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# STUDIES ON THE METABOLIC PATHWAY OF THE ACETYL GROUP FOR ACETYLCHOLINE SYNTHESIS

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**Abstract**—Glucose and pyruvate are the most effective precursors of the acetyl moiety of acetylcholine in mammalian brain; the metabolic intermediates between pyruvate and acetylcholine, however, are unknown. The following data suggest that citrate is not the sole intermediate of the acetyl group for acetylcholine synthesis in rat brain slices or synaptosomes: (1) 2.5 mM (−)-hydroxycitrate decreased acetylcholine synthesis from [U-$^{14}$C]glucose by only 25 per cent; (2) inhibition of citrate transport out of mitochondria by *n*-butylmalonate or 1,2,3-benzenetricarboxylate variably affected acetylcholine synthesis; and (3) high concentrations of nonradioactive citrate decreased the synthesis of acetylcholine but did not decrease the specific activity of the acetylcholine synthesized from [U-$^{14}$C]glucose, even though the uptake of citrate into the synaptosomes under these experimental conditions was approximately five times greater than the uptake of glucose. Other possible acetyl donors altered acetylcholine synthesis. Acetylcarnitine stimulated synthesis in brain slices, and carnitine stimulated synthesis by synaptosomes. The specific activity of the acetylcholine synthesized from [U-$^{14}$C]glucose by synaptosomes was decreased by *N*-acetyl-L-aspartate (10 mM), acetyl CoA (1 mM), and acetyl phosphate (10 mM) which is consistent with these compounds acting as direct acetyl donors. Acetate (10 mM) did not affect either the amount or specific activity of the acetylcholine synthesized. Further evidence of compartmentation of cytoplasmic acetyl CoA is presented. The cytoplasmic acetyl CoA for acetylcholine synthesis is distinguishable from the cytoplasmic acetyl CoA for lipid synthesis. (−)-Hydroxycitrate inhibited acetylcholine synthesis without inhibiting lipid synthesis from [U-$^{14}$C]glucose. However, when 3-hydroxy[3-$^{14}$C]butyrate was used as substrate, (−)-hydroxycitrate inhibited incorporation into lipids twice as much as incorporation into acetylcholine. [U-$^{14}$C]Glucose metabolism by infant brain slices was more sensitive than adult brain slices to (−)-hydroxycitrate. However, the response to the other compounds which interfere with citrate metabolism was similar in slices from adult and infant brains.

Acetylcholine is synthesized from acetyl CoA and choline in the cytoplasm. Glucose [1, 2] and pyruvate [3] are the preferred precursors of the acetyl carbons of acetylcholine in adult mammalian brain. Glucose is converted to pyruvate by glycolysis in the cytoplasm. The pyruvate is transported into the mitochondria and converted to acetyl CoA by pyruvate dehydrogenase. Acetoacetate [4] and 3-hydroxybutyrate [5] are good precursors of the acetyl group of acetylcholine in infant brain. 3-Hydroxybutyrate is converted to acetoacetate in the mitochondria. In the mitochondria, acetoacetate is converted to two acetyl CoAs. The acetyl moiety is then transported out of the mitochondria in an unknown molecular form and converted back to acetyl CoA which is used for lipid and acetylcholine synthesis [6]. Direct leakage of acetyl CoA occurs from ether-treated mitochondria [7] or from mitochondria contaminated with choline acetyltransferase, but not from normal mitochondria [8]. Citrate [9–11], carnitine [9, 12], and acetate [9, 13, 14] are all potential, but unproven, transport forms.

These studies, previously reported in abstract form [15, 16], examine acetylcholine synthesis from [U-$^{14}$C]glucose in synaptosomes and tissue slices after addition of compounds known to inhibit citrate transport out of the mitochondria, or cleavage, in a dose-dependent manner. The effect of adding nonradioactive potential acetyl group donors is also presented. If a compound is a donor, then the specific activity (d.p.m./nmole) of the acetylcholine synthesized from [U-$^{14}$C]glucose or 3-hydroxy[3-$^{14}$C]butyrate should be reduced after addition of a nonradioactive acetyl donor compound. This approach is analogous to the methods used by Watson and Lowenstein [17] for studying fatty acid synthesis by liver, except that their studies were done with liver mitochondria and a cell free supernatant solution. This approach allows glucose to be added to the incubation medium to help maintain tissue integrity and still evaluate the role of these donors in acetylcholine synthesis. In some studies on the evaluation of citrate and acetate as potential donors, glucose was not added [2, 18]. High concentrations of the potential acetyl donors were added to dilute all of the various metabolic pools, including the small, rapidly turning-over pool of acetylcholine [19]. Since acetylcholine metabolism varies with age [5], slices from infant and adult brains were used.

## MATERIALS AND METHODS

*Materials.* Unless specified, reagents were as described previously [5, 20–22]. The trisodium salt of (−)-hydroxycitrate was a gift from Dr. A. C. Sullivan, Roche Research Center, Hoffman–LaRoche, Inc., Nutley, NJ. *n*-Butylmalonate and 1,2,3-benzenetricarboxylate were from the Aldrich

Chemical Co., Inc., San Leandro, CA. Citric acid (trisodium salt), *N*-acetyl-L-aspartic acid, *N*-acetylglycine, *N*-acetyl-DL-aspartic acid, *N*-acetyl-DL-methionine, *N*-acetyl-L-glutamic acid, *N*-acetyl-L-methionine, acetyl phosphate, acetyl CoA, CoA, carnitine and acetylcarnitine were from the Sigma Chemical Co., St. Louis, MO. Reagents for the gas chromatography–mass spectrometry measurement of acetylcholine were described previously [23]. [U-$^{14}$C]Glucose (230 mCi/mmole) was from Amersham-Searle, Chicago, IL, and [1,5-$^{14}$C]citrate (100 mCi/mmole) and DL-3-hydroxy[3-$^{14}$C]butyric acid (5 mCi/mmole) were from New England Nuclear, Boston, MA.

