The Use of Choline Acetyltransferase for Measuring the Synthesis of Acetyl-Coenzyme A and its Release from Brain Mitochondria

By STANISLAV TUČEK*

Department of Physiology, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

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1. A method for measuring small amounts of acetyl-CoA synthesized in sub-cellular fractions of the brain from pyruvate and released from particles into the incubation medium has been developed by using placental choline acetyltransferase and choline in the incubation medium to transform acetyl-CoA into acetylcholine. Acetylcholine is measured by biological assay. Optimum conditions of incubation are described. 2. With fresh mitochondria, a decrease of acetyl-CoA output into the medium is observed in the presence of ATP or ADP, and an increase in the presence of calcium chloride or 2,4-dinitrophenol. Fluorocitrate and malonate have little or no effect. 3. After the mitochondria had been treated with ether, the release of acetyl-CoA into the medium is much larger; presumably, nearly all acetyl-CoA synthesized is then released and transformed into acetylcholine under the conditions used. The release of acetyl-CoA is diminished in the presence of Krebs-cycle intermediates and ADP. 4. Of all subcellular fractions, the highest acetyl-CoA production from pyruvate is found in the crude mitochondria; rates up to 51 μ moles of acetyl-CoA/g. of original tissue/hr. are observed in ether-treated samples. 5. The activities of acetyl-CoA synthetase and ATP citrate lyase found in homogenates and nerve-ending fractions of brain tissue are considerably lower than those of pyruvate oxidase complex and choline acetyltransferase. 6. The bearing of some of the findings on the question of the source of acetyl radicals for the synthesis of acetylcholine *in vivo* is discussed.

Pyruvate appears to be the most important source of acetyl-CoA in the brain. The oxidative decarboxylation of pyruvate occurs intramitochondrially (Sanadi, 1963). Mitochondrial membrane, however, is generally believed to be only slightly, if at all, permeable to acetyl-CoA, and the question of how intramitochondrially produced acetyl-CoA may be put at the disposal of extra-mitochondrial enzymes is of the utmost importance (Srere, 1965; Kornacker & Lowenstein, 1965; Hardwick, 1966).

In the present work an attempt has been made to devise a suitable method for measuring the amounts of acetyl-CoA synthesized from pyruvate inside the mitochondria and then released into the incubation medium. For this purpose, choline and choline acetyltransferase (acetyl-CoA-choline *O*-acetyltransferase, EC 2.3.1.6) were included in the incubation medium. Since the equilibrium of the

reaction catalysed by choline acetyltransferase is far in the direction of the formation of ACh† (Reisberg, 1957), most of the acetyl-CoA that passed through the mitochondrial membrane could be transformed into ACh during the incubation and measured as such by biological assay. A similar way of measuring low rates of acetyl-CoA synthesis from citrate and acetate in ether-treated subcellular fractions of the brain has already proved useful (Tuček, 1966a).

The results indicate that the use of choline acetyltransferase to convert acetyl-CoA into ACh may be a valuable means for measuring the passage of acetyl-CoA through the mitochondrial membranes.

METHODS

Subcellular fractions. Rabbit forebrains or sheep caudate nuclei were homogenized in 0.32M-sucrose and separated by centrifugation in a Servall type SS-1 angle centrifuge

* Present address: Department of Physiology, Charles University Medical Faculty, Plzeň, Czechoslovakia.

† Abbreviation: ACh, acetylcholine.

into three primary fractions: nuclear fraction P_1 (pellet at $900g_{\max}$ for 5 min.), crude mitochondrial fraction P_2 (pellet at $12000g_{\max}$ for 30 min.) and final supernatant S_2 . Fraction P_2 was separated into three subfractions, A, B and C, by centrifugation ($51000g_{\text{av}}$ for 120 min. in the SW 39 L rotor of the Beckman model L preparative ultracentrifuge) on a discontinuous density gradient composed of 0.8M-, 1.2M- and 1.5M-sucrose. Subfraction A was obtained from the top of the gradient and contained mainly myelin and microsomes; subfraction B, from the middle, contained mainly nerve endings; subfraction C, the bottom, contained mainly mitochondria and some nerve endings. The fractionation technique was similar to that described by Hebb & Whittaker (1958), Gray & Whittaker (1962), De Robertis, Pellegrino de Iraldi, Rodríguez de Lores Arnaiz & Salganicoff (1962) and Eichberg, Whittaker & Dawson (1964); details have been given elsewhere (Tuček, 1966c, 1967b).

