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Decreased Synthesis of Acetylcholine Accompanying Impaired Oxidation of Pyruvic Acid in Rat Brain Minces

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The relation between pyruvate utilization and acetylcholine synthesis was investigated in minces of adult rat brain. The flux of pyruvate to acetylcholine was less than 1% of that to CO_2; nevertheless, a number of agents which inhibited conversion of [1-^{14}C]-pyruvate or [2-^{14}C]pyruvate into $^{14}CO_2$ were associated with corresponding decreases in the conversion of [2-^{14}C]pyruvate into acetylcholine. The amount of acetylcholine produced by minces of whole rat brain, measured by g.l.c.–mass spectrometry, decreased similarly. Among the inhibitory compounds tested were 3-bromopyruvate, an irreversible inhibitor of pyruvate dehydrogenase; 2-oxobutyrate, a competitive inhibitor of pyruvate dehydrogenase; other 2-oxo acids; and amobarbital and pentobarbital. Linear-regression equations relating CO_2 production to acetylcholine synthesis gave correlation coefficients of 0.89–0.93 for the combined observations. The inhibition of acetylcholine synthesis could not be attributed to inhibition of choline acetyltransferase. Incorporation of [2-^{14}C]pyruvate into lipids, proteins and nucleic acids was effected less than that into acetylcholine. Under these experimental conditions, it was shown that pyruvate utilization can limit acetylcholine synthesis.

For decades, impaired carbohydrate metabolism has been known to cause neurological dysfunction (Kinnersley & Peters, 1929). More recently, neurological disorders have been associated with inherited deficiencies of specific enzymes of carbohydrate metabolism, including the pyruvate dehydrogenase complex (Blass *et al.*, 1970, 1971).

The precise biochemical mechanisms of brain damage in such conditions are still poorly understood. The obvious possibility was impaired production of energy in the form of ATP or energy charge potential (Atkinson, 1968), but MacMillan & Siesjo (1972) and Liao & Yatsu (1973) have shown that hypoxia can cause profound and irreversible brain damage despite normal or near-normal values for ATP concentration and energy charge. Another possibility is impaired synthesis of neurotransmitters, including specifically acetylcholine, because the acetyl group of acetylcholine is normally derived from glucose and pyruvate (Quastel *et al.*, 1936; Grewaal & Quastel, 1973). This possibility has generally been discounted since maximal synthesis of acetylcholine would require less than 1% of the normal carbohydrate utilization of the whole brain (McIlwain & Bachelard, 1971). However, we have found that the synthesis of acetylcholine by minces of rat brain was decreased in proportion to decrease in pyruvate oxidation (Gibson *et al.*, 1974). The details of those experiments are reported here.

Experimental

Reagents

Siliclad was from VWR Scientific, Los Angeles, Calif., U.S.A.; Aquasol was from New England Nuclear Corp., Boston, Mass., U.S.A.; Bio-Rex 9 and AG-1 ion-exchange resins were from Bio-Rad Laboratories, Richmond, Calif., U.S.A.; CM-cellulose t.l.c. plates were from Brinkman Instruments Inc., Burlingame, Calif., U.S.A.; sodium amobarbital and sodium pentobarbital (50mg in 1ml of 10% alcohol and 40% propylene glycol) were from the pharmacy of UCLA Medical School; acetylcholinesterase (EC 3.1.1.7; from electric eel; 200units/mg of protein) was from Sigma Chemical Co., St. Louis, Mo., U.S.A.; and paraoxon (diethyl 4-nitrophenyl phosphate) was from K & K Laboratories, Hollywood, Calif., U.S.A.

[*acetyl*-1-^{14}C]Acetyl-CoA (40–60Ci/mol), [*acetyl*-1-^{14}C]acetylcholine iodide (1–5Ci/mol), sodium [1-^{14}C]pyruvate (2–10Ci/mol) and sodium [2-^{14}C]-pyruvate (2–10Ci/mol) were obtained from New England Nuclear Corp. Because of the instability of radioactive pyruvate (Silverstein & Boyer, 1964), it was stored as desiccated crystals at −20°C and dissolved just before incubation.