Male Sprague–Dawley rats, weighing 180–220 g, were from the vivarium in the Department of Biology at the University of California at Los Angeles. Infant rats were 19-days-old and were obtained from the laboratories of Dr. Steven Zamenhof at the University of California at Los Angeles.

*Methods.* All incubations were in a modified Krebs–Ringer phosphate buffer (pH 7.4) exactly as described previously [4, 21, 22], containing 141 mM NaCl, 31 mM KCl, 2.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 10.3 mM $Na_2HPO_4$, 50 $\mu$M choline chloride and 40 $\mu$M paraoxon as the cholinesterase inhibitor. The 31 mM $K^+$ is used to maximally stimulate acetylcholine synthesis [1]. The substrate was 5 mM glucose in a final volume of 3 ml for the slice experiments, and 1.5 mM glucose in a final volume of 1 ml for the synaptosome experiments. In the synaptosomal experiments, the specific activity of the [U-$^{14}$C]glucose was increased from the 0.3 mCi/mmole used in the slice experiments to 4.7 mCi/mmole. This was necessary to get adequate incorporation into acetylcholine. The two anomers of glucose were allowed to equilibrate overnight. All inhibitors were added only after the media containing them were adjusted to pH 7.4 by the addition of either HCl or NaOH. Incubations with slices were for 1 hr at 37° and incubations with synaptosomes were for 30 min at 37°. All incubations were under 95% $O_2$/5% $CO_2$ to assure full oxygenation of the tissue. Incubations were terminated by the addition of $HClO_4$ to a final concentration of 0.2 N. Incorporation of [U-$^{14}$C]glucose into $^{14}CO_2$, lipids, proteins and nucleic acids was determined as described previously [20–22, 24]. The $^{14}$C in acetylcholine was determined as described previously for whole brain [19], except that the neutralization of the tissue extract was omitted. The acetylcholine was determined by gas chromatography–mass spectrometry (g.c.–m.s.) [19]. Acetylcholine specific activity was calculated by dividing the d.p.m. in acetylcholine from [U-$^{14}$C]glucose by the nmoles of acetylcholine measured by g.c.–m.s.

Rat brain slices were prepared and incubated exactly as described previously [5, 20–22]. Rat brain synaptosomes were prepared by the method of Verity [25]. After removal from the sucrose-ficoll gradient, the synaptosomes were rinsed twice with the Krebs–Ringer phosphate buffer except that the KCl was 5 mM. Incorporation of [U-$^{14}$C]glucose into acetylcholine and $CO_2$ was proportional to the time of incubation and to the amount of tissue added.

Uptake of glucose and citrate by the synaptosomes was determined after 1, 2 or 4 min of incubation. Synaptosomes were incubated with 1.5 mM glucose and 10 mM citrate. Either [U-$^{14}$C]glucose (1 $\mu$Ci) or [1,5-$^{14}$C]citrate (1 $\mu$Ci) was added to each flask. Incubation flasks were removed at appropriate time intervals and swirled for 20 sec in an ice–ethanol bath at −10°. The synaptosomes were immediately transferred to microfuge tubes (Eppendorf microfuge, Brinkman Instrument Co., Westbury, NY) and spun for 20 sec (16,500 *g*). The buffer was removed by suction and the synaptosomes were immediately resuspended in 1 ml of the incubation medium without $^{14}$C. The spin and rinse were repeated. The final synaptosome pellet was dissolved in 0.5 ml hyamine hydroxide, and the entire tube was placed in a liquid scintillation vial with 15 ml of counting mixture. The uptake of both isotopes was linear with time (1, 2 and 4 min), and synaptosomal protein was added. If the synaptosomal pellet was washed with water instead of buffer, all of the radioactivity was released. Blanks were determined by adding the isotope at the end of the incubation or by incubating the samples on ice. Since the incubation conditions for measuring uptake of glucose and citrate were identical except for the isotopes added, swelling of the synaptosomes would not influence the comparison of citrate and glucose. The nmoles taken up were calculated by the following equation:

$$\text{nmoles taken up} = (\text{d.p.m. in synaptosomes}) \frac{\text{nmoles substrate}}{\text{d.p.m. substrate}}$$

## RESULTS

High concentrations of compounds which interfere with citrate metabolism caused relatively small changes in acetylcholine synthesis. This was true in tissue slices and in synaptosomes where the glial contribution is minimized. (−)-Hydroxycitrate at concentrations (2.5 mM) 2500 times the $K_i$ for isolated citrate lyase (0.8 $\mu$M) [17, 26, 27] diminished acetylcholine synthesis by tissue slices from adults or infants (Fig. 1) or by synaptosomes (Table 1) by only 20–30 per cent, whether the precursor was [U-$^{14}$C]glucose or 3-hydroxy[3-$^{14}$C]butyrate. The total nmoles of acetylcholine produced by slices from adult rat brains (1.39 ± 0.07 to 1.07 ± 0.08 nmoles/mg of protein/hr) or by synaptosomes (Table 1) declined in the presence of (−)-hydroxycitrate. If ATP (5 mM) was added to activate citrate lyase [27], acetylcholine synthesis from glucose was reduced by 32.6 ± 3.6 per cent (N = 10) and the quantity as measured by g.c.–m.s. was reduced by 32.7 ± 3.5 per cent (N = 4). (−)-Hydroxycitrate affects the incorporation of [U-$^{14}$C]glucose and 3-hydroxy[3-$^{14}$C]butyrate into lipids in different ways. Although (−)-hydroxycitrate did not inhibit [U-$^{14}$C]glucose incorporation into lipids in slices from adult rats, lipid synthesis utilizing 3-hydroxy[3-$^{14}$C]butyrate was far more sensitive than acetylcholine synthesis to inhibition by (−)-hydroxycitrate (Fig. 1).