Incubation and assay. Subcellular fractions were incubated either with or without previous treatment with ether (Tuček, 1967a). Fractions not treated with ether were used within approx. 2 hr. after they had been prepared; ether-treated fractions were used either after a similar delay, or after being kept frozen at -18° for 1–2 days. If they had been treated with ether, no difference was found between fresh and frozen fractions. Small volumes (0.01–0.1 ml.) of the subcellular fractions were incubated for 1 hr. at 39° in a medium of 0.5 ml. final volume (adjusted to pH 7.4) and of the following final composition: 10 mM-potassium pyruvate, 0.5 mM-CoA, 10 mM-NAD $^+$, 1.2 mM-thiamine pyrophosphate, 1.25 mM-MnCl $_2$, 14 mM-choline chloride, 0.1 mM-*serine* sulphate, 12.5 mM-cysteine hydrochloride, 50 mM-potassium phosphate ($\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$) and 100 mM-KCl; partially purified choline acetyltransferase from human placenta (Morris, 1966) was added in a volume of 0.01 ml./0.5 ml. of final incubation volume. In the initial experiments, lactate dehydrogenase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) was added to the incubation mixture (3.6 units/ml. of incubation medium); later, it was found to have no significant effect and was therefore omitted. The incubation was stopped after 1 hr. and the samples were prepared for biological assay of ACh on frog rectus abdominis muscle as described by Morris & Tuček (1966).

RESULTS

Optimum concentrations of the components of the incubation medium. Experiments aimed at finding the optimum composition of the incubation medium were all performed with P_2 fractions treated with ether. Treatment with ether most probably caused the diffusion of substances like CoA or NAD $^+$ from mitochondria into the medium and a decrease of their concentration inside the mitochondria where the synthesis of acetyl-CoA from pyruvate occurred. It must be assumed, therefore, that lower concentrations of these substances in the incubation medium might have proved sufficient to obtain maximum values of acetyl-CoA synthesis and outward diffusion from the particles in those experiments in which mitochondria not treated with ether were used.

Fig. 1. Influence of the concentration of NAD $^+$ in the incubation medium on the synthesis of acetyl-CoA from pyruvate by ether-treated crude mitochondrial fraction from rabbit forebrains. Conditions of incubation were as described in the Methods section. Acetyl-CoA was measured as ACh, formed by choline acetyltransferase included in the incubation medium. The yield was expressed in $\mu\text{moles/g.}$ of original tissue/hr.

Fig. 2. Influence of the concentration of CoA in the incubation medium on the synthesis of acetyl-CoA from pyruvate by ether-treated crude mitochondrial fraction from rabbit forebrains. Conditions of incubation were as described in the Methods section; the concentration of NAD $^+$ in the incubation medium was 12 mM. Acetyl-CoA was measured and the yield expressed as in Fig. 1.

The effect of varying the concentration of NAD $^+$ in the incubation medium is shown in Fig. 1. The necessity for using high concentrations of NAD $^+$ may be due in some degree to the presence of NAD nucleosidase (NAD glycohydrolase, EC 3.2.2.5) in the brain (Kaplan, 1955). Addition of nicotinamide to the system was considered as a means of inhibiting NAD nucleosidase, but this modification was not

Fig. 3. Influence of the concentration of thiamine pyrophosphate in the incubation medium on the synthesis of acetyl-CoA from pyruvate by ether-treated crude mitochondrial fraction from rabbit forebrains. Conditions of incubation were as described in the Methods section; 6mM-NAD⁺, 2mM-lipoic acid and 0.1mM-FAD were present in the incubation medium. Acetyl-CoA was measured and the yield expressed as in Fig. 1.

adopted because the yield of acetyl-CoA was lower in a few experiments in which nicotinamide was tested.

