the concentration of acetylcholine or the incorporation of [$^2H_4$]choline to acetylcholine in the brains of adult animals (data not shown).

The reduction in incorporation of 3-hydroxy[3-^{14}C]butyrate into acetylcholine in these studies was proportional to the reduction in $^{14}CO_2$ production, even though the flux through oxidative pathways was over 100-fold that into acetylcholine (Fig. 6).

DISCUSSION

The results described above allow three conclusions. First, the proportionality between CO_2 production and acetylcholine synthesis which has been demonstrated with glucose or pyruvate as substrates [7–11] also occurs when the substrate is 3-hydroxybutyrate (Fig. 6). Second, optimal utilization of 3-hydroxybutyrate for acetylcholine synthesis requires the concomitant presence of physiological amounts of glucose. Third, cerebral metabolism of 3-hydroxybutyrate may be compartmentalized with respect to acetylcholine synthesis.

As shown in Fig. 6, inhibiting the oxidation of 3-hydroxybutyrate to CO_2 led to a proportional inhibition of the synthesis of acetylcholine from this substrate, even though the flux to CO_2 was over 100 times that to acetylcholine. Effects were qualitatively similar in brain slices from 19-day-old and adult rats. The relationship between inhibition of mitochondrial oxidation and acetylcholine synthesis with hydroxybutyrate as substrate is similar to that already reported with glucose or pyruvate as substrates [7–11]. With the carbohydrates, that relation has been shown to be due to compartmentation of metabolism with respect to acetylcholine synthesis [11]. It also appears to relate to changes in transmitochondrial membrane potentials [10].

The presence of glucose was necessary for optimal incorporation of 3-hydroxybutyrate into acetylcholine in slices of young or adult rat brain. The levels of glucose which were optimal were in the mM range, i.e. physiological concentrations for brain [24]. This observation agrees with the observations of others on a need for glucose for optimal synthesis from ketone bodies of CO_2 and amino acids [12–15] and lipids [13]. Itoh and Quastel [12] reported that glucose increased the incorporation of [3-^{14}C]acetoacetate into acetylcholine by 40 per cent in adult brain but had a negligible effect in infant