High concentrations of compounds which block different aspects of citrate transport out of mitochondria interfered with acetylcholine synthesis. 1,2,3-Benzenetricarboxylate inhibits mitochondrial

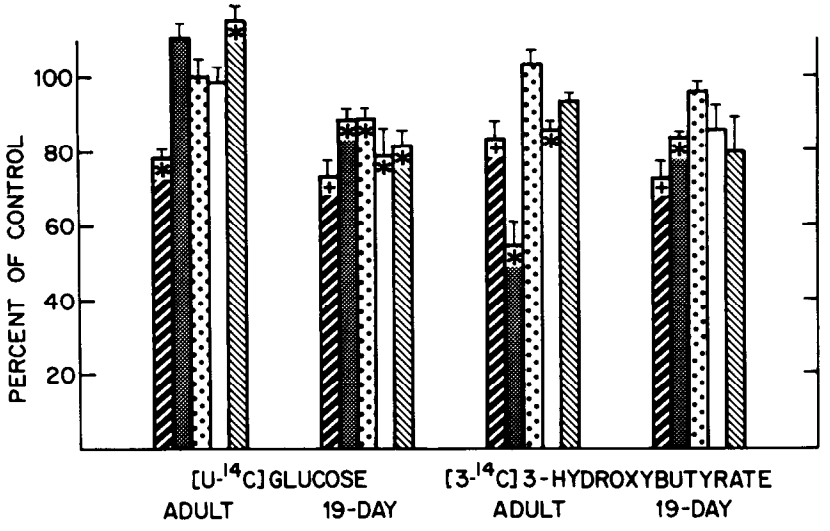


Fig. 1. (−)-Hydroxycitrate (2.5 mM) and the utilization of [U-$^{14}$C]glucose and 3-hydroxy[3-$^{14}$C]butyrate by brain slices. Slices were prepared and incubated as described under Methods. Slices were incubated either with 5 mM [U-$^{14}$C]glucose (0.3 $\mu$Ci/$\mu$mole) or 5 mM glucose + 2 mM 3-hydroxy[3-$^{14}$C]butyrate (1 $\mu$Ci/$\mu$mole; Refs. 19–21), and the incorporation of radioactivity into acetylcholine (▨), lipid (▩), $CO_2$ (▩), nucleic acids (□), and protein (▨) was determined. Each value is the percentage of control ± S.E.M. of at least two experiments in triplicate. Significate difference from control is indicated by an asterisk (*) ($P < 0.05$) or a cross (+) ($P < 0.01$). The following control values (nmoles of the $^{14}$C compound incorporated mg of protein/hr ± S.E.M.) with at least six determinations were obtained: adult slices utilizing [U-$^{14}$C]glucose, acetylcholine (0.51 ± 0.02), lipid (2.15 ± 0.08), $CO_2$ (111.4 ± 4.3), nucleic acids (0.64 ± 0.03) and protein (1.05 ± 0.03); infant slices utilizing [U-$^{14}$C]glucose, acetylcholine (0.43 ± 0.01), lipid (4.18 ± 0.10), $CO_2$ (80.8 ± 2.9), nucleic acids (0.48 ± 0.05) and protein (3.5 ± 0.4); adult slices utilizing 3-hydroxy[3-$^{14}$C]butyrate, acetylcholine (0.118 ± 0.004), lipid (0.33 ± 0.01), $CO_2$ (50.4 ± 2.0), nucleic acids (0.028 ± 0.002) and protein (0.103 ± 0.006); infant slices utilizing 3-hydroxy[3-$^{14}$C]butyrate, acetylcholine (0.23 ± 0.03), lipid (1.54 ± 0.14), $CO_2$ (62.2 ± 1.8), nucleic acids (0.024 ± 0.002) and protein (0.32 ± 0.03). To convert the d.p.m. in acetylcholine to nmoles of acetylcholine produced, the [U-$^{14}$C]glucose values should be multiplied by 3 and the 3-hydroxy[3-$^{14}$C]butyrate values by 2 [2, 20, 28, 29].

Table 1. Citrate metabolism and acetylcholine synthesis by synaptosomes*

| | Acetylcholine from [U-$^{14}$C]glucose | Acetylcholine by g.c.–m.s. (nmoles) | Acetylcholine specific activity | $^{14}CO_2$ from [U-$^{14}$C]glucose |
|---|---|---|---|---|
| Control | 100.0 ± 0.9 | 100.0 ± 2.5 | 100.0 ± 2.9 | 100.0 ± 0.7 |
| Citrate | 76.0 ± 1.8† | 85.3 ± 1.9† | 94.6 ± 2.2 | 126.4 ± 8.7† |
| Hydroxycitrate | 74.9 ± 2.5† | 66.4 ± 12.4† | 126.2 ± 19.3 | 127.0 ± 10.9† |
| 1,2,3-Benzene tricarboxylate | 75.5 ± 1.4† | 78.7 ± 4.9† | 97.5 ± 7.0 | 88.4 ± 3.2† |
| *n*-Butylmalonate | 114.5 ± 2.8† | 127.2 ± 5.7† | 90.9 ± 4.7 | 116.6 ± 6.2† |

* Synaptosomes were prepared and incubated as described in Methods. The media glucose was 1.5 mM and contained 7 $\mu$Ci [U-$^{14}$C]glucose. Citrate (10 mM), hydroxycitrate (2.5 mM), *n*-butylmalonate (5 mM) and 1,2,3-benzenetricarboxylate (10 mM) were added only after careful adjustment of the pH to 7.4. Acetylcholine specific activity was calculated by dividing the d.p.m. in acetylcholine from [U-$^{14}$C]glucose by the nmoles of acetylcholine measured by g.c.–m.s. Each value is the percentage of control ± S.E.M. of at least two experiments in quadruplicate. The following control values ± S.E.M. of at least three experiments in quadruplicate were obtained: 0.51 ± 0.03 nmoles acetylcholine/mg of protein/30 min from [U-$^{14}$C]glucose; 0.54 ± 0.04 nmoles acetylcholine/mg of protein/30 min, as measured by g.c.–m.s.; 1178 ± 51 d.p.m./nmole of acetylcholine; 21.8 ± 0.6 nmoles of [U-$^{14}$C]glucose converted to $CO_2$/mg of protein/30 min.

