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Utilization of Citrate, Acetylcarnitine, Acetate, Pyruvate and Glucose for the Synthesis of Acetylcholine in Rat Brain Slices

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Abstract: Slices of rat caudate nuclei were incubated in saline media containing choline, paraoxon, unlabelled glucose, and [1,5-^{14}C]citrate, [1-^{14}C-acetyl]carnitine, [1-^{14}C]acetate, [2-^{14}C]pyruvate, or [U-^{14}C]glucose. The synthesis of acetyl-labelled acetylcholine (ACh) was compared with the total synthesis of ACh. When related to the utilization of unlabelled glucose (responsible for the formation of unlabelled ACh), the utilization of labelled substrates for the synthesis of the acetyl moiety of ACh was found to decrease in the following order: [2-^{14}C]pyruvate > [U-^{14}C]glucose > [1-^{14}C-acetyl]carnitine > [1,5-^{14}C]citrate > [1-^{14}C]acetate. The utilization of [1,5-^{14}C]citrate and [1-^{14}C]acetate for the synthesis of [^{14}C]ACh was low, although it was apparent from the formation of $^{14}CO_2$ and ^{14}C-labelled lipid that the substrates entered the cells and were metabolized. The utilization of [1,5-^{14}C]citrate for the synthesis of [^{14}C]ACh was higher when the incubation was performed in a medium without calcium (with EGTA); that of glucose did not change, whereas the utilization of other substrates for the synthesis of ACh decreased. The results indicate that earlier (indirect) evidence led to an underestimation of acetylcarnitine as a potential source of acetyl groups for the synthesis of ACh in mammalian brain; they do not support (but do not disprove) the view that citrate is the main carrier of acetyl groups from the intramitochondrial acetyl-CoA to the extramitochondrial space in cerebral cholinergic neurons. **Key Words:** Acetylcholine synthesis—Acetylcarnitine—Citrate—Acetyl group carriers—Precursors. **Doležal V. and Tuček S.** Utilization of citrate, acetylcarnitine, acetate, pyruvate and glucose for the synthesis of acetylcholine in rat brain slices. *J. Neurochem.* **36**, 1323–1330 (1981).

In spite of much effort, the origin of acetyl groups in the acetyl-CoA which is used for the synthesis of acetylcholine (ACh) in mammalian brain has not been established with certainty. In experiments with different labelled precursors of acetyl groups, the highest incorporation of labelled atoms into ACh was observed from glucose and pyruvate; *in vivo* (Tuček and Cheng, 1970; 1974) and *in vitro* (Browning and Schulman, 1968; Nakamura et al., 1970; Sollenberg and Sörbo, 1970; Lefresne et al., 1973; 1977) approaches led to the same result. Since the pyruvate dehydrogenase complex is localized in the mitochondrial matrix, it is of great interest to discover how the acetyl groups of the pyruvate-generated intramitochondrial acetyl-CoA pass the inner and outer mitochondrial membranes and become available for the cytoplasmic synthesis of ACh. The inner mitochondrial membrane is probably little, if at all permeable for acetyl-CoA (Greville, 1969; cf. Tuček, 1967). Several compounds

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Abbreviations used: ACh, Acetylcholine; AChE, Acetylcholinesterase; Na-TPB, Sodium tetraphenylboron.

have been proposed to act as carriers of acetyl groups from the intramitochondrial acetyl-CoA to the extramitochondrial space; they should be formed from acetyl-CoA in the inner mitochondrial compartment and, after having passed the inner mitochondrial membrane, should be used for the synthesis of acetyl-CoA in the outer mitochondrial space or in the cytoplasm. Among the proposed carriers, citrate, acetylcarnitine, and acetate appear the most important (see Tuček, 1978, for review).

Evidence linking these three compounds with the synthesis of ACh in mammalian brain is controversial or negative. Radioactive label from acetate and citrate is incorporated into ACh much less than that from glucose or pyruvate (Tuček and Cheng, 1970; 1974; Nakamura et al., 1970; Lefresne et al., 1977; Cheng and Brunner, 1978). When [^{14}C]glucose is used as a precursor of [^{14}C]ACh, the incorporation of label into ACh cannot be diluted by the addition of unlabelled acetylcarnitine into the incubation medium (Lefresne et al., 1977). These findings contradict the idea that citrate, acetate, or acetylcarnitine act as carriers of acetyl groups for the synthesis of ACh. On the other hand, several pieces of recently published indirect evidence do point to citrate as the source of the extramitochondrial acetyl-CoA which is used for the synthesis of ACh (Sollenberg and Sörbo, 1970; Sterling and O'Neill, 1978; Hayashi and Kato, 1978; Szutowicz et al., 1979). Sterri and Fonnum (1980) observed that the degeneration of cholinergic nerve terminals in a brain region is accompanied by a marked decrease in the activity of carnitine acetyltransferase, which suggests that acetylcarnitine might play a specific role in the synthesis of ACh.

