A Study on the Precursors of the Acetyl Moiety of Acetylcholine in Brain Slices

OBSERVATIONS ON THE COMPARTMENTALIZATION OF THE ACETYL-COENZYME A POOL

By R. NAKAMURA, S.-C. CHENG AND H. NARUSE

New York State Research Institute for Neurochemistry and Drug Addiction, Ward's Island, New York, N.Y. 10035, U.S.A.

(Received 11 August 1969)

1. A method was devised for the determination of the specific radioactivity of the acetyl moiety of acetylcholine synthesized from various ^{14}C -labelled substrates. 2. The precursor for the acetyl moiety of acetylcholine was studied in slices of striatum and cerebral cortex from rat and guinea-pig brain. Incorporation of radioactivity into acetylcholine was determined after incubating the slices in the presence of $[2\text{-}^{14}\text{C}]\text{acetate}$, $[^{14}\text{C}]\text{bicarbonate}$, $[1,5\text{-}^{14}\text{C}]\text{citrate}$, DL- $[1\text{- or }5\text{-}^{14}\text{C}]\text{glutamate}$ or $[1\text{- or }2\text{-}^{14}\text{C}]\text{pyruvate}$. 3. After incubation for 1 h, acetylcholine was accumulated significantly in both striatum slices (4.1 nmol/mg of protein) and cerebral-cortex slices (0.57 nmol/mg of protein) from the rat. Final concentrations were about 11 and 5 times respectively the initial values. 4. With slices from rat striatum, rat cerebral cortex and guinea-pig cerebral cortex, the specific radioactivity of acetylcholine derived from $[2\text{-}^{14}\text{C}]\text{pyruvate}$ was very high, reaching approx. 30, 20 and 6% respectively of the initial specific radioactivity of added pyruvate in the medium. With the striatum slices this high value was reached after incubation for 15 min. Incorporation of radioactivity from $[2\text{-}^{14}\text{C}]\text{acetate}$ was only 1.25, 5.3 and 19.7% of that from $[2\text{-}^{14}\text{C}]\text{pyruvate}$ in rat striatum, rat cerebral-cortex and guinea-pig cerebral-cortex slices respectively. A small but definite incorporation was found from $[5\text{-}^{14}\text{C}]\text{glutamate}$. No incorporation was found from the other substrates. The findings suggest that pyruvate is the most important precursor for the synthesis of the acetyl moiety of acetylcholine in brain slices. 5. The specific radioactivity of acetylcholine relative to that of citrate when $[2\text{-}^{14}\text{C}]\text{pyruvate}$ was used compared with that obtained when $[2\text{-}^{14}\text{C}]\text{acetate}$ was used. A marked difference was found in all slices, suggesting metabolic compartmentation of the acetyl-CoA pool.

In studying the fixation of CO_2 by nervous tissue, we demonstrated the reversal of the tricarboxylic acid cycle at the tricarboxylic acid level (Waelsch, Cheng, Côté & Naruse, 1965; Nakamura, Cheng, Naruse & Waelsch, 1966–67). It was suggested that the citrate thus formed from glutamate could conceivably yield acetyl-CoA and ACh* through the combined action of citrate cleavage enzyme and choline acetylase. The acetyl moiety of acetyl-CoA has been thought to be derived from pyruvate (Quastel, Tennenbaum & Wheatley, 1936); however, acetate and citrate have also been suggested as the precursor (Lipton & Barron, 1946; Hebb, 1954; Tuček, 1967*a,b*). In this and the following paper (Cheng & Nakamura, 1970), we have re-investigated the problem by using ^{14}C -labelled substrates in brain slices and in isolated lobster

nerve, both of which synthesize ACh *in vitro* (Sattin, 1966; Dettbarn & Rosenberg, 1966). The two principal precursors were found to be pyruvate and acetate and their relative utilizations in the two kinds of nervous tissue were different. A preliminary report of this work has appeared (Nakamura & Cheng, 1967).

MATERIALS AND METHODS

Sources of material. MN300-cellulose was obtained from Macherey, Nagel and Co. Düren, Germany, and AG-1 (Dowex 1) (X4; Cl^- form; 200–400 mesh) and BioRex-70 (Na^+ form) were obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. The following chemicals were purchased from the sources named: ^{14}C -labelled substrates and tracers (New England Nuclear Corp., Boston, Mass., U.S.A.); GSH (Boehringer-Mannheim Corp., New York, N.Y., U.S.A.); CoA (grade II-A; Sigma Chemical Co.,

* Abbreviation: ACh, acetylcholine.

Table 1. *Concentration and radioactivity of added substrates in the incubation medium*

The efficiency of the counting system was approx. 32%.