† Significantly different from control ($P < 0.05$).

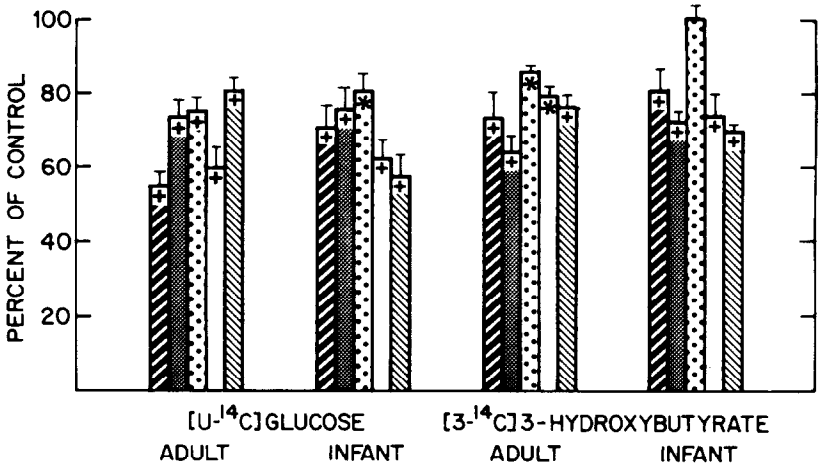


Fig. 2. 1,2,3-Benzenetricarboxylic acid (10 mM) and the utilization of [U-$^{14}$C]glucose and 3-hydroxy[3-$^{14}$C]butyrate by brain slices. Slices were prepared and incubated as described under Methods and in the legend to Fig. 1. Each value is the percentage of control ± S.E.M. of at least two experiments in triplicate for acetylcholine (▨), lipid (▩), $CO_2$ (▩), nucleic acids (□), or protein (▨). Significant difference from control is indicated by an asterisk (*) ($P < 0.05$) or a cross (+) ($P < 0.01$).

citrate transport by inhibiting the tricarboxylate exchange system [30, 31] without affecting other mitochondrial exchange systems. It was added at 10 mM ($K_i$ = 0.16 mM in liver mitochondria [31] or 0.37 mM in brain mitochondria [30]). Incorporation of [U-$^{14}$C]glucose into acetylcholine and all other fractions declined 25–45 per cent in the presence of 10 mM 1,2,3-benzenetricarboxylate (Table 1, Fig. 2). No change in the specific activity of acetylcholine occurred (Table 1). *n*-Butylmalonate inhibits mitochondrial citrate transport by inhibiting only the malate permease system [12, 31, 32]. With [U-$^{14}$C]glucose as precursor, *n*-butylmalonate (5 mM) either caused a slight stimulation (synaptosomes, Table 1) or no change (tissue slices, Fig. 3) in incorporation into acetylcholine. Specific activity was unchanged. Higher concentrations of *n*-butylmalonate (25 or 50 mM) inhibited incorporation of [U-$^{14}$C]glucose into acetylcholine by synaptosomes. In contrast to these results with glucose, incorporation of 3-hydroxy[3-$^{14}$C]butyrate into acetylcholine by brain slices was inhibited by 5 mM butylmalonate. Incorporation into lipids was depressed whether [U-$^{14}$C]glucose or 3-hydroxy[3-$^{14}$C]butyrate was used as substrate.

The specific activity of the acetyl moiety of acetylcholine derived from [U-$^{14}$C]glucose by synaptosomes or tissue slices did not change in the presence of 10 mM citrate; both synthesis from [U-$^{14}$C]glucose (Fig. 4 and Table 1) and total acetylcholine from tissue slices (1.39 ± 0.007 to 0.99 ± 0.10 nmoles/mg of protein/hr) and in synaptosomes (Table 1)

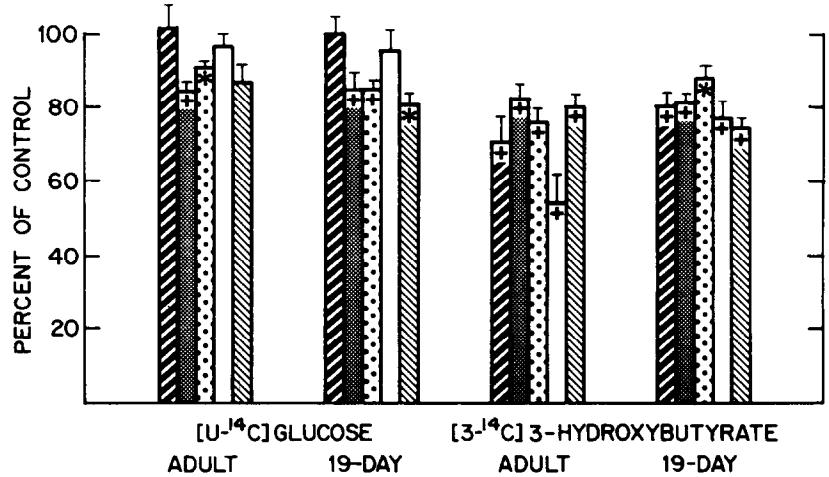


Fig. 3. *n*-Butylmalonate (5 mM) and the utilization of [U-$^{14}$C]glucose and 3-hydroxy[3-$^{14}$C]butyrate by brain slices. Slices were prepared and incubated as described under Methods and in the legend to Fig. 1. Each value is the percentage of control ± S.E.M. of at least two experiments in triplicate for acetylcholine (▨), lipid (▩), $CO_2$ (▩), nucleic acids (□) or protein (▨). Significant difference from control is indicated by an asterisk (*) ($P < 0.05$) or a cross (+) ($P < 0.01$).