In the present work, an attempt has been made to obtain further information regarding the roles of citrate and acetylcarnitine in the synthesis of ACh in the brain. It appeared that some of the contradictions concerning the role of citrate might be explained if it could be shown that the low incorporation of label from citrate into ACh is a consequence of a failure of exogenous citrate to enter the cells and to be metabolized; measurements of the metabolic utilization of citrate had not been made in previous studies concerning the role of citrate in the synthesis of ACh in brain tissue *in vitro*. In view of the fact that calcium ions are usually present in the incubation media and that the dissociation constant of calcium citrate is low, it appeared of interest to investigate the effect of the omission of Ca^{2+} from the incubation medium on the utilization of citrate for the synthesis of ACh.

With regard to the role of acetylcarnitine, the availability of [^{14}C-acetyl]carnitine (Tuček et al., 1978) permitted us to use a direct approach (not applied previously) and to measure the formation of labelled ACh during incubations of brain slices in the presence of labelled acetylcarnitine. The utilization of acetylcarnitine and other labelled substrates for the synthesis of ACh has been compared with the utilization of glucose.

Preliminary reports on the present work have been published (Doležal et al., 1980; Tuček et al., 1980).

MATERIALS AND METHODS

Reagents

[1-^{14}C]Acetate, [1-^{14}C-acetyl]choline, [1,5-^{14}C]citrate and [2-^{14}C]pyruvate were obtained from the Radiochemical Centre, Amersham, U.K.; [1,5-^{14}C]citrate was repurified by TLC on cellulose with pentanol–formic acid–water (48.8:48.8:2.4, by vol.) as solvent. [U-^{14}C]Glucose was from ÚVVVR, Prague; [1-^{14}C-acetyl]carnitine was synthesized from [1-^{14}C]acetyl chloride (ROTOP, Dresden) and (-)-carnitine chloride (Koch-Light, Colnbrook, U.K.) as described earlier (Tuček et al., 1978). Sodium tetraphenylboron (Na-TPB) was from VEB Laborchemie, Apolda, acetylcholinesterase (AChE, enzyme from electric eel, type VI-S) from Sigma, St. Louis, Missouri, hyamine hydroxide from Koch-Light, Colnbrook, U.K., EGTA (ethyleneglycol-bis(β-aminoethylether)-*N,N'*-tetraacetic acid) from Eastman, Rochester, New York, and paraoxon (Mintacol) from Bayer, Leverkusen.

Preparation of the Slices and Incubation

Wistar rats of both sexes, weighing 180–240 g, were killed by decapitation. The caudate nuclei were weighed and cut into slices using a McIlwain chopper (McIlwain and Rodnight, 1962) set at 0.4 mm. Slices obtained from one animal (70–80 mg) were incubated in 0.6 ml of the following medium: 123 mM-NaCl, 5 mM-KCl, 1.2 mM-$MgCl_2$, 2.5 mM-$CaCl_2$, 1.2 mM-NaH_2PO_4, 25 mM-$NaHCO_3$, 0.2 mM-choline chloride, 0.058 mM-paraoxon, 5 mM-glucose or [U-^{14}C]glucose, and other labelled substrates as indicated. [1-^{14}C]Acetate, [1,5-^{14}C]citrate and [2-^{14}C]pyruvate were used in concentrations close to 0.1 mM; [1-^{14}C-acetyl]carnitine was used in a concentration of 1 mM. Final specific radioactivities during incubations were 5.8–10.8 Ci/mol in experiments with [1-^{14}C]acetate, 15.4 Ci/mol with [1,5-^{14}C]citrate, 6.8–7.8 Ci/mol with [2-^{14}C]pyruvate, 0.23 Ci/mol with [1-^{14}C-acetyl]carnitine, and 0.23–0.24 Ci/mol with [U-^{14}C]glucose. In some experiments, $CaCl_2$ was replaced by 1 mM-EGTA (sodium salt, pH 7.4). Before incubation, the medium was gassed with 95% O_2 + 5% CO_2; the incubations were performed with shaking in an atmosphere of 95% O_2 and 5% CO_2 at 38°C for 60 min.

Measurement of the Production of $^{14}CO_2$

At the end of the incubation, the plastic cap of the incubation vial was pierced with an injection needle and 0.5 ml of 1 M-hyamine hydroxide in methanol was put into a small side-vessel in the vial; a strip of filter paper had been put into the side-vessel before the start of the incubation and arranged so as to project into the atmosphere of the main incubation compartment. After the addition of

hyamine, 1 ml of 0.05 M-HCl was added to the incubation medium, the hole in the cap was closed, and the incubation vials were shaken for 60 min in a water bath at 38°C. After that, hyamine from the side-vessel and the strip of filtration paper wetted by it were transferred to a scintillation vial and the radioactivity they contained, which corresponded to $^{14}CO_2$ was measured in a Nuclear Chicago Isocap 300 liquid scintillation spectrometer.