Substrate	Rat brain slices		Guinea-pig brain slices	
	Concn. (mM)	$10^{-6} \times$ Sp. radioactivity (c.p.m./ μ mol)	Concn. (mM)	$10^{-6} \times$ Sp. radioactivity (c.p.m./ μ mol)
[2- 14 C]Acetate	2.5	1.2	2.5	1.2
[14 C]Bicarbonate	15.5	0.6	—	—
[1,5- 14 C]Citrate	1.95	1.54	1.0	3.0
DL-[1- 14 C]Glutamate	0.50	6.0	4.39	0.34
DL-[5- 14 C]Glutamate	0.52	5.76	0.98	2.83
[1- 14 C]Pyruvate	1.43	2.09	2.0	1.5
[2- 14 C]Pyruvate	1.48	2.02	0.55	2.44

St Louis, Mo., U.S.A.); choline (Fisher Scientific Co., New York, N.Y., U.S.A.); acetylcholine (Eastman Kodak Co., Rochester, N.Y., U.S.A.); propionylcholine (Calbiochem, Los Angeles, Calif., U.S.A.); butyryl-, succinyl- and γ -aminobutyryl-choline (K & K Laboratories Inc., Plainview, N.Y., U.S.A.); acetylcarnitine (Nutritional Biochemicals Co., Cleveland, Ohio, U.S.A.); and cholinesterase (horse serum butyrylcholinesterase; Worthington Biochemical Corp., Freehold, N.J., U.S.A.).

Preparation and incubation of tissue. Male Wistar rats (300–400 g) were killed by decapitation. The brain was removed as fast as possible and placed in ice-cold phosphate medium of the following composition (final concentrations): 127.3 mM-NaCl, 5.1 mM-KCl, 2.7 mM- CaCl_2 , 1.3 mM- KH_2PO_4 , 1.3 mM- MgSO_4 , 10 mM-glucose, 1.0 mM-choline chloride and 9.9 mM- Na_2HPO_4 buffered to pH 7.4 with HCl. The striatum was cut out with a spatula on a cold platform above crushed ice and transferred into a preweighed bottle containing the same medium. Striata from three brains were pooled and weighed (usually 250–300 mg). Slices of the striatum (0.364 mm thick) were made with a tissue chopper (McIlwain & Rodnight, 1962). They were then transferred to a 50 ml Erlenmeyer flask containing the incubation medium described below.

The incubation flask contained 4.8 ml of bicarbonate medium of the following composition (final concentrations): 118.4 mM-NaCl, 4.7 mM-KCl, 2.5 mM- CaCl_2 , 1.2 mM- KH_2PO_4 , 1.2 mM- MgSO_4 , 15.5 mM- NaHCO_3 , 10 mM-glucose, 1.0 mM-choline chloride, 0.3 mM-GSH and 0.1 mM-CoA (Sattin, 1966). The last two compounds were added as solids and the medium was equilibrated at 37°C with $\text{O}_2 + \text{CO}_2$ (95:5). The slices were introduced and another 5 min later 0.2 ml of radioactive substrate was added (Table 1). Equilibration was continued for another 10 min (except in the [14 C]bicarbonate experiments) and then the flask was stoppered. The shaking rate was 152/min in a gyratory shaker. The incubation lasted 1 h unless otherwise stated.

Slices, 0.3–0.4 mm thick, were prepared from rat cerebral cortices (McIlwain & Rodnight, 1962). The first slice was used, with the second slice sometimes added to make up the weight. Slices from two rats usually weighing about 250–300 mg were used for one experiment. The cerebral-cortex slices were weighed and incubated in the same manner as the striatum slices.

Cerebral-cortex slices from guinea-pigs (about 400 g) were prepared similarly to those from the rat. Both the

first and second slices from each hemisphere were used, and sometimes a third slice was added to make up the weight, which was about 500 mg in these experiments.

Isolation of ACh. After incubation, the slices were collected on a Buchner funnel, quickly rinsed with 5 ml of phosphate medium and then transferred to an all-glass tissue homogenizer. The tissue was homogenized in ice-cold 0.3 M-trichloroacetic acid (2 ml/100 mg wet wt.). The homogenate was centrifuged and the supernatant was collected in a 25 ml cylinder. The precipitate was suspended in 5 ml of water and recentrifuged. The pooled supernatants, constituting fraction C (see below), were extracted with ether to remove trichloroacetic acid, then freed of ether and diluted to 15 ml/200 mg initial wet wt. The resulting fraction D was chromatographed to obtain the ACh fraction as described below.

