The Kinetic Properties of Human Placental Choline Acetyltransferase

By D. MORRIS,* A. MANECKJEE† AND CATHERINE HEBB

*Agricultural Research Council Institute of Animal Physiology,
Babraham, Cambridge, U.K.*

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1. Michaelis constants for human placental choline acetyltransferase were shown to be dependent on the concentration of the second substrate present. The primary plots indicate a sequential rather than a Ping Pong mechanism and are of the same type with 300 mM- and 500 mM-sodium chloride. 2. Similar results have been obtained with rabbit brain choline acetyltransferase. 3. Product inhibition of the forward reaction has been studied. CoA inhibits competitively with respect to acetyl-CoA and non-competitively with respect to choline. Acetylcholine inhibits competitively with respect to choline and non-competitively with respect to acetyl-CoA. No inhibition is given by acetylcholine when the enzyme is saturated with choline. 4. It is concluded that human placental choline acetyltransferase has an ordered mechanism of the Theorell-Chance type.

Choline acetyltransferase (acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6) catalyses the reversible transfer of acetyl groups between acetyl-CoA and choline, with an equilibrium constant the magnitude of which is dependent on salt concentration (Schuberth, 1966; Potter, Glover & Saelens, 1968), varying between 150 and 5000 in the direction of acetylcholine synthesis (but see Glover & Potter, 1971). An understanding of the mechanism of action is important in terms of the control of production of acetylcholine in the nervous system.

In a kinetic study of the human placental enzyme Schuberth (1966) obtained parallel double-reciprocal plots characteristic of a Ping Pong mechanism (Cleland, 1963) involving a central binary acetyl-enzyme complex formed from either acetyl-CoA or from acetylcholine, with subsequent release of the deacylated substrate. On the other hand Potter *et al.* (1968), in a study of rat brain choline acetyltransferase, obtained convergent double-reciprocal plots indicative of a sequential ternary-complex mechanism (Cleland, 1963). Although Potter *et al.* (1968) suggested that this fundamental difference in mechanism might reflect species variation this seems rather unlikely but, if true, deserves further careful confirmation.

Morris & Grewaal (1969) showed that isotopic exchange between acetylcholine and [*methyl*-¹⁴C]-

choline, catalysed by human placental choline acetyltransferase, was dependent on the presence of CoA. This finding does not support a Ping Pong mechanism for the placental enzyme and is in accord with a ternary-complex mechanism. Calf caudate nucleus choline acetyltransferase has been suggested to have a Theorell-Chance mechanism (White & Cavallito, 1970).

The present paper describes a reinvestigation of the placental enzyme kinetics by using a sensitive radiometric assay. Some of the measurements were made on rabbit brain enzyme also. A brief report of part of this work has appeared (Morris & Hebb, 1971).

MATERIALS AND METHODS

Enzymes. Human placental choline acetyltransferase, after homogenization in 50 mM-thioglycollate-0.154 M-NaCl, pH 6.4, was prepared as a freeze-dried powder from a 36-week-old placenta. The powder was extracted with cold 0.5 M-NaCl (100 mg of powder/ml) and centrifuged (12000g for 15 min). The supernatant was adjusted to pH 5.0 and (NH₄)₂SO₄ was added to a final concentration of 20% (w/v). The precipitated protein was extracted with 0.5 M-NaCl, centrifuged as before and the supernatant was dialysed overnight at 4°C against 0.5 M-NaCl. The preparation, which was stored at -18°C, had a specific activity of 19 mU of acetylcholine synthesized/mg of protein at 39°C, was nearly free of choline (see below) and contained no acetyl-CoA hydrolase activity (1 enzyme unit equals 1 μmol of product formed/min). Some choline was formed in the enzyme preparations over periods of months and from time to time they were monitored by incubation in the absence of added choline. On occasion the enzyme preparation was dialysed to decrease the choline content but in practice this was

* Present address: Department of Physiology and Biochemistry, University of Southampton, Southampton SO9 5NH, U.K.

† Present address: Department of Pharmacology, University of Ottawa, Ottawa 2, Ont., Canada.

rarely necessary, as the amount of free choline was not sufficient to affect the results. In the experiment illustrated in Fig. 4, for example, 20 pmol was found in the 10 μ l of enzyme added.