Extraction of ACh and Measurement of Radioactivity Incorporated into the Acetyl Groups of ACh

After the expulsion of CO_2, the acidified incubation medium and the sliced tissue were transferred to a centrifugation tube; the incubation vial was washed with 0.5 ml of water, which was added to the medium and the tissue. The tube was kept in a boiling water bath for 10 min; then it was centrifuged for 20 min at 1700 *g* (max). The supernatant solution was collected and its volume made up to 2 ml by adding water; this solution will hereafter be called the "ACh-supernate."

The content of ACh in the ACh-supernate was measured by bioassay on guinea-pig ileum (Tuček, 1973*a*); the Tyrode solution used contained morphine sulphate (5 mg/litre), eserine sulphate (20 μg/litre) and antistine (antazoline) (0.4 mg/litre).

The radioactivity present in the acetyl groups of ACh was calculated from the difference in the radioactivity which could be extracted from two aliquots of the ACh-supernate by sodium tetraphenylboron (Na-TPB) after one of the aliquots had been and the other had not been incubated with acetylcholinesterase (AChE). To remove paraoxon, the inhibitor of AChE, 1.9 ml of the ACh-supernate was at first extracted three times with 5 ml of ether; ether was discarded and its residues were removed from the water phase by a stream of N_2. The ACh-supernate was made alkaline to pH 8.0 with 0.5 M-Tris and divided into two aliquots. One aliquot was kept in ice and the other was incubated, after an addition of 5 units of AChE for 30 min at 38°C. ACh and choline contained in each aliquot were then extracted into 1.0 ml of a solution of TPB in butyl acetate (15 mg/ml); the radioactivity in the TPB/butyl acetate extract was measured by liquid scintillation spectrometry (Tuček, 1973*b*).

In each experiment the whole extraction procedure was performed with several samples of caudate nucleus slices which contained no radioactive substrate, but to which a known amount of [^{14}C]ACh was added. The results obtained with these control slices served (a) to confirm that the hydrolysis of ACh was complete (>95%) in aliquots incubated with AChE and (b) to indicate the degree of recovery of [^{14}C]ACh at the end of the extraction procedure. The results obtained with experimental samples were corrected to correspond to 100% recovery.

Incorporation of Radioactivity into Lipids

Tissue sediment remaining after the removal of the ACh-supernate was washed once with 5 ml of 155 mM-NaCl and recentrifuged; the supernate was discarded. The pellet was homogenized in 0.5 ml of a mixture of chloroform and methanol (1:1 v/v) and transferred to a tube containing 0.5 ml of 50 mM-NaCl. The homogenizer was washed twice with 0.75 ml of chloroform-methanol and the washings were added to the homogenate. The tube with the homogenate was shaken for 2 min and centrifuged for 20 min at 2500 *g* (max). The upper phase was discarded and the lower (chloroform) phase was used for scintillation counting.

RESULTS

Time-Course of the Synthesis of Acetylcholine, CO_2 and Lipids from [U-^{14}C]Glucose

In view of the restricted availability of radiolabelled substrates, the tissue slices were incubated in a small volume of the incubation medium (tissue/medium ratio of about 0.125). The incubation medium contained 5 mM-glucose in all experiments; the other substrates were used in a 0.1 mM or, in case of acetylcarnitine, 1.0 mM concentration. In accord with this, glucose was always the main substrate for the synthesis of the acetyl groups of ACh; this was evident from a comparison of the incorporation of labelled atoms from various substrates into ACh with the total synthesis of ACh, measured by bioassay. To be sure that, under the conditions used, the concentration of glucose was sufficient to support the synthesis of ACh, CO_2 and lipids during the whole incubation period, the time-course of the synthesis was measured in experiments in which 5 mM-[U-^{14}C]glucose was the only exogenous substrate and the formation of CO_2 from it was stimulated by the absence of Ca^{2+} from the medium. It may be seen from Fig. 1 that the synthesis of ACh, production of CO_2 and incorporation of ^{14}C into lipids proceeded during the whole incubation period of 60 min.

Comparison of the Incorporation of ^{14}C from [2-^{14}C]Pyruvate, [1,5-^{14}C]Citrate and [1-^{14}C]Acetate into Acetylcholine, CO_2 and Lipids

Figure 2 gives a summary of results obtained in experiments in which slices of the caudate nuclei were incubated in a complete incubation medium containing 2.5 mM-$CaCl_2$ and 5 mM-glucose, supplemented with 0.1 mM-[2-^{14}C]pyruvate, [1,5-^{14}C]citrate, or [1-^{14}C]acetate. The amount of [^{14}C]ACh synthesized was calculated from the incorporation of label into the acetyl groups of ACh as found in the experiments and from the known specific radioactivity of each substrate; in the case of [1,5-^{14}C]citrate it was assumed, however, that only the ^{14}C atom in position 5 has a reasonable chance to be incorporated into ACh, since in the reaction catalysed by ATP-citrate lyase (EC 4.1.3.8), only the carbon atoms from positions 4 and 5 of citrate are transferred to acetyl-CoA. The specific radioactivity of [1,5-^{14}C]citrate was, therefore,