Fraction D was added to a column of Dowex 1 (acetate form) to remove anions (including citrate) which were analysed as described elsewhere (Cheng & Waelsch, 1963; Naruse, Cheng & Waelsch, 1966). A final 10 ml water wash was also collected. The pH of this combined eluent (fraction E) was about 4.5. At this pH ACh was relatively stable and could be kept for some time without significant loss.

Fraction E from the Dowex 1 column was added to a column of a weak carboxylic acid resin, BioRex-70 (H^+ form). The column was washed with 10 ml of water (the combined eluent was designated fraction F) and then eluted with 5 ml portions of 5 mM-formic acid. The column was prepared by mixing 1 part of 100–200-mesh resin with 2 parts of 200–400-mesh resin. After being decanted in 2 M-HCl several times to remove fine particles, the mixture was poured into columns (11 mm outside diam.) to a capacity of 20 ml. Before use, each column was washed twice with 300 ml of water.

ACh appeared in fractions 25–35 (Fig. 1, which shows the elution pattern of several choline derivatives and also γ -aminobutyrate). There was only a very slight overlapping of ACh fraction from choline and propionylcholine. In later experiments it was found that the peaks could be sharpened slightly by using 15 mM-formic acid as the eluent and the separation became better. The fractions containing ACh were pooled, freeze-dried and kept frozen. The recovery was 89% (S.E.M. $\pm$ 3%) in 24 control experiments where authentic radioactive ACh was added to a non-radioactive extract (fraction E).

Purity of ACh fraction. Although the ACh fraction thus

Fig. 1. Elution pattern of ACh from a BioRex-70 column. The eluent was 5 mM-formic acid and the eluate was collected in 5 ml fractions. The total radioactivity in each tube was plotted for choline, acetylcholine and γ -aminobutyrate, and the total nmol in each tube were plotted for propionylcholine and butyrylcholine.

collected was relatively free of choline and propionylcholine, its purity was still in doubt. After pooling and concentration of the fractions containing ACh from a number of experiments, a sample was spotted on a thin-layer plate of MN300-cellulose. The plate was developed with a mixture of butan-1-ol-ethanol-acetic acid-water (8:2:1:3, by vol.) (Hemsworth & Morris, 1964). The chromatogram was first sprayed with 0.3% ninhydrin in acetone and then 1% iodine in ethanol (Randerath, 1966). Besides choline and ACh, ninhydrin-positive streaks were seen. The presence of choline was expected, since the pooled sample used had been frozen, thawed and freeze-dried several times. The other contaminants were found to be arginine and histidine whose elution patterns overlapped ACh and choline respectively; lysine was eluted before histidine and its peak overlapped with that of histidine. Among other substances tested, adrenaline, noradrenaline, 3,4-dihydroxyphenylalanine, and 3,4-dihydroxyphenethylamine were all eluted before ACh. Serotonin was eluted after ACh. Succinylcholine was eluted very late, apparently as a consequence of the ratio of two choline molecules to each succinic acid molecule. The monocholine ester was not tested. γ -Aminobutyrylcholine was also eluted very late and slowly decomposed on the column. Acetylcarnitine was assayed by its acetyl group which was not recovered from any column in several attempts. We thus assume that it is unstable on these columns. The only other compound tested and found contaminating the ACh fraction was tyramine, whose elution pattern overlapped slightly with that of ACh.

Determination of the specific radioactivity of ACh. The radioactivity in the final ACh sample after freeze-drying was determined with a low background planchet counter (Cheng & Waelisch, 1963). Sample contamination was corrected by the determination of the percentage of radioactivity in the acetyl group as follows: two identical portions of the final sample were added to two separate scintillation bottles. To one of them was added horse serum cholinesterase to hydrolyse the ACh in 0.5 ml of the following reaction mixture (final concentrations) at pH 7.4: 4 mM-tris, 40 mM-MgCl₂, 0.2 mg of cholinesterase and sample containing approx. 15 nmol of ACh. Cholinesterase was absent from the other bottle. Similarly a few standards were set up containing 15 nmol of ACh and various tracer amounts of acetyl-labelled ACh also with and without cholinesterase. After 2 h of incubation at room temperature, the reaction was stopped with 0.05 ml of 50% (w/v) trichloroacetic acid. A filter-paper disc was introduced into each bottle and the samples were dried over NaOH pellets in a vacuum desiccator. It was most important to dry the filter paper thoroughly so that the removal of acetic acid was complete. Scintillation fluid [6 ml of 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene] (Mans & Novelli, 1961) was added and the radioactivities of the samples were measured in a scintillation counter (Tri-Carb model 4322). An example is given in Table 2, which shows that enzymic hydrolysis was essentially complete under these conditions, in good agreement with the observation of Hestrin (1949, 1950). Complete recovery was found with either labelled choline or choline-labelled ACh. In