The dependence of acetyl-CoA synthesis on the concentrations of CoA and of thiamine pyrophosphate is shown in Figs. 2 and 3 respectively.

Since lipoic acid is known to form part of the pyruvate oxidase complex, possible need for its inclusion in the incubation medium (at least with ether-treated fractions) had to be considered. The results of an experiment with different concentrations of lipoic acid indicated that concentrations of lipoic acid up to 2.0mM had little or no effect and that 10mM-lipoic acid inhibited by about 40%. Similarly, adding FAD did not increase the yield of acetyl-CoA.

The effect of pyruvate concentration was investigated in the experiment described in Table 1.

Time-course. The time-course of ACh synthesis was linear for 60min. under the conditions used (Fig. 4).

Production of acetyl-CoA from pyruvate by different subcellular fractions. The values for the output of acetyl-CoA by different subcellular fractions incubated in the presence of pyruvate are given in Table 2. As might have been expected, by far the highest output was found with fraction P₂. When calculated per g. of original tissue, its output was higher than that found in the original homogenate. This might be due to the utilization or destruction of a proportion of acetyl-CoA by some enzymes present in the whole homogenate, but missing from fraction P₂. Another possibility was that more acetyl-CoA was utilized via the Krebs cycle in

Table 1. *Synthesis of acetylcholine by ether-treated crude mitochondrial fraction of rabbit brain homogenate in the presence of various concentrations of potassium pyruvate in the incubation medium*

Conditions of incubation were as given in the Methods section, except that placental choline acetyltransferase had not been included in the incubation medium and the synthesis of ACh from acetyl-CoA and choline was due to the choline acetyltransferase present in the nerve endings, which form one of the main constituents of brain crude mitochondrial fractions; 2mM-lipoic acid and 0.1mM-FAD were present in the incubation medium. All data are the means of two observations.

Conc. of potassium pyruvate (mM)	Synthesis of ACh (μmoles/ml. of fraction/hr.)
0	8
1	96
10	334
25	288

Fig. 4. Time-course of the synthesis of acetyl-CoA from pyruvate by ether-treated crude mitochondrial fraction from rabbit forebrains. Conditions of incubation were as described in the Methods section; the concentration of NAD⁺ was 12mM. Acetyl-CoA was measured and the yield expressed as in Fig. 1.

experiments with the homogenate, since more intermediates might be present; however, dialysis of the homogenate did not improve the yield of acetyl-CoA.

In experiments with ether-treated fractions, the sum of activities found in subfractions A, B and C was much lower than the activity in fraction P₂ before its fractionation in the density-gradient centrifugation (Table 2). The loss of activity could have been due to changes in the structure of mitochondria under hyperosmotic conditions. It does

Table 2. *Output of acetyl-CoA from the particles into the incubation medium in subcellular fractions from rabbit forebrains and sheep caudate nuclei incubated in the presence of pyruvate*

Acetyl-CoA was measured as ACh, formed by choline acetyltransferase included in the incubation medium. Conditions of incubation as described in the Methods section; the concentration of NAD⁺ was 12mM in the experiment with rabbit brain and 7.2mM in the experiment with sheep caudate nuclei; 0.5mM-lipoic acid was present in the incubation medium in the experiment with rabbit brain. All data are the means of two observations.