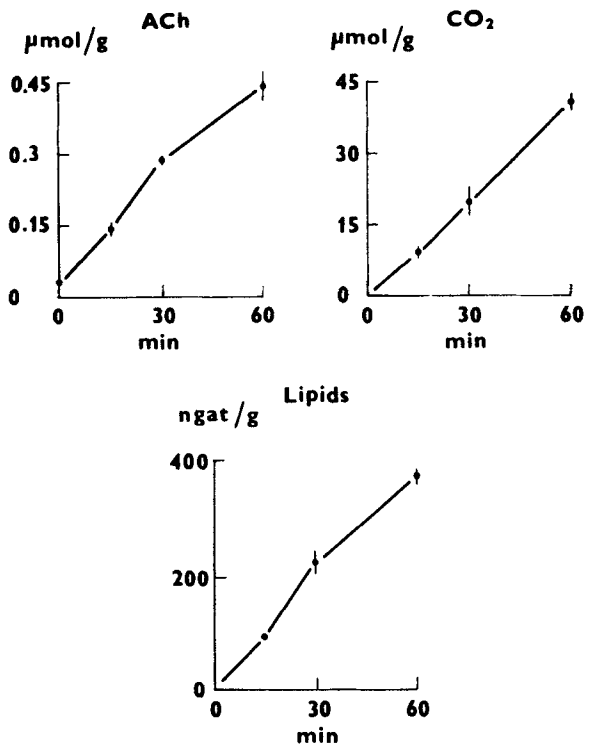

FIG. 1. The time-course of the synthesis of ACh (measured by bioassay), production of CO_2 (calculated from the incorporation of radioactivity into $^{14}CO_2$) and synthesis of lipids (expressed in nanogram-atoms of ^{14}C incorporated into lipids per g tissue) during the incubation of slices of rat caudate nuclei in a medium without Ca^{2+}, with 5 mM-[U-^{14}C]glucose and 1 mM-EGTA (see Methods). Most points are means (±S.E.M.) of three observations.

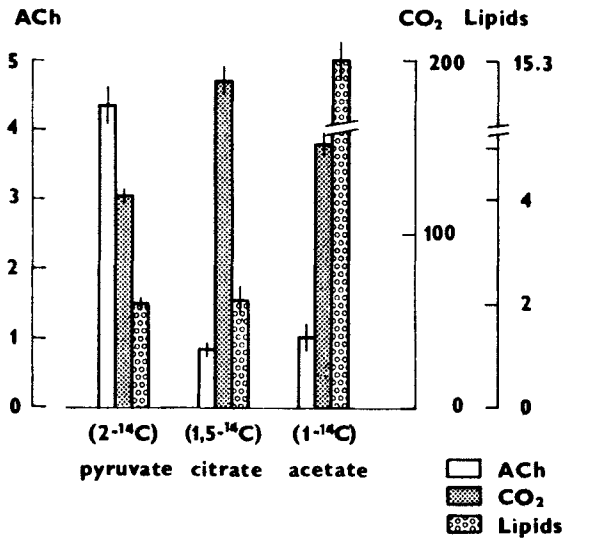

FIG. 2. Comparison of the incorporation of ^{14}C into ACh, CO_2 and lipids during 60 min incubation of slices of rat caudate nuclei in the presence of 5 mM-glucose and of 0.1 mM-[2-^{14}C]pyruvate, [1,5-^{14}C]citrate, or [1-^{14}C]acetate, respectively. The incorporation into ACh is expressed as nmol [^{14}C]ACh synthesized/g tissue/h; into CO_2 as nmol $^{14}CO_2$ produced/g tissue/h; into lipids as nanogram-atoms of ^{14}C incorporated/g tissue/h. Data are means (±S.E.M.) of 4–11 observations.

halved before being taken for calculations of [^{14}C]ACh synthesis.

As may be seen from Fig. 2, pyruvate was utilized for the synthesis of ACh five times as much as citrate and four times as much as acetate. At the same time, however, the incorporation of ^{14}C from [2-^{14}C]pyruvate and from [1,5-^{14}C]citrate into lipids was about the same; the incorporation from [1-^{14}C]acetate was more than five times higher. The highest incorporation of label into CO_2 was from [1,5-^{14}C]citrate; that from [1-^{14}C]acetate was 19% and from [2-^{14}C]pyruvate 35% lower.

Utilization of [U-^{14}C]Glucose and [1-^{14}C-Acetyl]Carnitine for the Synthesis of Acetylcholine; Comparison with Other Substrates

Whereas [2-^{14}C]pyruvate, [1,5-^{14}C]citrate and [1-^{14}C]acetate were used in a comparatively low concentration of 0.1 mM, [U-^{14}C]glucose and (1-^{14}C-acetyl]carnitine were applied in 5 mM and 1 mM concentrations, respectively. The reason was that glucose served as the main substrate for the metabolism of the brain slices and concentrations higher than 1.5 mM appear necessary to support it adequately (McIlwain and Bachelard, 1971). In the case of [1-^{14}C-acetyl]carnitine, 1 mM rather than 0.1 mM solution was used to obtain sufficiently high values of incorporation, as our labelled reagent was of a low specific radioactivity.