Table 2. *Enzymic hydrolysis of commercial acetylcholine*

The assay system in a scintillation vial contained 4 μ mol of tris, 40 μ mol of $MgCl_2$, 14 nmol of unlabelled ACh and trace amounts of labelled ACh, and cholinesterase (0.2 mg) in a total volume of 0.5 ml, pH 7.4. Incubation was for 2 h at room temperature. The reaction was stopped with 0.5 ml of 50% (w/v) trichloroacetic acid. A filter paper disc was introduced into each bottle and the contents were dried overnight *in vacuo*. A scintillation fluid was added and the samples were counted in a Packard Tri-Carb scintillation counter at approx. 80% efficiency.

Substrate	Sample volume (ml)	Cholinesterase	Radioactivity (c.p.m.)	Hydrolysis (%)
Choline, methyl-labelled	0.10	—	1243	—
	0.10	+	1303	0
ACh, methyl-labelled	0.10	—	607	—
	0.10	+	631	0
ACh, acetyl-labelled	0.10	—	770	—
	0.10	+	13	98.3
	0.07	+	8	98.5
	0.04	+	6	98.0
	0.02	+	3	98.0

Table 3. *Concentration of ACh in brain slices*

	Rat striatum		Rat cerebral cortex		Guinea-pig cerebral cortex	
	(nmol/mg of protein)	(nmol/g wet wt.)	(nmol/mg of protein)	(nmol/g wet wt.)	(nmol/mg of protein)	(nmol/g wet wt.)
Sattin (1966)						
no incubation	0.40	47				
Takahashi & Aprison (1964)						
no incubation		38		7.9		
Mann <i>et al.</i> (1938)						
no incubation				10		
1 h incubation				41		
Browning & Schulman (1969)						
1 h incubation				27		
This paper						
no incubation	0.36	31	0.12	10.5	—	14.9
1 h incubation	4.08	172	0.57	26.7	0.19	13.0

experimental samples the percentage of labile acetyl group times the total counts in the ACh fraction gave corrected total counts in ACh.

The amounts of ACh in the freeze-dried samples were assayed with the frog rectus abdominis muscle (Chang & Gaddum, 1933). No interfering substance was found by the Feldberg technique (Feldberg & Vogt, 1948).

RESULTS

Concentration of ACh in slices. The concentrations of ACh in rat striatum and cerebral-cortex slices are shown in Table 3. Without incubation the results were in general agreement with those reported in the literature (Mann, Tennenbaum & Quastel, 1938; Takahashi & Aprison, 1964; Sattin, 1966). After incubation the ACh content in the

striatum slices increased some 11–12-fold from the resting value of 0.36 nmol/mg of protein to 4.1 ± 0.1 nmol/mg of protein ($n = 24$). This compared favourably with the reported increase of 14–15-fold (Sattin, 1966). Although the increase in the cerebral-cortex slices was less, only fivefold to 0.57 ± 0.02 nmol/mg of protein ($n = 14$), it was also in line with that reported by Mann *et al.* (1938). The ACh concentration in slices after incubation in media containing various labelled substrates in addition to glucose remained the same.

Incorporation of radioactivity into the acetyl group of ACh. Slices from both the striatum and cerebral cortex incorporated C-2 of pyruvate into the acetyl moiety of ACh to a great extent (Table 4). In the slices from the rat striatum and cerebral cortex, the specific radioactivity of ACh reached approx. 30%

Table 4. Incorporation of radioactivity into ACh from various ^{14}C -labelled substrates

Substrate	Rat striatum slices			Rat cerebral-cortex slices			Guinea-pig cerebral cortex slices		
	Total radioactivity (c.p.m./mg of protein)	Hydrolysis (%)	Sp. radioactivity of acetyl group (c.p.m./ μmol)	Total radioactivity (c.p.m./mg of protein)	Hydrolysis (%)	Sp. radioactivity of acetyl group (c.p.m./ μmol)	Total radioactivity (c.p.m./mg of protein)	Hydrolysis (%)	Sp. radioactivity of acetyl group (c.p.m./ μmol)
$[2\text{-}^{14}\text{C}]\text{Acetate}$	428	88	7500	222	64	20800	133	82	27000
$[^{14}\text{C}]\text{Bicarbonate}$	16	52	175	7	38	527	—	—	—
$[1,5\text{-}^{14}\text{C}]\text{Citrate}$	47	29	229	85	5	695	32	45	1920
$[1\text{-}^{14}\text{C}]\text{Glutamate}$	72	3	87	134	7	1300	9	38	520
$[5\text{-}^{14}\text{C}]\text{Glutamate}$	337	25	1670	436	3	1770	109	21	1540
$[1\text{-}^{14}\text{C}]\text{Pyruvate}$	14	25	51	15	2	21	1	—	0
$[2\text{-}^{14}\text{C}]\text{Pyruvate}$	24500	99	599000	3450	96	390000	1220	98	137000