For experiments in which the inhibitory action of acetylcholine was being studied a more pure preparation was obtained by gel-filtration through a Sephadex G-75 column (84 cm $\times$ 1.9 cm) and then, after freeze-drying and re-extraction with 0.3 M-NaCl, through Sephadex G-100 (same column dimensions). This procedure considerably decreased the amount of cholinesterase activity; as measured by the technique of Ellman, Courtney, Andres & Featherstone (1961) no acetyl- or acyl-cholinesterase could be detected in the extract retained for use. Nevertheless, some small trace of a choline ester-hydrolysing enzyme must have remained, since in the acetylcholine inhibition experiments with this enzyme preparation the results indicated that some release of choline was occurring even in the presence of eserine. To prevent this the enzyme preparation was preincubated at room temperature with an equal volume of 10 μ M-di-isopropyl phosphorofluoridate. The treated enzyme was added to the incubation medium, which also contained 10 μ M-di-isopropyl phosphorofluoridate. This was sufficient to prevent the release of choline from added acetylcholine.

Rabbit brain choline acetyltransferase was extracted from acetone-dried powders of rabbit brain as detailed by Hemsworth & Morris (1964) and partially purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation as described above for the placental enzyme. The specific activity was not determined but the preparation had an activity of 32 mU/ml at 39°C, contained less than significant amounts of choline, and was free of acetyl-CoA hydrolase activity.

Citrate synthase (EC 4.1.3.7) was obtained from Boehringer Corporation (London) Ltd., London W.5, U.K.

Chemicals. CoA (87% pure), oxaloacetic acid and 5,5'-dithiobis-(2-nitrobenzoic acid) were the products of the Sigma Chemical Co., St Louis, Mo., U.S.A. Acetyl-CoA was prepared from CoA by treatment with acetic anhydride (Simon & Shemin, 1953). [$1\text{-}^{14}\text{C}$]Acetic anhydride, obtained from The Radiochemical Centre, Amersham, Bucks., U.K., was used to prepare [$1\text{-}^{14}\text{C}$]acetyl-CoA in a modified procedure in which thioglycollate was omitted and in which the residual [$1\text{-}^{14}\text{C}$]acetic acid was removed by successive extractions with ether at pH 1.0; 12 such extractions usually sufficed to decrease the radioactivity in the final ether extract to less than 1% of that in the aqueous phase containing the acetyl-CoA, but 15 extractions were done as a routine. To avoid high blank values in the radiometric choline acetyltransferase assay procedure (see below) it was necessary to purify the product. The radioactive acetyl-CoA (4–6 ml) was applied to a DEAE-cellulose column (30 cm $\times$ 2 cm) equilibrated with 3 mM-HCl and eluted with a linear NaCl gradient (0–0.15 M) in 3 mM-HCl. Two main radioactive peaks with an extinction at 254 nm were obtained. The first one, which was not fully retained by the anion-exchange resin used in the choline acetyltransferase assay procedure, was rejected and the second (major) peak was concentrated to 2 ml by rotary evaporation at 40°C. The product was finally desalted by gel filtration on a column (100 cm $\times$ 1.7 cm) of Sephadex G-10; this step also served to remove the last traces of [$1\text{-}^{14}\text{C}$]acetic acid and a small amount of CoA. The appropriate fractions

were combined, freeze-dried, dissolved in 0.1 M-HCl that was saturated with N_2 and stored at -18°C . Acetyl-CoA was assayed by measuring the release of CoA in the presence of an excess of oxaloacetate, citrate synthase and 5,5'-dithiobis-(2-nitrobenzoic acid) as described by Chase (1967). CoA solutions were similarly assayed in terms of their thiol content, citrate synthase and oxaloacetate being omitted from the assay system.

Choline chloride and eserine sulphate were obtained from British Drug Houses Ltd., Poole, Dorset, U.K.; choline chloride was dried *in vacuo* over P_2O_5 before use. Choline solutions were assayed in terms of their chloride content with a chloride electrode. Acetylcholine chloride, obtained from Le Matte et Boinot, Paris, France, and from Roche Products Ltd., Welwyn Garden City, Herts., U.K., was found to be contaminated with an unacceptably high concentration of free choline. Fractional crystallization (by Dr Dennis Sharman, to whom we owe thanks) was tried but even then the choline content was too high for experiments in which the inhibitory effect of acetylcholine was being tested in the presence of low choline concentrations. Acetylcholine iodide was synthesized by D. M. from dimethylaminoethyl acetate by quaternization with methyl iodide and was found to contain, by assay with placental choline acetyltransferase, only 0.1% of choline. This preparation was used because it was a simple matter to calculate the adjustment to be made for each concentration of choline in the different concentrations of acetylcholine used in the inhibition experiments. The adjustment was done by adding (from a 10 μ l Hamilton syringe) volumes of choline decreased by the amount of choline present in the acetylcholine added.

DEAE-cellulose was a Whatman product (H. Reeve Angel and Co. Ltd., London E.C.4, U.K.). 2,5-Diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Sephadex gel-filtration media were obtained from Pharmacia, Uppsala, Sweden. All other chemicals were of AnalaR quality or of the highest purity available.