Absolute values of the incorporation of ^{14}C from [U-^{14}C]glucose and [1-^{14}C-acetyl]carnitine into ACh, CO_2 and lipids are given in Tables 1–3. The tables also contain numerical data on the incorporation of ^{14}C from the other substrates examined and serve to show the effect of the omission of Ca^{2+} on the incorporation; this aspect will be discussed in the next subsection.

The fact that all labelled substrates except glucose were always used in the presence of a nonlabelled substrate, and that their concentrations were not identical, poses the difficult problem of comparing the utilization of various substrates in quantitative terms. One way of doing it is by calculating the relative preferences which the tissue displayed for various labelled substrates in comparison with the nonlabelled substrate (glucose). The relative preference (RP) for a ^{14}C-labelled substrate is given by the ratio:

$$RP = \frac{\dfrac{\text{Synthesis of } [^{14}C]\text{ACh}}{\text{Total synthesis of ACh}}}{\dfrac{\text{Concentration of } [^{14}C]\text{substrate}}{\text{Total concentration of substrates}}}$$

where the concentration of glucose is taken as twice its real value, since 1 mole of glucose yields 2 moles of acetyl-CoA. The use of relative preferences for

comparison of different substrates is based on the obvious assumption that, if one labelled and one nonlabelled substrate are being used by the neurons for the synthesis of ACh with the same affinity, the proportion between the synthesis of the labelled ACh and of total ACh will be equal to the proportion between the concentration of the labelled substrate and the total concentration of substrates in the medium.

For the calculation of relative preferences (Fig. 3), the total synthesis of ACh was measured by bioassay; it was taken as the difference between the amount of ACh found in the tissue plus the medium after 60 min of incubation, and the amount found in the sliced tissue at time zero.

It may be seen from Fig. 3 that the value of the relative preference for [U-^{14}C]glucose, which would be expected to equal 1, is indeed close to, although slightly higher than, unity. The relative preference for [2-^{14}C]pyruvate is equal to 1.465 ± 0.080, whereas the relative preferences for [1,5-^{14}C]citrate and [1-^{14}C]acetate are considerably lower than unity (0.289 and 0.182, respectively). The relative preference for [1-^{14}C-acetyl]carnitine is comparatively high—0.633 ± 0.053; this suggests that the slices of the caudate nuclei utilize acetylcarnitine for the synthesis of ACh with nearly two-thirds of the affinity with which they utilize unlabelled glucose.

Effect of the Omission of Ca^{2+} on the Utilization of [1,5-^{14}C]Citrate and Other Substrates

It is well known that citrate forms a nonionized complex with calcium (Schubert and Lindenbaum, 1952). Possibly, this might affect its penetration into the cells and its utilization in the metabolism. Experiments were therefore performed in which 1 mM-EGTA was included in the incubation medium instead of $CaCl_2$. The omission of Ca^{2+} should preclude its binding to labelled carboxylic acids, but it could also alter the permeability of cell membranes for the substrates (Davson, 1970). Data on the utilization of [1,5-^{14}C]citrate and of other substrates for the synthesis of ACh, CO_2 and lipids in the absence of Ca^{2+} are summarized in Tables 1–3.

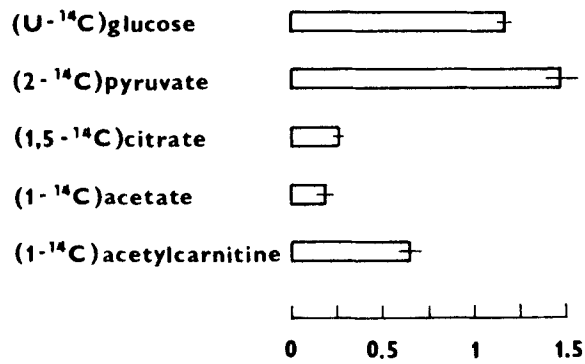

FIG. 3. Relative preference displayed by slices of rat caudate nuclei for ^{14}C-labelled substrates with regard to their utilization for the synthesis of ACh. For calculation of the value of relative preference, see text. Data are means ± S.E.M.; the number of observations was 12 for [U-^{14}C]glucose, 6 for [2-^{14}C]pyruvate, 10 for [1,5-^{14}C]citrate, 4 for [1-^{14}C]acetate, and 5 for [1-^{14}C-acetyl]carnitine. The value of relative preference for pyruvate given in the graph (1.465) was calculated from the results of experiments with 0.1 mM-[2-^{14}C]pyruvate; in experiments with 1.0 mM-[2-^{14}C]pyruvate, the relative preference for pyruvate was calculated to be 1.401 ± 0.091 (mean of 6 experiments ± S.E.M.).