The specific radioactivities in the acetyl group of ACh were calculated by multiplying the total c.p.m. by the percentage hydrolysis/total μmol of ACh.

and 19% respectively of that of pyruvate in the medium, whereas in the guinea-pig cerebral-cortex slices it was only 5.6%. With $[2\text{-}^{14}\text{C}]\text{acetate}$ as the precursor much less incorporation was found; in the same order as above, the specific radioactivities of ACh were 0.6, 1.7 and 2.4% respectively of that of acetate in the medium. Cerebral-cortex slices utilized acetate better than striatum slices whereas the reverse was true for pyruvate. There seemed to be an inverse relationship between pyruvate and acetate utilization in the three kinds of slices.

The total radioactivity and the percentage of hydrolysis in Table 4 illustrated the reliability of the specific radioactivity calculated from these results. A low percentage of hydrolysis (less than 20%) in any experiment or a low total radioactivity rendered the corrected specific radioactivity invalid. Thus the $[5\text{-}^{14}\text{C}]\text{glutamate}$ experiments in both the rat striatum slices and the guinea-pig cerebral cortex slices showed significant, although low, incorporation and the results from $[1,5\text{-}^{14}\text{C}]\text{citrate}$ experiments in both tissues were of doubtful significance. All other experiments with $[^{14}\text{C}]\text{bicarbonate}$, $[1,5\text{-}^{14}\text{C}]\text{citrate}$, $[1\text{-}^{14}\text{C}]\text{glutamate}$, $[5\text{-}^{14}\text{C}]\text{glutamate}$ and $[1\text{-}^{14}\text{C}]\text{pyruvate}$ did not show any significant incorporation of radioactivity into the acetyl group of ACh.

Incorporation of radioactivity into ACh with time. The incorporation of radioactivity into the acetyl moiety of ACh from $[2\text{-}^{14}\text{C}]\text{pyruvate}$ and $[2\text{-}^{14}\text{C}]\text{acetate}$ with respect to time was further investigated in the rat striatum slices (Table 5). As expected, the concentration of ACh increased with the duration of incubation, but most of the synthesis took place in the first half hour, as was observed by Satin (1966). The specific radioactivity in the acetyl moiety of ACh was much larger with $[2\text{-}^{14}\text{C}]\text{pyruvate}$ as the substrate than that with $[2\text{-}^{14}\text{C}]\text{acetate}$. Further, the specific radioactivities in the acetate experiments did not reach a steady value until after an hour of incubation, whereas in the pyruvate experiment a high value was reached within 15 min. Another difference lay in the percentage of hydrolysable acetyl group. In the pyruvate experiments all radioactivity was in the acetyl moiety, but this was not so in the acetate experiments.

Relative radioactivities of ACh and citrate. Since acetyl-CoA is the immediate precursor for both ACh and citrate syntheses, the specific radioactivities of ACh and citrate were compared (Table 6) to assess the precursor-product relationships. Particularly in view of the compartmentation of the intermediates of the tricarboxylic acid cycle in the brain (Berl & Clarke, 1969), it is relevant to ask whether the acetyl-CoA pool is also compartmentalized. The relative specific radioactivity ratio (specific radioactivity of citrate/specific

Table 5. *Specific radioactivity of ACh in rat striatum slices incubated for various times in media containing two different radioactive substrates*

The specific radioactivities in the acetyl group of ACh were calculated as indicated in Table 4.

Substrate	Incubation time (min)	ACh(nmol/ mg of protein)	Hydrolysis (%)	Sp. radioactivity of acetyl group (c.p.m./ μ mol)
None	0	0.36		
[2- ¹⁴ C]Pyruvate	15	2.85	100	541 000
	30	3.89	100	534 000
	60	3.98	100	612 000
	90	4.57	97	372 000
[2- ¹⁴ C]Acetate	15	2.62	42	2420
	30	3.33	87	5940
	60	4.20	86	7280
	90	4.54	77	7510

Table 6. *Specific radioactivities of ACh and citrate and their ratios derived from [2-¹⁴C]pyruvate and [2-¹⁴C]acetate*

Values for citrate in parentheses are in c.p.m./mg of protein; all others and those for ACh are in c.p.m./ μ mol. Values in the last column are dimensionless, except for those in parentheses. Results with the lobster nerve are taken from the following paper (Cheng & Nakamura, 1970).