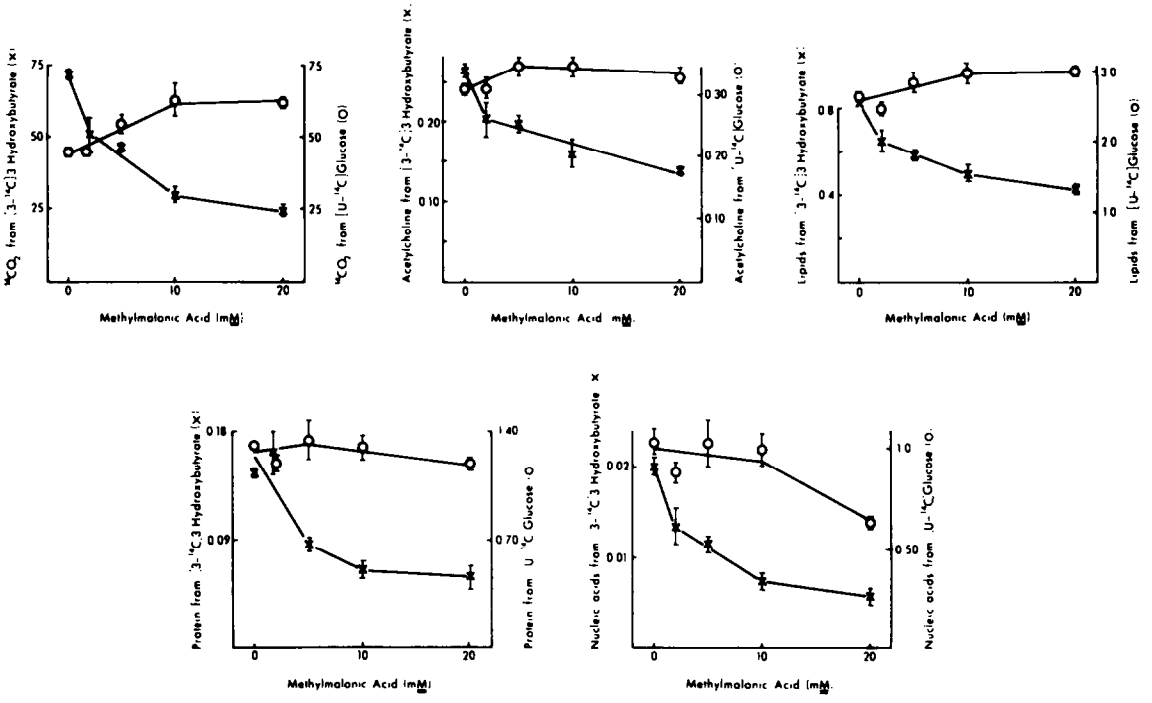

Fig. 5. Effect of methylmalonic acid on the utilization of [U-^{14}C]glucose or 3-hydroxy[3-^{14}C]butyrate. Slices from 19-day-old rats were incubated with 5 mM glucose and 2 mM 3-hydroxybutyrate and trace amounts of either [U-^{14}C]glucose or 3-hydroxy[3-^{14}C]butyrate. The final concentration of methylmalonic acid is indicated. The results are the means ± S.E.M. of at least six incubations. Values are expressed in nmoles of the radioactive substrate incorporated into the respective fractions/mg of tissue protein/hr.

brain. They concluded, however, that the unlabeled glucose was probably masking the effect on acetylcholine due to dilution of the label from acetoacetate. We found more profound and consistent effects on acetylcholine synthesis from 3-hydroxy[3-^{14}C]butyrate: a 7-fold increase in adult brain and a doubling in young rat brain. The requirement for glucose may represent a requirement for oxaloacetate derived from carbohydrates, which can condense with acetyl-CoA derived from ketone bodies to form citrate and then other biosynthetic intermediates as well [12].

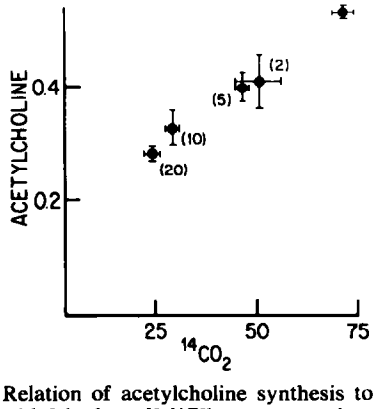

Fig. 6. Relation of acetylcholine synthesis to CO_2 production with 3-hydroxy[3-^{14}C]butyrate as substrate. Slices from 19-day-old rats were incubated with 5 mM glucose, 2 mM 3-hydroxybutyrate and trace amounts of 3-hydroxy[3-^{14}C]butyrate, and with the concentrations of methylmalonic acid indicated by the numbers in parentheses. The values are expressed in nmoles of 3-hydroxy-[3-^{14}C]butyrate incorporated/mg of tissue protein/hr and are the means ± S.E.M. of at least six incubations.

Several lines of evidence suggest that metabolism of 3-hydroxybutyrate may be compartmentalized with respect to acetylcholine synthesis in rat brain. First, this is a simple explanation for the proportionality between CO_2 production and acetylcholine synthesis from 3-hydroxybutyrate discussed above. Second, addition of glucose had a much more profound effect on conversion of 3-hydroxybutyrate to acetylcholine than to CO_2 in brain slices both from young animals (a doubling in acetylcholine production vs no effect on CO_2 production) and from adults (a 7-fold increase in acetylcholine production vs a doubling of CO_2). A straightforward way to interpret this differential effect is to assume a geographic and biochemical distinction between the structures where most of the CO_2 is produced and the structures where acetylcholine is made, i.e. compartmentation of metabolism with respect to acetylcholine synthesis. Third, 3-hydroxybutyrate effectively diluted the pool of acetyl groups going to acetylcholine but not that going to lipids in brain slices from 19-day-old rats incubated with radioactive glucose, again consistent with compartmentation of acetyl groups derived from hydroxybutyrate. Although these three lines of evidence do not provide definitive proof for compartmentation of 3-hydroxybutyrate metabolism with respect to acetylcholine synthesis, they do provide strong inferential evidence.

The utilization of ketone bodies for acetylcholine

synthesis is of practical as well as of theoretical interest. A number of patients are known who have inborn deficiences of the pyruvate dehydrogenase complex and neurological disease [29]. Some of them have been found to improve on a ketonemic diet, which provided a substrate which bypasses the deficient step [30]. Because of biochemical and pharmacological evidence that cholinergic systems are particularly sensitive to conditions which impair carbohydrate catabolism [7–11], others of these patients have been treated with a cholinergic agonist and they also seemed to get better [31]. The demonstration that acetylcholine synthesis in the brains of ketonemic animals is normal provides biochemical evidence supporting the use of ketonemic diets in patients with pyruvate dehydrogenase deficiences.

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