Methods

The whole brains of male Sprague–Dawley rats (200–300g) were removed and placed in ice-cold

modified Krebs–Ringer phosphate, pH 7.4: 141 mM-NaCl, 31 mM-KCl, 2.3 mM-$CaCl_2$, 1.3 mM-$MgSO_4$ and 10.3 mM-Na_2HPO_4 (Itoh & Quastel, 1970). Whole brains were chopped into prisms with a McIlwain chopper (Brinkman Instrument Co.) set for 0.3 mm intervals. The resulting mince was triturated with a wide-tipped plastic pipette, allowed to settle and the excess of fluid removed. Mince, containing 5–10 mg of protein, was added to the incubation media, to a final volume of 3 ml, in silicone-treated 25 ml Erlenmeyer flasks. The incubation medium was the modified Krebs–Ringer phosphate containing 2 mM-choline chloride, 40 μM-paraoxon, 5 mM-sodium pyruvate and either 1 μCi of [2-^{14}C]pyruvate or 0.14 μCi of [1-^{14}C]pyruvate. Other compounds were included at the final concentrations indicated in the legends. After addition of the mince, O_2+CO_2 (95:5) was blown on to the suspended minces for 15 s. The vessels were sealed with serum stoppers with suspended Kontes cups containing 0.2 ml of 1 M-Hyamine on filter paper, to trap the $^{14}CO_2$. After incubation for 60 min at 37°C, 0.5 ml of ice-cold 1.4 M-$HClO_4$ – 0.035 M-tetraethylammonium chloride was injected and the flasks were kept on ice for 10 min. The serum caps were removed and Kontes cups were added to 15 ml of scintillant, consisting of toluene–methanol (2:1, v/v), 0.018 M-2,5-diphenyloxazole and 0.00025 M-1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene. The acidified contents of the flasks were transferred with silicone-treated Pasteur pipettes to polypropylene centrifuge tubes (10 mm × 16 mm). The flasks were rinsed twice with 0.5 ml of 0.2 M-$HClO_4$ and 0.005 M-tetraethylammonium chloride. The combined $HClO_4$ extracts were centrifuged for 10 min at 25000g at 0°C (International Centrifuge model B-20 rotor number 874). The pellet was washed with 1 ml of 0.2 M-$HClO_4$, 5 mM-tetraethylammonium chloride.

Acetylcholine was isolated from the $HClO_4$ extract by a modification of the procedure of Hanin & Jenden (1969). Ice-cold saturated ammonium reineckate (3.5 ml) adjusted to pH 2 with conc. $HClO_4$, was added to the 5.5 ml of the $HClO_4$ acid extract obtained above to precipitate the acetylcholine. The precipitation was allowed to proceed for 1 h. After centrifugation for 20 min at 25000g, the precipitate was washed twice with 3.5 ml of the ammonium reineckate. The final pellet was resuspended in 2 ml of methanol adjusted to pH 4.5 with conc. acetic acid and containing 0.075 g of Bio-Rex 9-Cl/ml. These suspensions were then added to Pasteur pipette columns which contained glass wool, a layer of pyrollized sand, and 75 mg of Bio-Rex 9-Cl in 1 ml of acidified methanol. The acetylcholine chloride was eluted into scintillation vials with 4 × 1 ml of methanol, pH 4.5, and counted for radioactivity after addition of 10 ml of Aquasol. Values for acetylcholine were calculated on the assumption that the specific radioactivity of acetylcholine was equal to the specific radioactivity of the precursor pyruvate (Browning & Schulman, 1968; Grewaal & Quastel, 1973; LeFresne *et al.*, 1973). The recovery of acetylcholine was measured by adding [*acetyl*-1-^{14}C]-acetylcholine to flasks without [^{14}C]pyruvate at the beginning of the isolation procedure. Acetylcholine was also measured by g.l.c.–mass spectrometry (Jenden *et al.*, 1974).

Lipids, nucleic acids and proteins were extracted from the $HClO_4$-insoluble pellet by the method of Glazer & Weber (1971), except that RNA and DNA were extracted together with two 20 min incubations at 70°C in 0.5 M-$HClO_4$. The E_{265} of the combined supernatants was measured in a Unicam SP. 1800 recording u.v. spectrophotometer and the mass calculated by using $\varepsilon = 12.4 \times 10^{-3} mol^{-1} \cdot cm^{-1}$. Samples were counted for radioactivity by liquid scintillation, in Aquasol.