Tissue	Substrate	Sp. radioactivity		Citrate/ACh sp. radioactivity ratio
		Citrate	ACh	
Rat striatum	Pyruvate	1 300 000	599 000	2.2
	Acetate	184 000	7500	24.5
Rat cerebral cortex	Pyruvate	1 310 000	390 000	3.4
	Acetate	263 000	20 800	12.7
Guinea-pig cerebral cortex	Pyruvate	(684)	139 000	(0.0049)
	Acetate	(2260)	28 250	(0.0800)
Lobster nerve	Pyruvate	119 200	20 690	5.76
	Acetate	160 100	111 300	1.44

radioactivity of ACh) from labelled acetate was larger than that from pyruvate in the rat striatum and cerebral-cortex slices. With the guinea-pig cerebral-cortex slices, the quoted ratio may not be valid since the citrate values are expressed as c.p.m./mg of protein rather than as c.p.m./ μ mol of citrate, which was not determined.

DISCUSSION

Pyruvate as the principal substrate for ACh synthesis. The extent of radioactivity incorporation into the acetyl moiety of ACh from [2-¹⁴C]pyruvate was high (Table 4). In the striatum slices, the specific radioactivity reached 30% of that of the precursor in less than 15 min (Table 5). Such speed and extent of incorporation suggested an extremely rapid turnover of acetyl-CoA. [2-¹⁴C]Acetate was also a substrate, but the extent and speed of

incorporation were far less. Thus pyruvate can probably be considered as the normal substrate for ACh synthesis in brain slices.

While this manuscript was being prepared, Browning & Schulman (1969) published results on ACh synthesis from glucose, pyruvate and acetate. They found a transfer of radioactivity to ACh from [U-¹⁴C]glucose, [1- or 6-¹⁴C]glucose and [2-¹⁴C]pyruvate but not from [2-¹⁴C]acetate. Unfortunately, no observations were made with labelled citrate or glutamate. No glucose was present in the acetate experiments to support the slice metabolism, and thus no labelled ACh was synthesized from [2-¹⁴C]acetate. The incorporation of radioactivity from [3-¹⁴C]pyruvate has been reported by Cheney, Gubler & Jaussi (1969). The amounts of radioactivity incorporated were highly variable, which was probably due to the manner in which the labelled pyruvate was introduced. To study brain metabolism after subcutaneous injection of a sub-

strate encounters two difficulties, namely the participation of liver metabolism and the penetration of the blood-brain barrier.

On comparison of pyruvate and acetate as the substrate in the two kinds of rat brain slices, a difference in radioactivity incorporation into ACh emerged. In relative but not in absolute terms striatum slices utilized pyruvate better than did cerebral-cortex slices, but the contrary was observed with acetate. Perhaps various parts of the brain use these substrates differently [see Berl & Frigyesi (1969) for analogous observations on leucine incorporation]. We found that in a different nervous tissue, the lobster nerve, acetate was utilized far better than pyruvate (Cheng & Nakamura, 1970). The much lower incorporation of radioactivity from acetate and citrate could be a reflexion of the relatively low activity of acetate thiokinase and of the citrate-cleavage enzyme respectively in the brain (Tuček, 1967b). The lower permeability of these two acids may be a further reason for their lower incorporation compared with pyruvate. Also, the proportion of total incorporated radioactivity entering into ACh was quite different for the various precursors. From [2-¹⁴C]acetate, [1,5-¹⁴C]citrate and [2-¹⁴C]pyruvate the proportions of radioactivity in ACh as a percentage of the total incorporated radioactivity were respectively 0.22, 0.002 and 1.7% for rat striatum slices, 0.04, 0.07 and 0.15% for rat cerebral-cortex slices and 0.06, 0.001 and 0.14% for the guinea-pig cerebral-cortex slices. Clearly pyruvate was a better substrate for ACh synthesis.

[5-¹⁴C]Glutamate as substrate and the reversal of the tricarboxylic acid cycle. A small but definite incorporation of radioactivity into ACh from [5-¹⁴C]glutamate was found in rat striatum slices and guinea-pig cerebral-cortex slices. There was more than sufficient total radioactivity in the samples to be determined accurately and a value for hydrolysable radioactivity of over 20% was consistently obtained. With rat cerebral-cortex slices the low percentage of hydrolysable radioactivity made the results unreliable. These results confirmed the series of reactions leading from glutamate to α -oxoglutarate, citrate, acetyl-CoA and finally ACh (Waelsch *et al.* 1965). In the rat the specific radioactivity of ACh was approx. 0.5 and 0.3% of that of citrate in striatum slices and in cerebral-cortex slices respectively. These values indicated that the rate of conversion was small, which served to confirm the low activity of citrate-cleavage enzyme (Tuček, 1967b). However, the values of 0.5 and 0.3% should be compared with 0.003 and 0.002% observed with exogenous added citrate (S.-C. Cheng and R. Nakamura unpublished work). These large differences probably arose from metabolic compartmentation of citrate (S.-C.