The absence of Ca^{2+} did not cause any change in the synthesis of ACh from glucose. This was evident both from the measurements of the incorporation of ^{14}C from [U-^{14}C]glucose into the acetyl moiety of ACh (Table 1), and from the measurements of total ACh synthesis by bioassay (results not given). The incorporation of ^{14}C from [1,5-^{14}C]citrate, however, was by 106% higher in the absence of Ca^{2+}. The utilization of [2-^{14}C]pyruvate, [1-^{14}C]acetate and [1-^{14}C-acetyl]carnitine for the synthesis of [^{14}C]ACh was diminished.

The production of $^{14}CO_2$ (Table 2) from [1,5-^{14}C]citrate, [2-^{14}C]pyruvate, [1-^{14}C-acetyl]carnitine and [U-^{14}C]glucose was increased when Ca^{2+} was omitted. Particularly great increases were observed in experiments with [1,5-^{14}C]citrate (+195%) and [1-^{14}C-acetyl]carnitine (+281%). The incorporation of ^{14}C into lipids was diminished from all substrates tested except [1-^{14}C]acetate in the absence of Ca^{2+} (Table 3); the greatest change (a decrease of 66%) was observed when [1,5-^{14}C]citrate was the labelled substrate.

DISCUSSION

The present results are in good accord with earlier work showing that, in comparison with pyruvate or glucose, citrate is considerably less utilized as a source of acetyl groups for the synthesis of ACh in rat brain slices (Nakamura et al., 1970; Lefresne et al., 1977; Cheng and Brunner, 1978). Since there is indirect evidence favouring a role for citrate in the synthesis of ACh (Sollenberg and Sörbo, 1970; Sterling and O'Neill, 1978; Hayashi and Kato, 1978; Szutowicz et al., 1979), the possibility has to be taken into account that the failure to observe high incorporation of label from citrate into ACh might be due to a failure of citrate to enter the cells and to be metabolized. Such a possibility has been tested (and excluded) in earlier work regarding the synthesis of ACh in the brain *in vivo* (Tuček and Cheng, 1974), but not in studies of ACh synthesis *in vitro*.

A comparison of the utilization of [2-^{14}C]pyruvate, [1,5-^{14}C]citrate, and [1-^{14}C]acetate for the synthesis of ACh, CO_2 and lipids, as shown in Fig. 2, suggests that citrate is simply not metabolized under the conditions used—unlikely, even when it

TABLE 1. *Synthesis of [^{14}C]ACh in slices of rat caudate nuclei incubated with ^{14}C-labelled substrates and unlabelled glucose in the presence or absence of calcium ions*

Substrate(s)	+Ca^{2+}	+EGTA	Change in the absence of Ca^{2+} (%)
[1,5-^{14}C]citrate (0.1 mM) + glucose (5 mM)	0.84 ± 0.09 (10)	1.73 ± 0.05 (11)	+106.0
[2-^{14}C]pyruvate (0.1 mM) + glucose (5 mM)	4.33 ± 0.31 (6)	2.17 ± 0.27 (5)	−49.9
[1-^{14}C]acetate (0.1 mM) + glucose (5 mM)	1.06 ± 0.20 (4)	0.68 ± 0.26 (4)	−35.8
[1-^{14}C]acetylcarnitine (1 mM) + glucose (5 mM)	24.81 ± 2.97 (5)	17.08 ± 1.59 (5)	−31.2
[U-^{14}C]glucose (5 mM)	448.19 ± 11.51 (12)	452.23 ± 15.46 (6)	+0.9

The slices were incubated for 60 min as described in Methods. The incubation medium contained either 2.5 mM-$CaCl_2$ or 1 mM-EGTA (sodium salt). The results are expressed as nmol of [^{14}C]ACh per g fresh weight (mean ± S.E.M.); the number of experiments is given in parentheses.

is taken into account that ^{14}C from position 1 of citrate is converted to $^{14}CO_2$ faster than ^{14}C from the other positions and substrates used. Unfortunately, it is impossible with the present approach to distinguish whether citrate enters all cells including the cholinergic neurons, or only some of them, e.g., the glia, in which it is preferentially metabolized (Cheng, 1973).

A new feature of the present experiments was the use of [1-^{14}C-acetyl]carnitine for the evaluation of its contribution to the synthesis of brain ACh. As can be seen from Fig. 3, acetylcarnitine was utilized less than pyruvate and glucose, but more than citrate and acetate. It might be argued that the use of acetylcarnitine in a higher concentration than citrate and acetate could distort the comparison between these substrates. Such distortion would, however, lead only to underestimation of the role of acetylcarnitine. If the 1 mM concentration of acetylcarnitine were too high and the enzymes and carriers could not cope with it efficiently, then even higher estimates of the utilization of acetylcarnitine (relative to glucose) would have been obtained if acetylcarnitine had been used at lower concentrations. As indicated in the legend to Fig. 3, the value of relative preference for pyruvate was nearly the same when [2-^{14}C]pyruvate was used, either in a 0.1 or 1.0 mM concentration. Although the observed high degree of utilization of acetylcarnitine for the synthesis of ACh does not prove that this compound is the physiological precursor of brain ACh, it indicates that acetylcarnitine has to be considered a serious candidate for that role. The results with acetylcarnitine are in good accord with data published by Sterri and Fonnum (1980) indicating a specific association of carnitine acetyltransferase with the cholinergic nerve endings in the brain. At present, we find it difficult to explain the failure of unlabelled acetylcarnitine to cause any dilution of radioactivity in labelled acetylcholine produced from labelled glucose in the experiments on synapsomes described by Lefresne et al. (1978).