Experiment	Fraction	Yield of ACh (μ moles/g. of original tissue/hr.)	% of total recovered activity
Rabbit forebrains (fractions not treated with ether)	Homogenate	2.12	
	P ₁	0.14	4.8
	P ₂	2.53	87.6
	S ₂	0.22	7.6
	Recovery...	2.89 (136%)	
	A	0.01	0.3
	B	0.21	5.7
	C	3.47	94.0
	Recovery...	3.69 (146%)	
	Homogenate	33.60	
Rabbit forebrains (fractions treated with ether)	P ₁	1.56	3.5
	P ₂	43.40	96.1
	S ₂	0.19	0.4
	Recovery...	45.15 (134%)	
	A	1.05	5.9
	B	5.44	30.3
	C	11.46	63.8
	Recovery...	17.95 (41%)	
	Homogenate	27.30	
	P ₁	6.89	17.1
Sheep caudate nuclei (fractions treated with ether)	P ₂	33.10	82.4
	S ₂	0.20	0.5
	Recovery...	40.19 (147%)	
	A	0.18	2.0
	B	1.17	13.1
	C	7.60	84.9
	Recovery...	8.95 (27%)	

not seem probable that it was due to spatial separation of the components of the pyruvate oxidase complex: when the fractions obtained on the density gradient were combined to reconstitute the original fraction P₂, the activity still remained low (Table 3).

Effect of ether on the yield of acetyl-CoA measured as acetylcholine. Fractions treated with ether generally produced much more ACh in the incubation system used; this may be seen from the results in Table 4, and similar observations were made repeatedly. It should be stressed that this effect was not dependent on an activation of choline acetyltransferase present in the subcellular particles (Hebb & Smallman, 1956), since an excess of purified and fully active enzyme had been added to the system. The increase in the yield of ACh was probably due to easier diffusion of acetyl-CoA from the mitochondria, but the present experiments do not show whether or not ether treatment had any influence on mitochondrial acetyl-CoA synthesis as such.

Effect of some substances on the output of acetyl-

CoA from mitochondria into the incubation medium.

In the living cell, an important proportion of acetyl-CoA produced by oxidative decarboxylation of pyruvate enters the Krebs cycle of tricarboxylic acids. If the Krebs cycle were still working under the conditions of present experiments, the diffusion of acetyl-CoA from mitochondria into the incubation medium should be considerably decreased by the utilization of a proportion of acetyl-CoA for the formation of citrate. The possibility that the diffusion of acetyl-CoA into the incubation medium might be increased by blocking the Krebs cycle was therefore considered. As shown by the results in Table 4 this expectation has not been fulfilled: except for just one experiment, the output of acetyl-CoA into the incubation medium either remained very close to the control values (within $\pm 6\%$), or it was decreased in the presence of malonate, fluorocitrate or fluoroacetate.

Of other substances used, calcium chloride and 2,4-dinitrophenol increased the output of acetyl-CoA from untreated mitochondria (Table 5), and ADP, ATP, citrate and methylene blue decreased

Table 3. *Effect of combining the fractions obtained by density-gradient centrifugation on the recovery of activity of pyruvate oxidase complex*

The crude mitochondrial fraction (P_2) from sheep caudate nuclei was separated on a sucrose density gradient into three subfractions, A, B and C. The activity in the original fraction P_2 , the sum of activities in subfractions A, B and C and the activity in a reconstituted fraction P_2 obtained by combining subfractions A, B and C in appropriate proportions were compared; all fractions were treated with ether before incubation. The activity of the pyruvate oxidase complex was assessed according to the yield of acetyl-CoA in the incubation medium; it was measured as ACh formed by choline acetyltransferase contained in the medium. Conditions of incubation as described in the Methods section. All data are the means of two observations.

Fraction	Yield of ACh (μ moles/g. of original tissue/hr.)
P_2	33.10
A + B + C incubated separately	8.95
A + B + C combined in appropriate proportions to reconstitute P_2 and incubated simultaneously	5.29

the acetyl-CoA output from untreated mitochondria.

In mitochondria treated with ether, both calcium chloride and 2,4-dinitrophenol had no effect (Table 5); ATP and ADP decreased the acetyl-CoA output, and the decrease caused by ADP was not diminished by 2,4-dinitrophenol. All intermediates of the Krebs cycle used (citrate, α -oxoglutarate, succinate, oxaloacetate) decreased acetyl-CoA output; it was lowest in the presence of oxaloacetate. Methylene blue also caused a decrease of acetyl-CoA output from ether-treated mitochondria.