All radioactive measurements were done in a Packard liquid-scintillation counter. C.p.m. were converted into d.p.m. by the sample channels-ratio method (Wang & Willis, 1965).

To identify acetylcholine, the radioactive product was hydrolysed by incubation for 60 min at 37°C in 0.22 ml containing 0.1 mg of acetylcholinesterase, 0.005 M-acetylcholine, 0.1 M-NaCl, 0.02 M-$MgCl_2$ and 0.2 M-NaH_2PO_4, pH 7.0. The radioactive acetate was removed by column chromatography and the degree of hydrolysis estimated by the method of Wilson *et al.* (1972). Paper chromatography (Marchbanks & Israel, 1971) was on Whatman no. 1 filter paper and t.l.c. (Wilson *et al.*, 1972) on commercial CM-cellulose plates; radioactivity was located with a Packard strip scanner no. 7201.

Choline acetyltransferase (EC 3.1.1.7) was measured by the method of Wilson *et al.* (1972).

Results

Several criteria established the identity of the radioactive material in the acetylcholine fraction. Acetylcholinesterase hydrolysed the radioactive product by 93 ± 1.2% as shown by ten determinations with the products of five incubations. By comparison, commercial [*acetyl*-1-^{14}C]acetylcholine was hydrolysed 99.3 ± 0.2%. Standard acetylcholine and the radioactive product migrated similarly on paper and thin-layer chromatography. If the cholinesterase inhibitor, paraoxon, was omitted from the incubation medium, no radioactive product was detected. When flasks were incubated without tissue, radioactivities in the acetylcholine fraction were about 10% of those in the presence of mince. Recovery of standard [^{14}C]acetylcholine was greater than 95%.

Incorporation of radioactivity from [2-^{14}C]pyruvate into lipids and nucleic acids was not strictly proportional to time, whereas incorporation into

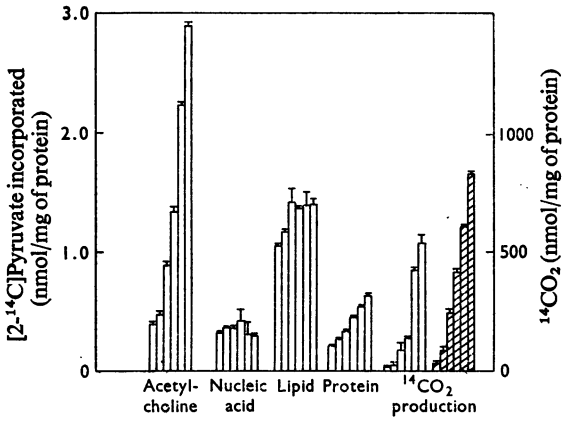

Fig. 1. *Pyruvate utilization with time*

Each bar represents incorporation of radioactivity from [2-^{14}C]pyruvate into the indicated fraction, except for the hatched bars on the right which indicate $^{14}CO_2$ from [1-^{14}C]pyruvate. In this Figure, mg of protein is a measure of the amount of brain tissue added to the flask. Values are means for three determinations ± S.E.M.; from left to right, they indicate values at 5, 10, 20, 40, 60 and 80min. Each flask contained approx. 6mg of protein. See the text for experimental details. Note that the magnitudes of the axes differ.

the other fractions and production of $^{14}CO_2$ was (Fig. 1). Incorporation of radioactivity into all fractions was proportional to the amount of protein added. About 200 times as much radioactivity from pyruvate was converted into CO_2 as was incorporated into acetylcholine (Fig. 1).

Structural analogues of pyruvate inhibited the production of acetylcholine from [2-^{14}C]pyruvate, and did so in proportion to the inhibition of pyruvate oxidation. Bromopyruvic acid was quantitatively more effective than 2-oxobutyric acid (Fig. 2) or 2-oxo-4-methylpentanoic acid (cf. Fig. 4). Even slight inhibition of pyruvate utilization decreased acetylcholine synthesis: 0.1mM-bromopyruvate inhibited $^{14}CO_2$ production by 7% and acetylcholine production by 12%. Parallel inhibition of $^{14}CO_2$ production from [1-^{14}C]pyruvate and [2-^{14}C]pyruvate occurred with bromopyruvate or 2-oxobutyric acid; 2-oxo-4-methylpentanoic acid was a more effective inhibitor of $^{14}CO_2$ production from [2-^{14}C]pyruvate than from [1-^{14}C]pyruvate.