The omission of Ca^{2+} ions from the incubation media and their replacement by EGTA did not alter

TABLE 2. *Production of $^{14}CO_2$ in slices of rat caudate nuclei incubated with ^{14}C-labelled substrates and unlabelled glucose in the presence or absence of calcium ions*

Substrate(s)	+Ca^{2+}	+EGTA	Change in the absence of Ca^{2+} (%)
[1,5-^{14}C]citrate (0.1 mM) + glucose (5 mM)	188 ± 8 (11)	554 ± 42 (13)	+194.7
[2-^{14}C]pyruvate (0.1 mM) + glucose (5 mM)	122 ± 5 (6)	212 ± 19 (6)	+73.8
[1-^{14}C]acetate (0.1 mM) + glucose (5 mM)	152 ± 9 (4)	103 ± 7 (5)	−32.2
[1-^{14}C]acetylcarnitine (1 mM) + glucose (5 mM)	213 ± 16 (5)	812 ± 70 (5)	+281.2
[U-^{14}C]glucose (5 mM)	41,900 ± 950 (12)	56,800 ± 1870 (6)	+35.6

The results are expressed as nmol of $^{14}CO_2$ per g fresh weight (mean ± S.E.M.). For additional information see legend to Table 1.

TABLE 3. *Incorporation of ^{14}C into lipids in slices of rat caudate nuclei incubated with ^{14}C-labelled substrates and unlabelled glucose in the presence or absence of calcium ions*

Substrate(s)	$+Ca^{2+}$	+EGTA	Change in the absence of Ca^{2+} (%)
[1,5-^{14}C]citrate (0.1 mM) + glucose (5 mM)	2.04 ± 0.29 (11)	0.69 ± 0.04 (12)	−66.1
[2-^{14}C]pyruvate (0.1 mM) + glucose (5 mM)	2.00 ± 0.08 (6)	0.91 ± 0.04 (6)	−54.5
[1-^{14}C]acetate (0.1 mM) + glucose (5 mM)	15.28 ± 0.38 (4)	15.25 ± 0.53 (5)	−0.2
[1-^{14}C]acetylcarnitine (1 mM) + glucose (5 mM)	9.16 ± 1.29 (5)	6.74 ± 0.73 (4)	-26.4
[U-^{14}C]glucose (5 mM)	867.00 ± 40.2 (13)	628.5 ± 36.0 (6)	−27.5

The results are expressed as nanogram-atoms of ^{14}C incorporated into lipids per g fresh weight (mean ± S.E.M.). For additional information see legend to Table 1.

the total amount of ACh synthesized and the incorporation of label from [U-^{14}C]glucose into ACh, but did alter the utilization of the other substrates tested as precursors of ACh. In general, the omission of Ca^{2+} would be expected to increase the permeability of cell membranes for substrates (Davson, 1970), and the increase in $^{14}CO_2$ synthesis from [U-^{14}C]glucose, [2-^{14}C]pyruvate, [1,5-^{14}C]citrate and [1-^{14}C-acetyl]carnitine (Table 2) observed in the absence of Ca^{2+} is probably at least partly a consequence of this. At the same time, however, the omission of Ca^{2+} must have been followed by a redistribution in the intensity of metabolic flow through various metabolic pathways. This is suggested by the fact that, after the removal of Ca^{2+}, the utilization of each single substrate for the synthesis of CO_2, ACh and lipids was changed in various directions (Tables 1–3).

The utilization of [1,5-^{14}C]citrate for the synthesis of [^{14}C]ACh was increased by 106% after the omission of Ca^{2+} and that for the synthesis of $^{14}CO_2$ by 195%; in contrast, its utilization for the synthesis of ^{14}C-labelled lipids was diminished by 66%. Apparently, experiments performed with media containing Ca^{2+} ions do not reveal the full capacity which the cholinergic neurons have for the metabolic conversion of citrate. Its conversion is probably restricted by the low permeability of the membranes of the nerve terminals for citrate. In experiments *in vitro*, problems of restricted permeability are usually overcome by the use of very high concentrations of substrates; it will be shown in the accompanying study (Tuček et al., 1981), however, that an increase in the concentration of citrate in the medium has a negative effect on the total synthesis of ACh in brain slices. In view of the not fully resolved problem of the permeability of nerve terminal membranes, caution is required in making conclusions about the role that the substrates applied to the extracellular fluid play inside the nerve terminals.

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REFERENCES

Browning E. T. and Schulman M. P. (1968) (^{14}C)Acetylcholine synthesis by cortex slices of rat brain. *J. Neurochem.* **15**, 1391–1405.