Synthesis of acetylcholine from pyruvate as acetyl donor by endogenous choline acetyltransferase of the nerve endings. The crude mitochondrial fraction of rabbit brain may synthesize considerable amounts of ACh (without any external supply of choline acetyltransferase) in the presence of choline and pyruvate. The use of pyruvate as acetyl donor for the synthesis of ACh was dependent to a large extent on the presence of NAD^+ (Fig. 5). Maximum synthesis of ACh in the experiment described in Fig. 5 was very near to the maximum activity of choline acetyltransferase present in the fraction;

Table 4. *Effects of metabolic inhibitors on the output of acetyl-CoA into the incubation medium from crude mitochondrial fractions (P_2) incubated in the presence of pyruvate*

Tubes containing the incubation medium as described in the Methods section served as controls. Other tubes contained the same incubation medium supplemented with one or more substances as indicated below. No cysteine was present in the incubation medium in Expt. 1. Acetyl-CoA was measured as ACh, formed by choline acetyltransferase included in the incubation medium.

Expt. no.	Fraction	Incubation medium	Yield of ACh (μ moles/g. of original tissue/hr.)	Yield of ACh (% of that in control tubes)
1	P_2 from rabbit forebrains, not treated with ether	Control	2.47	100
		Potassium malonate (40 mM)	2.36	96
		Sodium fluorocitrate (5 mM)	1.59	64
		Sodium fluoroacetate (10 mM)	2.43	98
2	P_2 from rabbit forebrains, not treated with ether	Control	1.94	100
		Sodium malonate (4 mM)	1.94	100
3	P_2 from rabbit forebrains, treated with ether	Control	51.2	100
		Potassium malonate (40 mM)	38.7	76
		Sodium fluorocitrate (5 mM)	44.5	87
		Sodium fluorocitrate (5 mM)	39.0	76
		+ potassium malonate (40 mM)		
		Sodium fluoroacetate (2 mM)	60.0	117
		Sodium fluoroacetate (10 mM)	49.3	96
		Sodium fluoroacetate (10 mM)	40.4	79
		+ potassium malonate (40 mM)		
		Sodium fluoroacetate (10 mM)	27.1	53
		+ potassium ATP (5 mM)		
		Sodium fluoroacetate (10 mM)	5.9	12
		+ potassium ATP (5 mM)		
		+ potassium oxaloacetate (10 mM)		
4	P_2 from rabbit forebrains, treated with ether	Control	45.6	100
		Sodium malonate (4 mM)	47.8	105
		Sodium fluorocitrate (2 mM)	48.3	106
		Sodium fluoroacetate (2 mM)	45.6	100

Table 5. *Effects of some substances on the output of acetyl-CoA into the incubation medium from crude mitochondrial fractions (P₂) incubated in the presence of pyruvate*

Tubes containing the incubation medium as described in the Methods section served as controls. Other tubes contained the same incubation medium supplemented with one or more substances as indicated below. No cysteine was present in the incubation medium in Expt. 1. Acetyl-CoA was measured as ACh, formed by choline acetyltransferase included in the incubation medium.