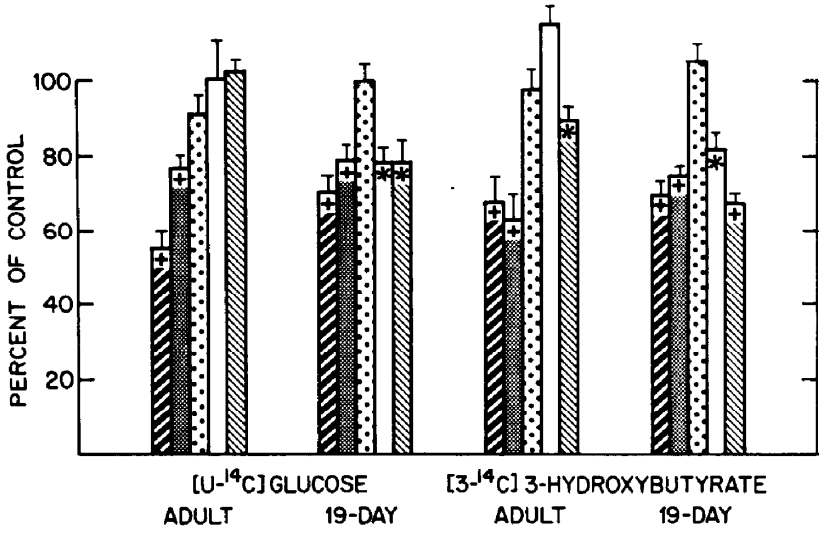


Fig. 4. Citrate (10 mM) and the utilization of [U-$^{14}$C]glucose and 3-hydroxy[3-$^{14}$C]butyrate by brain slices. Slices were prepared and incubated as described under Methods and in the legend to Fig. 1. Each value is the percentage of control ± S.E.M. of at least two experiments in triplicate for acetylcholine (▨), lipid (▩). $CO_2$ (▩), nucleic acids (□) or protein (▩). Significant difference from control is indicated by an asterisk (*) ($P < 0.05$) or a cross (+) ($P < 0.01$).

declined. McLennan and Elliott [33] also found inhibition of acetylcholine synthesis by citrate. The results with tissue slices were similar whether experiments were done with [U-$^{14}$C]glucose or 3-hydroxy[3-$^{14}$C]butyrate or with slices from adult or infant rats. Chelation of calcium has qualitatively similar effects on acetylcholine metabolism, but different effects on glycolysis. EDTA (2 mM) inhibited $CO_2$ production by 24.1 ± 0.8 per cent and acetylcholine production from [U-$^{14}$C]glucose by 46.6 ± 8.2 per cent, but did not affect the specific activity of the acetylcholine (99.6 ± 4 per cent of control). Since $^{14}CO_2$ production was stimulated by citrate (Table 1), it is unlikely that the effect of citrate on acetylcholine metabolism was due to a simple effect on glycolysis. Citrate does enter synaptosomes. The uptake of glucose and of citrate was determined as described in Methods. The uptake of [1,5-$^{14}$C]citrate was 10.7 ± 1.4 nmoles/mg of protein and the uptake of [U-$^{14}$C]glucose was 1.8 ± 0.2 nmoles/mg of protein. Values are the means of three experiments in quadruplicate ± S.E.M. after a 1-min incubation under the conditions in Table 1.

Other compounds which are known to be active acetyl group donors in other biochemical processes altered acetylcholine synthesis. With tissue slices (Table 2), acetylcarnitine stimulated [U-$^{14}$C]glucose incorporation into acetylcholine by 38 per cent but did not decrease the specific activity. The data were also consistent with the hypothesis that *N*-acetyl-L-aspartate, acetyl CoA and acetyl phosphate are direct acetyl donors, since the specific activity of

Table 2. Possible acetyl-group donors and the synthesis of acetylcholine from [U-$^{14}$C]glucose by tissue slices*

| | Acetylcholine from [U-$^{14}$C]glucose (nmoles/mg protein/hr) | [U-$^{14}$C]glucose converted to $^{14}CO_2$ (nmoles/mg protein/hr) |
|---|---|---|
| Control | 1.41 ± 0.14 | 91.4 ± 2.4 |
| *N*-Acetyl-L-aspartic acid | 1.48 ± 0.12 | 88.0 ± 2.7 |
| *N*-Acetylglycine | 1.31 ± 0.19 | 85.6 ± 2.3 |
| *N*-Acetyl-DL-aspartic acid | 1.23 ± 0.06 | 83.0 ± 2.4 |
| *N*-Acetyl-DL-methionine | 1.39 ± 0.10 | 84.8 ± 3.0 |
| *N*-Acetyl-L-glutamic acid | 1.22 ± 0.08 | 78.5 ± 8.5 |
| *N*-Acetyl-L-methionine | 1.28 ± 0.24 | 82.0 ± 3.1† |
| Acetyl phosphate | 1.33 ± 0.34 | 89.1 ± 3.6 |
| Acetylcarnitine | 1.94 ± 0.18† | 78.2 ± 3.0† |
| Carnitine | 1.56 ± 0.18 | 87.4 ± 1.7 |

* Slices were prepared and incubated as described in Methods and in the legend to Fig. 1. All compounds listed were at 10 mM. Each value is the percentage of control ± S.E.M. of at least two experiments in quadruplicate.

† Significantly different from the control ($P < 0.05$).