Amobarbital at a concentration of 1mM inhibited acetylcholine synthesis and $^{14}CO_2$ production to similar extents (Fig. 3), but 0.05mM-amobarbital slightly stimulated acetylcholine synthesis ($p<0.01$) although it slightly decreased $^{14}CO_2$ production from either [1-^{14}C]pyruvate or [2-^{14}C]pyruvate ($P<0.02$). Pentobarbital had a similar biphasic effect.

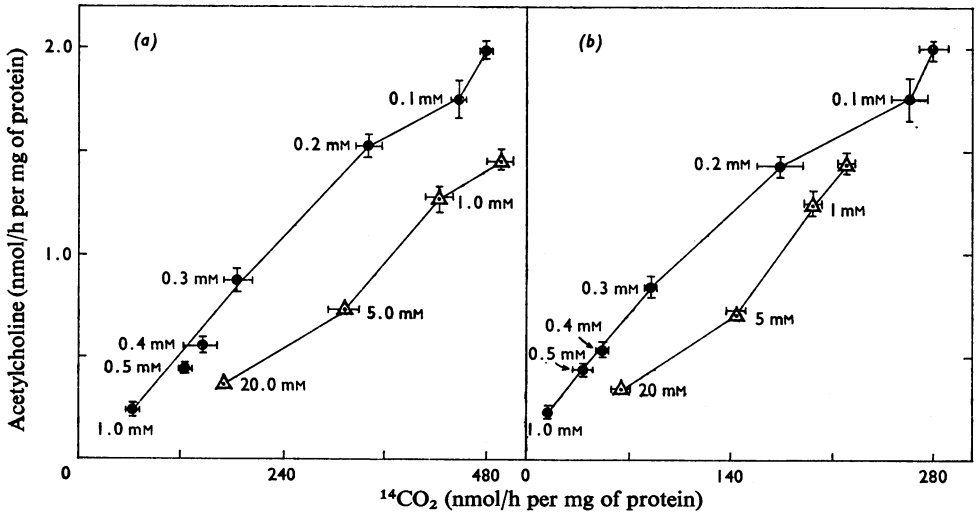

Fig. 2. *Inhibition of pyruvate oxidation and of acetylcholine synthesis by bromopyruvate* (●) *and 2-oxybutyrate* (△)

Incorporation of radioactivity from [2-^{14}C]pyruvate into acetylcholine is indicated on the vertical axis. Production of $^{14}CO_2$ is indicated on the horizontal axis, from [1-^{14}C]pyruvate (*a*) or from [2-^{14}C]pyruvate (*b*). The mM values refer to the final concentrations of inhibitor. Each value is the mean of at least two experiments done in triplicate, ±S.E.M. See the text for experimental details. Note the differences in magnitude of the vertical and horizontal axes.