Expt. no.	Fraction	Incubation medium	Yield of ACh (μ moles/g. of original tissue/hr.)	Yield of ACh (% of that in control tubes)
1	P ₂ from rabbit forebrains, not treated with ether	Control	2.47	100
		2,4-Dinitrophenol (0.05 mM)	3.00	121
		2,4-Dinitrophenol (0.05 mM) + sodium fluoroacetate (10 mM)	2.87	116
		CaCl ₂ (0.1 mM)	3.30	134
		CaCl ₂ (1.0 mM)	6.00	243
		Potassium ADP (5 mM)	0.17	7
		Potassium ATP (5 mM)	0.35	14
		Potassium ATP (5 mM) + CaCl ₂ (1 mM)	0.29	12
		Potassium ATP (5 mM) + potassium oxaloacetate (4 mM)	0.21	9
		Potassium ATP (5 mM) + potassium citrate (30 mM)	0.21	9
		Potassium citrate (30 mM)	0.79	32
		Potassium citrate (30 mM) + 2,4-dinitrophenol (0.05 mM)	1.14	46
		Potassium citrate (30 mM) + CaCl ₂ (1 mM)	1.47	59
		Control	1.94	100
		Methylene blue (2 mM)	0.97	50
		Carnitine (2 mM)	1.66	86
		Control	51.2	100
		2,4-Dinitrophenol (0.05 mM)	47.9	94
3	P ₂ from rabbit forebrains, treated with ether	CaCl ₂ (0.1 mM)	47.0	92
		CaCl ₂ (1.0 mM)	46.2	90
		Potassium ADP (5 mM)	9.1	18
		Potassium ADP (5 mM) + 2,4-dinitrophenol (0.05 mM)	8.4	16
		Potassium ATP (5 mM)	22.0	43
		Potassium citrate (30 mM)	28.5	56
		Potassium oxaloacetate (5 mM)	6.7	13
		Control	45.6	100
		Methylene blue (2 mM)	15.1	33
4	P ₂ from rabbit forebrains, treated with ether	Control	33.2	100
		Potassium α -oxoglutarate (10 mM)	22.8	69
5	P ₂ from sheep caudate nuclei, treated with ether	Sodium succinate (10 mM)	22.6	68
		Potassium oxaloacetate (10 mM)	5.7	17

choline acetyltransferase appeared to be the rate-limiting factor in the presence of saturating NAD⁺ concentrations.

Comparison of activities of acetyl-CoA synthetase, ATP citrate lyase, pyruvate oxidase complex and choline acetyltransferase in the brain. The activities of acetyl-CoA synthetase, ATP citrate lyase, pyruvate oxidase complex and choline acetyltransferase were compared in the homogenates and nerve-ending (B) fractions of sheep caudate nuclei and rabbit forebrains (Fig. 6). The activities of

acetyl-CoA synthetase and ATP citrate lyase were considerably lower than those of pyruvate oxidase complex and choline acetyltransferase.

DISCUSSION

The use of choline acetyltransferase for transforming acetyl-CoA into ACh may prove advantageous for measuring small amounts of acetyl-CoA, particularly under conditions of work with long incubation times and slow acetyl-CoA formation or

Fig. 5. Influence of the concentration of NAD⁺ on the synthesis of ACh by ether-treated crude mitochondrial fraction from rabbit forebrains with pyruvate as acetyl donor. Conditions of incubation were as described in the Methods section for measuring acetyl-CoA synthesis from pyruvate, except that no placental choline acetyltransferase had been added to the incubation medium, and the synthesis of ACh was due to the endogenous choline acetyltransferase present in the nerve endings, which form one of the main constituents of the brain crude mitochondrial fractions; 0.2mM-FAD and 2mM-lipoic acid were present in the incubation medium.

diffusion from particulate material. The relative irreversibility of the reaction catalysed by choline acetyltransferase and the high sensitivity of the biological assay of ACh both favour the use of this method. Further, it seems of advantage that choline is unlikely to influence oxidative processes in the mitochondria.

Experiments with intact mitochondria have confirmed that the diffusion of acetyl-CoA into the medium is very small compared with its diffusion from mitochondria after their treatment with ether. It is not certain, however, whether the rate of diffusion observed with untreated organelles is entirely negligible in terms of cellular economy. Most of the factors that caused changes of acetyl-CoA output from intact mitochondria (calcium chloride, ATP, ADP) are known to influence mitochondrial contraction and swelling (Lehninger, 1959; Vignais, Vignais & Lehninger, 1964; Wlodawer, Parsons, Williams & Wojtczak, 1966). It is tempting to explain their effect on acetyl-CoA output in the same way. Thus the decrease of acetyl-CoA output brought about by ADP or ATP would be due to mitochondrial contraction, and the increase of acetyl-CoA release induced by calcium chloride would be due to mitochondrial swelling. 2,4-Dinitrophenol does not fit this scheme; it is known to counteract the swelling and, at the same time, was observed to increase the output of acetyl-CoA from fresh mitochondria in the present experiments. The observation that 2,4-dinitrophenol does not abolish the effect of ADP on ether-