Table 3. Metabolism of the acetyl group of acetylcholine by synaptosomes*

| | Acetylcholine from [U-$^{14}$C]glucose | Acetylcholine by g.c.–m.s. | Acetylcholine specific activity | $^{14}CO_2$ from [U-$^{14}$C]glucose |
|---|---|---|---|---|
| Control | 100.0 ± 1.2 | 100.0 ± 4.6 | 100.0 ± 3.2 | 100.0 ± 3.0 |
| *N*-Acetyl-L-aspartate | 110.1 ± 3.7† | 130.3 ± 10.7† | 85.0 ± 6.4† | 101.5 ± 1.7 |
| *N*-Acetyl-DL-aspartate | 104.0 ± 4.0 | 116.0 ± 7.7 | 90.8 ± 3.4† | 100.1 ± 2.6 |
| *N*-Acetylglycine | 108.8 ± 3.6† | 104.6 ± 3.9 | 102.4 ± 3.3 | 100.3 ± 5.2 |
| *N*-Acetyl-L-methionine | 105.6 ± 3.7 | 107.1 ± 2.3 | 100.1 ± 4.8 | 109.5 ± 4.0 |
| *N*-Acetyl-L-glutamate | 110.1 ± 3.0 | 118.6 ± 8.4 | 99.4 ± 7.1 | 97.3 ± 2.7 |
| Acetyl CoA | 89.5 ± 6.6 | 127.5 ± 9.3† | 63.5 ± 5.6† | 118.8 ± 2.3† |
| CoA | 99.7 ± 4.1 | 104.0 ± 2.5 | 98.5 ± 4.4 | 98.4 ± 2.2 |
| Acetyl phosphate | 75.7 ± 3.0† | 109.8 ± 7.0 | 65.6 ± 5.0† | 114.6 ± 3.5† |
| Acetylcarnitine | 93.9 ± 3.1 | 85.5 ± 9.4 | 105.1 ± 10.8 | 102.3 ± 1.3 |
| Carnitine | 92.4 ± 3.7† | 160.4 ± 21.7† | 68.5 ± 9.5† | 94.3 ± 2.2† |
| Acetate | 106.7 ± 3.0 | 107.4 ± 3.0 | 99.2 ± 1.4 | 106.2 ± 4.3 |

* Incubation conditions were the same as those given in legend of Table 1. The final concentration of all compounds was 10 mM except for acetyl CoA (1 mM). Each value is the percentage of control ± S.E.M. of at least two experiments in quadruplicate.

† $P < 0.05$, compared to the control.

acetylcholine decreased when they were added to the medium (Table 3). Acetate did not affect the synthesis or the specific activity of the acetylcholine synthesized by synaptosomes (Table 3).

## DISCUSSION

Acetylcholine synthesis is linked to mitochondrial and cytoplasmic metabolism. Several approaches have been used to study cytosolic mitochondrial interaction (cf. Refs. 21 and 22), but all of these require some compromise (e.g. if mitochondria are isolated before they are studied, their metabolic state and their bathing medium are altered). One compromise we have chosen in these studies is to assume that an inhibitor [(−)-hydroxycitrate] of an isolated enzyme (citrate lyase) acts similarly whether the enzyme is isolated or in the synaptosome. In order to minimize the compromise, we added the inhibitor at concentrations three orders of magnitude higher than the $K_i$. An analogous approach has been used for the studies with inhibitors of mitochondrial transport.

Glucose and pyruvate are the most effective precursors of the acetyl moiety for acetylcholine synthesis in adult mammalian brain [1, 2, 34]. Ketone bodies [4, 5] are also effective precursors, particularly in the infant. Although acetate is a good precursor in lobster nerve [14] and corneal epithelium [35], it is a poor precursor of acetylcholine in mammalian brain [Table 3; Refs. 2, 7, 9, 18, 36, 37]. The metabolic pathway which furnishes the acetyl moiety from these precursors for acetylcholine synthesis is unknown. Lefresne *et al.* [38, 39] have suggested that cytoplasmic conversion of pyruvate to acetyl CoA provides the acetyl moiety for acetylcholine synthesis. Their evidence is indirect and, as they suggest [39], is equally compatible with mitochondrial compartmentation. A cytoplasmic pyruvate dehydrogenase coupled to acetylcholine synthesis is difficult to reconcile with four observations: (i) the close coupling of acetylcholine synthesis and mitochondrial metabolism [21, 22], (ii) the inability of synaptoplasmic fractions to synthesize acetylcholine [8], (iii) experiments showing that solubilization of mitochondria increase acetylcholine synthesis by synaptosomes [40], and (iv) the utilization of ketone bodies for acetylcholine synthesis [5] (Figs. 1–4) not occurring via a cytoplasmic pyruvate dehydrogenase.

Citrate has generally been regarded as the transporter of acetyl groups from the mitochondria by analogy with the liver [17, 41]. Experiments in which brain slices were incubated with glucose in carbon-6 labeled with both $^{3}$H and $^{14}$C gave the same labeling pattern in citrate and acetylcholine—the $^{3}H/^{14}C$ ratio was reduced by a third [10, 11]. However, this ratio may vary in the presence of (−)-hydroxycitrate or *n*-butylmalonate [11]. The level of citrate lyase also appears to be adequate to maintain acetylcholine synthesis [18, 42]. However, most other results [9], including those reported in this paper, are inconsistent with citrate being the sole donor. Our results demonstrate that the interference with citrate metabolism does affect acetylcholine metabolism under extreme conditions. The relatively small effect of high concentrations of (−)-hydroxycitrate (2.5 mM; $K_i$ for citrate lyase is 0.8 μM; Refs. 17 and 42) and the variable effects of the citrate transport inhibitors both support this conclusion. Although it is difficult to evaluate directly the effects of these compounds in the presence of both mitochondria and synaptoplasm, the concentrations are excessive for either the isolated enzyme or purified mitochondria respectively. Szutowicz *et al.* [27] were unable to show citrate accumulation in the presence of hydroxycitrate unless citrate lyase was activated by adding ATP:Mg. Although many of their incubation conditions were different than ours (i.e. they used