Cheng S.-C. (1973) Compartmentation of tricarboxylic acid cycle intermediates and related metabolites, in *Metabolic Compartmentation in the Brain* (Balazs R. and Cremer J. E., eds), pp. 107–118. Macmillan, London.

Cheng S.-C. and Brunner E. A. (1978) Alteration of tricarboxylic acid cycle metabolism in rat brain slices by halothane. *J. Neurochem.* **30**, 1421–1430.

Davson H. (1970) *A Textbook of General Physiology*, pp. 454–455. J. and A. Churchill, London.

Doležal V., Tuček S., and Herbrychová A. (1980) Utilization of citrate, acetylcarnitine, pyruvate and glucose for the synthesis of acetylcholine in the brain tissue *in vitro*. *Physiol. Bohemoslov.* **29**, 260.

Greville G. D. (1969) Intracellular compartmentation and the citric acid cycle, in *Citric Acid Cycle* (Lowenstein J. M., ed), pp. 1–136. Marcel Dekker, New York.

Hayashi H. and Kato T. (1978) Acetyl-CoA synthesizing enzyme activities in single nerve cell bodies of rabbit. *J. Neurochem.* **31**, 861–869.

Lefresne P., Guyenet P., and Glowinski J. (1973) Acetylcholine synthesis from (2-^{14}C)pyruvate in rat striatal slices. *J. Neurochem.* **20**, 1083–1097.

Lefresne P., Hamon M., Beaujouan J. C., and Glowinski J. (1977) Origin of the acetyl moiety of acetylcholine synthesized in rat striatal synaptosomes. *Biochimie* **59**, 197–215.

McIlwain H. and Bachelard H. S. (1971) *Biochemistry and the Central Nervous System*, p. 71. Churchill Livingstone, Edinburgh.

McIlwain H. and Rodnight R. (1962) *Practical Neurochemistry*, pp. 126–129. J. and A. Churchill, London.

Nakamura R., Cheng S.-C., and Naruse H. (1970) A study on the precursors of the acetyl moiety of acetylcholine in brain slices. *Biochem. J.* **118**, 443–450.

Schubert J. and Lindenbaum A. (1952) Stability of alkaline earth-organic acid complexes measured by ion exchange. *J. Am. Chem. Soc.* **74**, 3529–3532.

Sollenberg J. and Sörbo B. (1970) On the origin of the acetyl

moiety of acetylcholine in brain studied with a differential labelling technique using ^{3}H-^{14}C-mixed labelled glucose and acetate. *J. Neurochem.* **17**, 201–207.

Sterling G. H. and O'Neill J. J. (1978) Citrate as the precursor of the acetyl moiety of acetylcholine. *J. Neurochem.* **31**, 525–530.

Sterri S. H. and Fonnum F. (1980) Acetyl-CoA synthesizing enzymes in cholinergic nerve terminals. *J. Neurochem.* **35**, 249–254.

Szutowicz A., Łysiak W., and Angielski S. (1979) ATP-citrate lyase in cholinergic system of rat brain, in *Progress in Brain Research, Vol. 49: The Cholinergic Synapse* (Tuček S., ed), p. 494. Elsevier, Amsterdam.

Tuček S. (1967) The use of choline acetyltransferase for measuring the synthesis of acetyl-coenzyme A and its release from brain mitochondria. *Biochem. J.* **104**, 749–756.

Tuček S. (1973*a*) Choline acetyltransferase activity in skeletal muscles after denervation. *Exp. Neurol.* **40**, 23–35.

Tuček S. (1973*b*) An improved solvent system for liquid scintillation counting in choline acetyltransferase activity assays by Fonnum's method. *J. Neurochem.* **20**, 901–903.

Tuček S. (1978) *Acetylcholine Synthesis in Neurons*, pp. 1–259. Chapman and Hall, London.

Tuček S. and Cheng S.-C. (1970) Precursors of acetyl groups in acetylcholine in the brain *in vivo*. *Biochim. Biophys. Acta* **208**, 538–540.

Tuček S. and Cheng S.-C. (1974) Provenance of the acetyl group of acetylcholine and compartmentation of acetyl-CoA and Krebs cycle intermediates in the brain *in vivo*. *J. Neurochem.* **22**, 893–914.

Tuček S., Havránek M., and Ge I. (1978) Synthesis of (acetyl-^{14}C)carnitine and the use of tetraphenylboron for differential extraction of (acetyl-^{14}C)choline and (acetyl-^{14}C)carnitine. *Anal. Biochem.* **84**, 589–593.

Tuček S., Říčný J., and Doležal V. (1980) Supply of acetyl groups for the synthesis of acetylcholine, in *Ontogenesis and Functional Mechanisms of Peripheral Synapses* (Taxi J., ed), pp. 77–89. Elsevier/North Holland, Amsterdam.

Tuček S., Doležal V., and Sullivan A. C. (1981) Inhibition of the synthesis of acetylcholine in rat brain slices by (-)-hydroxycitrate and citrate. *J. Neurochem.* **36**, 1331–1337.