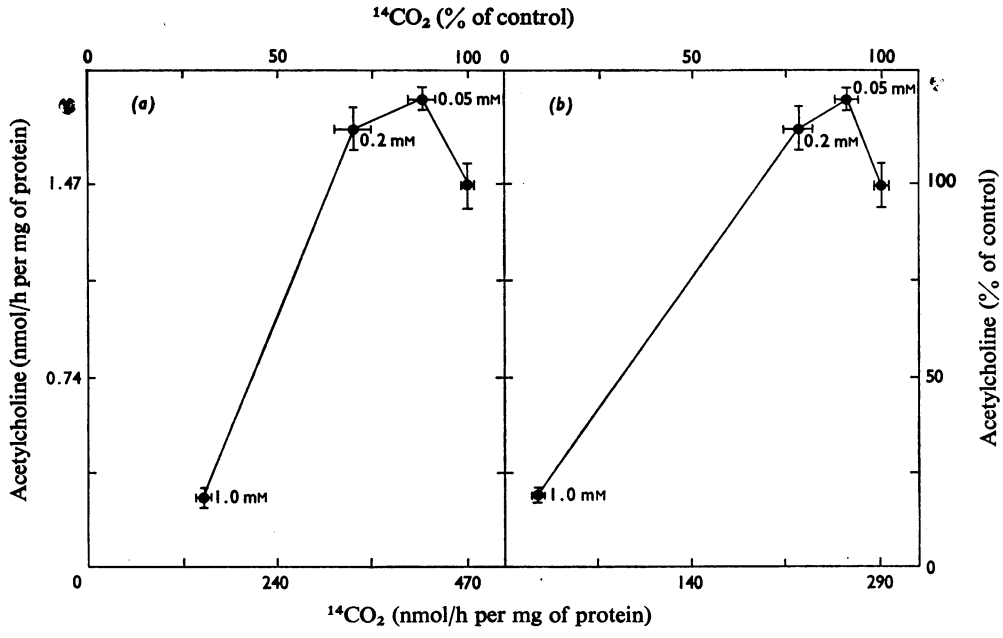

Fig. 3. *Effect of amobarbital on pyruvate oxidation and acetylcholine production*

See the legend of Fig. 2 for description of the conventions. Values are the means of at least three experiments done in triplicate, ±S.E.M.

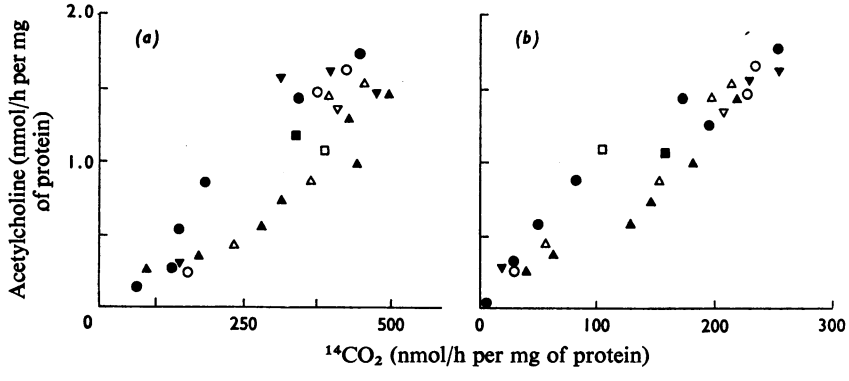

Fig. 4. *Inhibition of acetylcholine synthesis and of* [1-^{14}C]*pyruvate oxidation* (*a*) *and* [2-^{14}C]*pyruvate oxidation* (*b*) *by several compounds*

Each value is the mean of at least two experiments done in triplicate. Inhibitors were: 5mM-leucine (▽); 5mM-2-oxo-3-methylpentanoic acid (□); 5mM-2-oxo-3-methylbutanoate (■); 0.05, 0.2 and 1.0mM-pentobarbital (○); 1, 5 and 20mM-2-oxo-4-methylpentanoate (△); bromopyruvate, at all concentrations shown in Fig. 2 (●); 2-oxobutyrate, at concentrations shown in Fig. 2 (▲); and amobarbital, at concentrations shown in Fig. 3 (▼).

A summary of the relation of acetylcholine synthesis to the production of $^{14}CO_2$ from [1-^{14}C]-pyruvate is presented in Fig. 4(*a*). In addition to the above-mentioned data, results are shown for 5mM-2-oxo-3-methylbutanoic acid, 5mM-2-oxo-3-methylpentanoic acid and 5mM-leucine. A comparison of the axes shows that the flux to $^{14}CO_2$ is 250 times greater than the acetylcholine production. A regression equation relating the two processes was calculated by the method of least squares (Steele &

Torrie, 1960). The two processes are related by the equation

$$\frac{d[\text{acetylcholine}]}{dt} = 3.6\times10^{-3}\frac{d^{14}CO_2}{dt} - 0.11.$$

The correlation coefficient is 0.89. Acetylcholine synthesis and production of $^{14}CO_2$ from [2-^{14}C]-pyruvate (Fig. 4*b*) are related by the equation

$$\frac{d[\text{acetylcholine}]}{dt} = 6.2\times10^{-3}\frac{d^{14}CO_2}{dt} + 0.066.$$

The correlation coefficient is 0.92.