Fig. 6. Comparison of activities of acetyl-CoA synthetase (acetate-activating enzyme, AAE), ATP citrate lyase (citrate-cleavage enzyme, CCE), pyruvate oxidase complex (PO) and choline acetyltransferase (ChAc) in ether-treated homogenates (a) and nerve-ending fractions (b) from sheep caudate nuclei and rabbit forebrains. The activities of AAE, CCE and PO were measured by the yield of acetyl-CoA transformed into ACh by placental choline acetyltransferase, which was present in excess in the incubation mixture. Conditions of incubation for AAE, CCE and ChAc were as described in previous papers (Tuček, 1966a,c, 1967b), for PO as described in the Methods section of this paper. All activities were expressed in μmoles/g. of original tissue/hr.

treated mitochondria agrees with Lehninger's (1959) observation that 2,4-dinitrophenol does not alter the effect of ATP on mitochondrial swelling and with his assumption that both ATP and ADP affect the swelling in a basically similar way, independent of phosphorylating respiration.

The synthesis of acetyl-CoA by ether-treated crude mitochondrial fractions was near to 40 μmoles (33–51 μmoles)/g. of original tissue/hr. This value is very near to the value of 48 μmoles/g./hr. given by McIlwain (1966) as the rate of oxidative decarboxylation of pyruvate by mammalian brain tissue *in vitro*. Hence it seems likely that by far the greatest proportion of acetyl-CoA formed from pyruvate in fact diffused outwards from ether-treated mitochondria and was transformed into ACh. This view would be favoured both by the

failure of calcium chloride, 2,4-dinitrophenol, fluorocitrate and malonate to increase the output of acetyl-CoA of ether-treated mitochondria, and by the great decrease of acetyl-CoA output when Krebs-cycle intermediates were added to the incubation medium.

The main source of acetyl groups that are used for the synthesis of ACh by the tissue of the brain *in vivo* has not yet been established; it is beyond doubt that acetyl-CoA is the immediate precursor (Balfour & Hebb, 1952). In experiments with brain homogenates or extracts of dried brain tissue, acetate, citrate and acetoacetate served as acetyl donors for the synthesis of ACh (Stedman & Stedman, 1937; Feldberg & Hebb, 1947; Balfour & Hebb, 1952; Ebashi, 1954). Pyruvate inhibited the synthesis of ACh in experiments by Stedman & Stedman (1937), Nachmansohn & John (1945), McLennan & Elliott (1950) and Kumagai, Ebashi & Takeda (1954). Some of the present results may have a bearing on the problem of ACh precursors. It has been noted in previous work (Tuček, 1966b, 1967b) that the activities of acetyl-CoA synthetase and ATP citrate lyase are very low compared with the activities of choline acetyltransferase both in the nerve-ending fraction and in whole homogenates of brain tissue. In spite of the fact that the pyruvate oxidase complex lost a great proportion of its activity consequent on the density-gradient centrifugation, the results shown in Fig. 6 indicate that pyruvate may be much more efficiently used than citrate or acetate as a source of acetyl-CoA, not only by the homogenate, but even by its nerve-ending fraction. This underlines the importance of the problem of finding out how intramitochondrial acetyl-CoA is transferred to meet extramitochondrial needs, the synthesis of ACh possibly being one of them.

The failure of previous authors to obtain higher rates of ACh synthesis by brain homogenates when pyruvate was used as the source of acetyl groups may be explained, at least in part, by its steep dependence on the concentration of NAD^+ in the incubation medium (Fig. 5). Although a small inhibitory effect of pyruvate on choline acetyltransferase could be observed in most experiments in which this question was tested, actual observations indicate that such inhibition was due to impurities or condensation products of pyruvate. The inhibitory effect of pyruvate solutions, which had been kept frozen at -18° for several days,

was much higher than that of solutions freshly prepared from crystalline sodium pyruvate. Therefore only fresh solutions were used throughout.

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