Cheng & R. Nakamura, unpublished work; see also Berl & Clarke, 1969).

A comparison was made of the incorporation of radioactivity from acetate and pyruvate into ACh and citrate, which shared acetyl-CoA as their common precursor. If acetate and pyruvate were entering into a common pool of acetyl-CoA, and at the same time ACh and citrate were derived from the same common pool of acetyl CoA, then the relative specific radioactivity ratio would remain the same, irrespective of whether acetate or pyruvate was the source of radioactivity in acetyl-CoA. Results in Table 6 suggested that this was not so. The ratio was always higher in brain slices when labelled acetate was the precursor. Those from acetate were approx. 11 and 3.7 times those from pyruvate for rat striatum and cerebral cortex respectively. A high ratio was also obtained with guinea-pig cerebral-cortex slices, but here total radioactivity instead of specific radioactivity was used in the calculation. This deviation from unity suggested compartmentation of acetyl-CoA in the brain slices (as suggested by Hebb, 1957; Berl & Frigyesi, 1968) and could only be explained by assuming that: (a) there were at least two pools of acetyl-CoA; (b) pyruvate and acetate entered the two pools differently; (c) ACh and citrate utilized acetyl-CoA from the two pools differentially. In the lobster nerve, the ratio for acetate was less than that for pyruvate. We tentatively suggest, although no direct experimental measurements have been made, that these metabolic compartments reside in different cellular structures. In the lobster nerve, these compartments could be located in motor, sensory or inhibitory fibres. For example, a high concentration of γ -aminobutyrate is only found in the inhibitory fibres (Kravitz & Potter, 1965). In brain, they could be located in neurones, glia, nerve fibres or nerve endings (Rose, 1968; Chakrin & Whittaker, 1969; Neidle, van den Berg & Grynbaum, 1969; Berl & Clarke, 1969). The compartmentation of acetyl-CoA in brain could also be linked to the compartmentation of tricarboxylic acid cycle as a whole. Evidence for this assumption has been communicated elsewhere (Nakamura & Cheng, 1969).

Transport of acetyl-CoA from mitochondria. Since it is generally agreed that choline acetylase is extra-mitochondrial (see Fonnum, 1968) and acetyl-CoA is produced inside the mitochondria the problem arises as to how acetyl-CoA is transported from the inside of mitochondria to the sites of ACh synthesis. In liver, four possible mechanisms for acetyl-CoA transport have been proposed, involving (a) a direct transport of acetyl-CoA, (b) its transport via acetyl-carnitine, (c) its transport via acetate, and (d) its transport via citrate, which was considered the most likely route (Srere, 1965; Spencer &

Lowenstein, 1966; Greville, 1969). In the present studies with brain slices, no significant radioactivity was found in ACh from [1,5-¹⁴C]citrate, a finding not consistent with the citrate mechanism postulated for liver. On the other hand, a small amount of radioactivity was transferred from [5-¹⁴C]glutamate to ACh giving weak support to the oxoglutarate shunt hypothesis (D'Adamo & D'Adamo, 1968). Acetyl thio kinase is located primarily in the mitochondria in the brain (see Nakamura & Cheng, 1969), so the acetyl-CoA synthesized from acetate would be intramitochondrial. This cannot serve as an acetate-mediated transport mechanism where intramitochondrial acetate-CoA is required to be deacetylated at the inside of the mitochondrial membrane and the resultant acetate combines with another CoA on the outside. The possibility of either a direct transport of acetyl-CoA or its transport via the acetyl carnitine mechanism cannot be assessed here. Other possibilities also exist such as its transport in the form of acetylhistidine or acetylgarginine (Erspamer, Roseghini & Anastasi, 1965; Ohkusu & Mori, 1969). Its transport as acetylaspartate or acetylglutamate should not be likely since these ions would be more charged and their metabolic turnover was reported to be slow (Reichelt & Kvamme, 1967; Marcucci, Airolidi & Mussini, 1969).

The authors thank the U.S. Public Health Service for financial support (Grant NB-05869), Mr B. Arenberg for his technical assistance, and Dr A. Lajtha for his encouragement in this work. R.N. was a Fulbright Scholar and H.N. a Rockefeller Foundation Fellow from the University of Tokyo, Japan.