pyruvate as substrate), if this is also true in our synaptosomes, considerable acetylcholine synthesis occurs in the absence of activated citrate lyase. Furthermore, 10 mM citrate did not decrease the specific activity of acetylcholine from [U-$^{14}$C]glucose even though citrate readily penetrated the synaptosome. If citrate is an intermediate, it is not rate limiting, since high levels did not increase acetylcholine levels. Inhibition of the malate permease component of citrate transport by *n*-butylmalonate did not inhibit synthesis. High concentrations (60 times $K_i$) of the citrate transport inhibitor 1,2,3-benzenetricarboxylate decreased total acetylcholine and synthesis from [U $^{14}$C]glucose by 25–45 per cent; it had no effect on pecific activities. Moreover, the changes in activ.'y of citrate lyase with development do not correlate with changes in choline acetyltransferase or acetylcholine biosynthesis [43, 44]. Tucek and Cheng [9] showed citrate to be a poor precursor of acetylcholine *in vivo*, even though it was incorporated into lipids and demonstrated the same subcellular distribution as [2-$^{14}$C]pyruvate. In their studies, only trace amounts of radioactive compounds were injected. Since exogenous compounds are metabolized in metabolic pools different from those of endogenous compounds [45], we attempted to flood all of the pools by adding high concentrations of potential acetyl donors. The data indicate citrate is not the sole intermediate. Walter and Soling [46] also concluded that citrate was not the sole transport form of acetyl groups for fatty acid synthesis in liver. Patel and Owen [26] concluded that extramitochondrial citrate is only one of at least two sources of acetyl groups for lipid synthesis in infant rats. These results with citrate and (−)-hydroxycitrate also suggest that the oxogluturate or glutamate shunt is not important in acetylcholine synthesis, since the final step in such a pathway is citrate [47–49].

Carnitine and acetylcarnitine variably affect acetylcholine metabolism. Acetylcarnitine did not appear to be a direct donor or transport form of acetyl groups. It stimulated synthesis of acetylcholine from glucose in slices, but did not decrease the specific activity. It was without effect on acetylcholine synthesis in synaptosomes. Carnitine was without effect in slices, but stimulated synthesis in synaptosomes from non-glucose sources (i.e. the specific activity decreased). Carnitine decreases incorporation of acetate into acetylcholine *in vivo* [9]. It may act by stimulating fatty acid conversion through acetoacetate to acetyl precursors. Acetylcarnitine stimulates acetylcholine synthesis in acetone powders from mammalian brain [40]. Acetylcarnitine is released from mitochondria supplemented with carnitine, but it is not coupled with acetylcholine synthesis [31].

*N*-Acetyl-L-aspartate and acetyl phosphate appear to donate acetyl groups (i.e. they increase the synthesis of acetylcholine and decrease the specific activity); this probably includes the transfer of the acetyl group to CoA. *N*-Acetyl-L-aspartate may be a source of acetyl groups for fatty acid synthesis [48, 49]; it has been disregarded as a precursor of acetylcholine *in vivo* because of its charge and large, slowly turning-over pool [37]. It is the only compound added in near physiological concentrations. Acetylaspartate and the other *N*-acetylated amino acids tested can be utilized by acetone powders of brain for acetylcholine synthesis [50], but have not been examined previously in brain slices or synaptosomes. Although acetyl phosphate has received considerable attention in microbial chemistry [51], its role in mammalian biochemistry, including acetylcholine synthesis, has not been clarified. Although it has not been identified in mammalian tissues, high levels of acetyl phosphatases make its isolation difficult [51]. Utilization of acetyl phosphate by the nervous system has been established [52].

Cellular acetyl groups exist in at least two compartments; one labels lipids and one labels acetylcholine [3, 5, 16, 19, 21, 22, 37, 53]. *In vivo*, exogneously added citrate labels lipids, but not acetylcholine [9]. In these studies, incorporation into lipid and acetylcholine was affected differentially by inhibitors. (−)-Hydroxycitrate did not affect incorporation of [U-$^{14}$C]glucose into lipids by slices from adult brains. Hydroxycitrate decreases incorporation of 3-hydroxy[3-$^{14}$C]butyrate into lipids by 50 per cent, whereas incorporation of either precursor into acetylcholine is inhibited by only 20 per cent. The inhibitors do not affect incorporation of 3-hydroxybutyrate and glucose similarly. *n*-Butylmalonate inhibits incorporation of [U-$^{14}$C]glucose into lipids but not into acetylcholine. However, *n*-butylmalonate inhibits incorporation of 3-hydroxy[3-$^{14}$C]butyrate into lipids and acetylcholine. *In vivo*, carnitine increases the incorporation of label from [1-$^{14}$C]acetate into brain lipids, but lowers its incorporation into acetylcholine [9].

Although (−)-hydroxycitrate and citrate inhibit the metabolism of [U-$^{14}$C]glucose by the infant brain more than by the adult brain, the metabolism of the infant and adult brain slices responds similarly to the other perturbations, even though the contribution of 3-hydroxybutyrate to acetylcholine synthesis is much greater in the infant brain [4, 5].

There is a close link between oxidative metabolism and acetylcholine synthesis [1, 16, 20–22, 53, 54]. A decrease in acetylcholine synthesis accompanies a decrease in oxidative metabolism. The decrease is linearly related to a decrease in the transmitochondrial membrane potential [21, 53]. The molecular basis of the linkage between acetylcholine synthesis and oxidative metabolism may be that the transport of the acetyl moiety for acetylcholine synthesis from mitochondria is linked to the transmitochondrial membrane potential [21, 53]. This hypothesis cannot be tested until the transport form of the acetyl moiety is established.

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## REFERENCES

1. J. H. Quastel, M. Tennenbaum and A. H. M. Wheatley, *Biochem. J.* **33**, 1668 (1936).
2. E. T. Browning and M. P. Schulman, *J. Neurochem.* **15**, 1391 (1968).