Inhibitors of pyruvate utilization decreased not only the incorporation of radioactivity into acetylcholine but also the mass of acetylcholine present as measured by g.l.c.–mass spectrometry (Table 1). The radiochemical method detected 76±6% (S.E.M., eight experiments) of the acetylcholine detected by g.l.c.–mass spectrometry. This difference between radiochemical and mass measurements of acetylcholine agrees with the observations of Guyenet *et al.* (1973).

Table 1. *Comparison of radiometric and g.l.c.–mass-spectrometric measurements of acetylcholine*

Values are the means for eleven determinations for the controls and six for each inhibitor, ±S.E.M. See the text and Jenden *et al.* (1974) for details of the methods.

	Acetylcholine (nmol/h per mg of protein)	
	G.l.c.–mass spectrometric method	Radiometric method
Control	1.62±0.05	1.09±0.04
+2-Oxobutyrate (20mM)	0.45±0.01	0.18±0.02
+2-Oxo-4-Methylpentanoate (20mM)	0.45±0.01	0.19±0.01
+3-Bromopyruvate (0.5mM)	0.40±0.02	0.20±0.02
+Amobarbital (1.0mM)	0.27±0.01	0.16±0.02
+Pentobarbital (1.0mM)	0.18±0.01	0.12±0.01

The incorporation of [2-^{14}C]pyruvate into nucleic acids, lipids and protein was inhibited less than its incorporation into acetylcholine (Table 2).

Bromopyruvate was the only compound that inhibited acetylcholine production in the mince and also has more than a slight effect on choline acetyltransferase, as measured in a triton-treated homogenate (Fig. 5). Inhibition of acetylcholine synthesis and $^{14}CO_2$ production in the mince declined together, whereas the inhibition of choline acetyltransferase activity in the homogenate followed a different pattern. 2-Oxobutyric acid (20mM) and 2-oxo-4-methylpentanoic acid (20mM) inhibited choline acetyltransferase by 15 and 18% respectively. Pentobarbital (1mM) and amobarbital (1mM) had

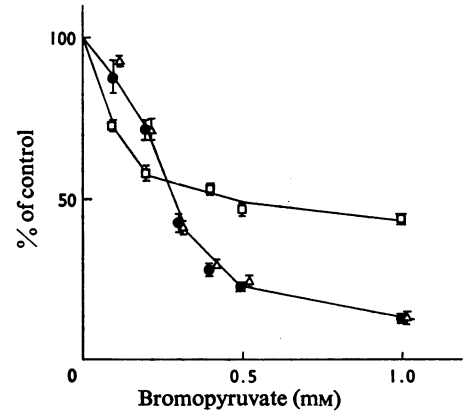

Fig. 5. *Comparison of the effects of bromopyruvate on choline acetyltransferase in homogenates and on pyruvate and acetylcholine metabolism in brain minces*

Choline acetyltransferase activity (□) was measured in cell-free rat brain homogenates (Wilson *et al.*, 1972). Acetylcholine production (●) from [2-^{14}C]pyruvate and $^{14}CO_2$ production from [1-^{14}C]pyruvate (△) were measured in minces as described in the text. Values are means of at least two triplicate experiments, ±S.E.M.

Table 2. *Inhibition by several compounds of the incorporation of [2-^{14}C]pyruvate into lipids, proteins, nucleic acids and acetylcholine*

Values are percentages of control values. Each value is the mean of at least two experiments done in triplicate ±S.E.M. Values in parentheses are final concentrations of the inhibitor.

	Nucleic acids	Protein	Lipid	Acetylcholine
2-Oxobutyrate (20mM)	82.3±7.4	53.7±4.8	58.9± 4.4	25.8±1.8
2-Oxo-4-methylpentanoate (20mM)	62.8±6.7	49.9±2.0	45.4± 3.2	27.0±3.9
3-Bromopyruvate (0.5mM)	56.8±3.5	79.3±3.8	39.4± 2.8	15.8±2.3
Amobarbital (1.0mM)	110.0±6.2	46.2±2.1	81.3±11.1	19.1±2.1
Pentobarbital (1.0mM)	120.1±7.9	47.0±3.1	84.0± 8.1	24.0±4.8

no significant effect. The following compounds at 5mM concentration gave no inhibition of choline acetyltransferase: 2-oxo-3-methylbutanoic acid, 2-oxo-3-methylpentanoic acid, leucine, valine, isoleucine and pyruvate.