REFERENCES

- Berl, S. & Clarke, D. D. (1969). In *Handbook of Neurochemistry*, vol. 2, p. 447. Ed. by Lajtha, A. New York: Plenum Press Inc.
- Berl, S. & Frigyesi, T. L. (1968). *J. Neurochem.* **15**, 965.
- Berl, S. & Frigyesi, T. L. (1969). *J. Neurochem.* **16**, 405.
- Browning, E. T. & Schulman, M. P. (1969). *J. Neurochem.* **15**, 1391.
- Chakrin, L. W. & Whittaker, V. P. (1969). *Biochem. J.* **113**, 97.
- Chang, C. H. & Gaddum, J. H. (1933). *J. Physiol., Lond.*, **79**, 255.
- Cheney, D. L., Gubler, C. J. & Jaussi, A. W. (1969). *J. Neurochem.* **16**, 1283.
- Cheng, S.-C. & Nakamura, R. (1970). *Biochem. J.* **118**, 451.
- Cheng, S.-C. & Waelsch, H. (1963). *Biochem. Z.* **338**, 643.
- D'Adamo, A. F. & D'Adamo, A. P. (1968). *J. Neurochem.* **15**, 315.
- Dettbarn, W.-D. & Rosenberg, P. (1966). *J. gen. Physiol.* **50**, 447.
- Erspamer, V., Roseghini, M. & Anastasi, A. (1965). *J. Neurochem.* **12**, 123.
- Feldberg, W. & Vogt, M. (1948). *J. Physiol., Lond.*, **107**, 372.
- Fonnum, F. (1968). *Biochem. J.* **109**, 389.
- Greville, G. D. (1969). In *Citric Acid Cycle: Control and Compartmentation*, p. 1. Ed. by Lowenstein, J. M. New York: Marcel Dekker.
- Hebb, C. O. (1954). *Pharmac. Rev.* **6**, 39.
- Hebb, C. O. (1957). *Physiol. Rev.* **37**, 2.
- Hemsworth, B. A. & Morris, D. (1964). *J. Neurochem.* **11**, 793.
- Hestrin, S. (1949). *J. biol. Chem.* **180**, 879.
- Hestrin, S. (1950). *Biochim. biophys. Acta*, **4**, 310.
- Kravitz, E. A. & Potter, D. D. (1965). *J. Neurochem.* **12**, 323.
- Lipton, M. A. & Barron, E. S. G. (1946). *J. biol. Chem.* **166**, 367.
- McIlwain, H. & Rodnight, R. (1962). *Practical Neurochemistry*, p. 109. Boston: Little Brown and Co.
- Mann, P. J. G., Tennenbaum, M. & Quastel, J. H. (1938). *Biochem. J.* **32**, 243.
- Mans, R. J. & Novelli, G. D. (1961). *Archs Biochem. Biophys.* **94**, 48.
- Marcucci, F., Airolidi, L. & Mussini, E. (1969). *J. Neurochem.* **16**, 272.
- Nakamura, R. & Cheng, S.-C. (1967). *Proc. 1st int. Meet. int. Soc. Neurochemistry, Strasbourg*, p. 160.
- Nakamura, R. & Cheng, S.-C. (1969). *Life Sci.* **8**, 657.
- Nakamura, R., Cheng, S.-C., Naruse, H. & Waelsch, H. (1966-67). *Brain Res.* **3**, 384.
- Naruse, H., Cheng, S.-C. & Waelsch, H. (1966). *Expl Brain Res.* **1**, 40.
- Neidle, A., van den Berg, C. J. & Grynbaum, A. (1969). *J. Neurochem.* **16**, 225.
- Ohkusu, H. & Mori, A. (1969). *J. Neurochem.* **16**, 1485.
- Quastel, J. H., Tennenbaum, M. & Wheatley, A. H. M. (1936). *Biochem. J.* **30**, 1668.
- Randerath, K. (1966). *Thin Layer Chromatography*, p. 151. New York: Academic Press Inc.
- Reichelt, K. L. & Kvamme, E. (1967). *J. Neurochem.* **14**, 987.
- Rose, S. P. R. (1968). *J. Neurochem.* **15**, 1415.
- Sattin, A. (1966). *J. Neurochem.* **13**, 515.
- Spencer, A. F. & Lowenstein, J. M. (1966). *Biochem. J.* **99**, 760.
- Srere, P. A. (1965). *Nature, Lond.*, **205**, 766.
- Takahashi, R. & Aprison, M. H. (1964). *J. Neurochem.* **11**, 887.
- Tuček, S. (1967a). *J. Neurochem.* **14**, 519.
- Tuček, S. (1967b). *J. Neurochem.* **14**, 531.
- Waelsch, H., Cheng, S.-C., Côté, L. & Naruse, H. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 1249.