3. R. Nakamura and S. C. Cheng, *Life Sci.* **8**, 657 (1969).
4. T. Itoh and J. H. Quastel, *Biochem. J.* **116**, 641 (1970).
5. G. E. Gibson and J. P. Blass, *Biochem. Pharmac.* **28**, 133 (1979).
6. E. T. Browning, in *Biology of Cholinergic Function* (Eds. A. M. Goldberg and I. Hanin), p. 187. Raven Press, New York (1976).
7. S. Tucek, *Biochem. J.* **104**, 749 (1967).
8. R. L. Polak, P. C. Molenaar and P. Braggaar-Schaap, in *Cholinergic Mechanisms and Psychoparmacology* (Ed. D. J. Jenden), p. 511. Plenum Press, New York (1978).
9. S. Tucek and S. C. Cheng, *J. Neurochem.* **22**, 893 (1974).
10. J. Sollenberg and B. Sorbo, *J. Neurochem.* **17**, 201 (1970).
11. G. H. Sterling and J. J. O'Neill, *J. Neurochem.* **31**, 525 (1978).
12. W. D. Thomitzek and E. Strack, *Acta biol. med. germ.* **13**, 110 (1964).
13. J. A. Watson, M. Fang and J. M. Lowenstein, *Archs Biochem. Biophys.* **135**, 209 (1969).
14. S. C. Cheng and R. Nakamura, *Biochem. J.* **118**, 451 (1970).
15. G. E. Gibson and M. Shimada, *Trans. Am. Soc. Neurochem.* **8**, 120 (1977).
16. G. E. Gibson and J. P. Blass, *Trans. Am. Soc. Neurochem.* **9**, 1 (1978).
17. J. A. Watson and J. M. Lowenstein, *J. biol. Chem.* **245**, 5993 (1970).
18. S. Tucek, *J. Neurochem.* **14**, 519 (1967).
19. G. E. Gibson and J. P. Blass, *J. Neurochem.* **30**, 71 (1978).
20. G. E. Gibson, R. Jope and J. P. Blass, *Biochem. J.* **148**, 17 (1975).
21. G. E. Gibson and J. P. Blass, *J. biol. Chem.* **251**, 4127 (1976).
22. G. E. Gibson and J. P. Blass, *J. Neurochem.* **26**, 1073 (1976).
23. J. J. Freeman, R. L. Choi and D. J. Jenden, *J. Neurochem.* **24**, 729 (1975).
24. R. I. Glazer and G. Weber, *J. Neurochem.* **18**, 1569 (1971).
25. M. A. Verity, *J. Neurochem.* **19**, 1305 (1972).
26. M. S. Patel and O. E. Owen, *Biochem. J.* **156**, 603 (1976).
27. A. Szutowicz, W. Lysiak and S. Angielski, *J. Neurochem.* **29**, 375 (1977).
28. D. Grewaal and J. H. Quastel, *Biochem. J.* **132**, 1 (1973).
29. P. Lefresne, P. Guyenet and J. Glowinski, *J. Neurochem.* **20**, 1083 (1973).
30. M. S. Patel, *Brain Res.* **98**, 607 (1975).
31. B. H. Robinson, G. R. Williams, M. L. Halperin and C. C. Leznoff, *Eur. J. Biochem.* **20**, 65 (1971).
32. B. H. Robinson and J. B. Chappell, *Biochem. biophys. Res. Commun.* **28**, 249 (1967).
33. H. McLennan and K. A. C. Elliott, *Am. J. Physiol.* **163**, 605 (1950).
34. J. H. Quastel, in *Cholinergic Mechanisms and Psychoparmacology* (Ed. D. J. Jenden), p. 411. Plenum Press, New York (1978).
35. G. G. Fitzgerald and J. R. Cooper, *Fedn Proc.* **26**, 651 (1968).
36. P. J. G. Mann, M. Tennenbaum and J. H. Quastel, *Biochem. J.* **32**, 243 (1938).
37. R. Nakamura, S. C. Cheng and H. Naruse, *Biochem. J.* **118**, 443 (1970).
38. P. Lefresne, J. C. Beaujouan and J. Glowinski, *Biochimie* **60**, 479 (1978).
39. P. Lefresne, M. Hamon, J. C. Beaujouan and J. Glowinski, *Biochimie* **59**, 197 (1977).
40. P. Lefresne, P. Guyenet, J. C. Beaujouan and J. Glowinski, *J. Neurochem.* **25**, 415 (1975).
41. A. F. Spencer and J. M. Lowenstein, *J. biol. Chem.* **237**, 3640 (1962).
42. A. Szutowicz, M. Stepien, W. Lysiak and S. Angielski, *Acta biochim. pol.* **23**, 227 (1976).
43. A. Szutowicz, W. A. Frazier and R. A. Bradshaw, *J. biol. Chem.* **251**, 1516 (1975).
44. P. C. Marchisio and G. Giacobini, *Brain Res.* **15**, 301 (1969).
45. P. C. Molenaar and R. L. Polak, *J. Neurochem.* **26**, 95 (1976).
46. U. Walter and J. Soling, *Fedn Eur. Biochem. Soc.* **63**, 260 (1976).
47. H. Waelsch, S. C. Cheng, L. Cote and H. Naruse, *Proc. natn. Acad. Sci. U.S.A.* **54**, 1249 (1965).
48. A. F. D'Adamo and F. M. Yatsu, *J. Neurochem.* **13**, 961 (1966).
49. A. F. D'Adamo, Jr. and A. P. D'Adamo, *J. Neurochem.* **15**, 315 (1968).
50. H. C. Buniatian, V. S. Hovhannissian and G. V. Aprlkian, *J. Neurochem.* **12**, 695 (1965).
51. F. Lipman, *Adv. Enzymol.* **6**, 231 (1946).
52. A. Mizutani and K. Sugimura, *Acta Histochem. Cytochem.* **10**, 150 (1977).
53. G. E. Gibson and J. P. Blass, *J. Neurochem.* **27**, 37 (1976).
54. G. E. Gibson, M. Shimada and J. P. Blass, *J. Neurochem.* **31**, 757 (1978).