Discussion

In the rat brain minces described above, the production of radioactive acetylcholine was proportional to the oxidation of radioactive pyruvic acid, even though less than 0.5% of the pyruvate utilized was converted into acetylcholine. LeFresne *et al.* (1973) found that the initial rate of acetylcholine production was dependent on pyruvate concentration. Michalek *et al.* (1971) observed that triperidol inhibited the oxidation of glucose and pyruvate and the synthesis of acetylcholine. These observations together suggest that acetylcholine synthesis may be closely linked to pyruvate oxidation, despite the differences in fluxes. Low concentrations of barbiturates were the only conditions we have tested in which pyruvate oxidation could be inhibited without decreasing acetylcholine synthesis. The biphasic effect of barbiturates on acetylcholine synthesis, first reported by McLennan & Elliott (1951), may be useful for defining the minimal mechanisms needed to link acetylcholine production to pyruvate oxidation.

Acetylcholine and $^{14}CO_2$ production were decreased by several different types of inhibitors of pyruvate oxidation. Bromopyruvate is an irreversible inhibitor of the pyruvate dehydrogenase complex (Maldonado *et al.*, 1972), but it also acts on succinate dehydrogenase (EC 1.3.99.1; Sanborn *et al.*, 1971), and, like other alkyl halides (Hebb, 1972), on choline acetyltransferase (Fig. 5). The 2-oxobutyric acid is a competitive inhibitor (and alternative substrate) of purified preparations of the pyruvate dehydrogenase complex (Kanzaki *et al.*, 1969; Blass & Lewis, 1973). The other 2-oxo acids used inhibit the 2-oxoglutarate dehydrogenase complex, some preparations of the pyruvate dehydrogenase complex (Kanzaki *et al.*, 1969; Blass & Lewis, 1973) and the transport of pyruvate into mitochondria (J. Clark & J. Land, personal communication; Halestrap *et al.*, 1974). These 2-oxo acids, unlike bromopyruvate, inhibit $^{14}CO_2$ production from [2-^{14}C]pyruvate more than from [1-^{14}C]pyruvate. They may act on a step in the tricarboxylic acid cycle after the condensation of acetyl-CoA with oxaloacetic acid. The barbiturates presumably act on electron transport, but beyond that the mechanism of their effect on pyruvate oxidation is not resolved.

Of the compounds tested, 2-oxo-3-methylbutanoic acid, 2-oxo-3-methylpentanoic acid, 2-oxo-4-methylpentanoic acid, leucine, and, to a lesser extent, 2-oxobutyric acid, characteristically accumulate in the tissues of patients with Maple-Syrup-Urine Disease (Menkes, 1959). All lead to inhibition of acetylcholine synthesis from pyruvate. The concentrations of these acids and of pyruvic acid used with the mince are one or two orders of magnitude higher than those occurring *in vivo*, but the ratios of 2-oxo acid to pyruvic acid in our experiments are similar to those found in the tissues of some of these patients.

Our observations raise the question of whether carbohydrate utilization might be a significant factor in the control of acetylcholine synthesis under more physiological conditions. The regulation of acetylcholine synthesis is poorly understood (Hebb, 1972). Choline acetyltransferase is present in a ninefold excess of that required for turnover of acetylcholine (Schuberth *et al.*, 1970) in rat brain. Mass action of substrates and products may control the activity of this enzyme (Kaita & Goldberg, 1969; Glover & Potter, 1971). Inhibition of high-affinity choline uptake has been reported to inhibit acetylcholine synthesis in rat striatal synaptosomes, suggesting that the availability of choline can be rate-limiting for acetylcholine synthesis (Guyenet *et al.*, 1973). It remains to be determined whether utilization of carbohydrate can also limit acetylcholine synthesis under physiological conditions.

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