Choline acetyltransferase assay. For determination of activity with both substrates present in a saturating concentration the assay system comprised: sodium phosphate buffer, pH 7.5, 33 mM; NaCl, 300 mM or 500 mM; eserine sulphate, 0.13 mM; [$1\text{-}^{14}\text{C}$]acetyl-CoA, 0.5 mM; choline chloride, 10 mM; choline acetyltransferase, 20 μ l of placental enzyme (diluted 1:4 with 0.15 M-NaCl or 1:1 with 10 μ M-di-isopropyl phosphorofluoridate) or 25 μ l of rabbit brain enzyme; and water to 120 μ l. In the acetylcholine inhibition experiments the incubation medium contained 10 μ M-di-isopropyl phosphorofluoridate instead of eserine. The incubation was usually begun by the addition of enzyme (but occasionally with choline) after the other components had been warmed to 39°C for 20 s. After a further 10 min at 39°C the incubation was stopped by the addition of 0.15 M-HClO₄ (80 μ l) and rapid cooling. The incubate was transferred to the drained surface of a column (10 cm $\times$ 0.65 cm) of De-Acidite FF-IP SRA 63 (100–200 mesh; Cl⁻ form; Permutit Co. Ltd., London W.4, U.K.). The incubation tube was washed out with water (3 $\times$ 0.4 ml) and the washings were transferred to the column, which was allowed to drain to the surface; the eluate was discarded. The column was finally washed with a volume of water (2.4 ml with one batch of resin, but later and as a routine with 1.6 ml)

sufficient to elute all the labelled acetylcholine; the eluate was collected in a scintillation vial containing 15 ml of scintillation fluid (see below).

Measurement of radioactivity. The scintillation fluid contained diphenyloxazole (7 g), 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.3 g) and recrystallized naphthalene (100 g) in 1 litre of AnalaR dioxan. Both the radioactive acetylcholine from choline acetyltransferase incubations and [^{14}C]acetyl-CoA were determined in 15 ml of scintillation fluid plus 2.4 ml (or 1.6 ml, see above) of water with a Packard automatic liquid-scintillation spectrometer at an efficiency of about 85%. The specific radioactivities of the preparations of [^{14}C]acetyl-CoA used in the experiments were about 6.5×10^5 and 5.7×10^6 c.p.m./ μmol respectively. Acetyl-CoA standards were always counted for radioactivity at the same time as the experimental samples.

Kinetics. The incubation medium was the same as described above except that the concentrations of acetyl-CoA and of choline were varied. Substrate solutions were assayed on the day of the experiment, as described above. Initial rates of acetylcholine synthesis were obtained in 10 min incubations and the results were expressed as double-reciprocal plots (Lineweaver & Burk, 1934).

RESULTS

Initial rates of acetylcholine synthesis by the placental enzyme at a range of choline concentrations in the presence of four fixed concentrations of acetyl-CoA, and vice versa, were obtained. The experiments were performed at two different concentrations of sodium chloride (300 mM and 500 mM). The results obtained with 300 mM-sodium chloride, expressed as double-reciprocal plots, are shown in Fig. 1. The plots were linear over the range of sub-

strate concentrations studied and were convergent with the point of intersection to the left of the ordinate and below the abscissa; the apparent K_m values of one substrate were thus dependent on the concentration of the second substrate. Similar plots were obtained with 500 mM-sodium chloride although the derived kinetic constants were different. The limiting values of the apparent K_m as the second substrate decreases to zero were derived from the points of intersection of the primary plots and are shown in Table 1. From the primary plots secondary plots of intercept on the ordinate against the reciprocal of the concentration of the second substrate were made, to derive the values of K_m (Table 1) when the second substrate was saturating. The secondary plot derived from Fig. 1(a) is shown in Fig. 2.

For comparison with the placental enzyme similar kinetic experiments were performed with rabbit brain choline acetyltransferase, when convergent plots were again obtained. In all subsequent experiments only placental enzyme was used and the final concentration of sodium chloride in the incubation medium was 300 mM.

Product inhibition of the forward reaction. Inhibition of acetylcholine synthesis by the products, CoA and acetylcholine itself, was examined in further kinetic experiments.

Inhibition by CoA in the presence of a fixed non-saturating concentration of choline was found to be competitive with respect to acetyl-CoA (Fig. 3a). Inhibition by CoA was non-competitive with respect to choline in the presence of a fixed non-saturating concentration of acetyl-CoA (Fig. 3b).

Fig. 1. Effect of varying the second substrate on reciprocal plots for choline and acetyl-CoA with placental choline acetyltransferase in the presence of 300 mM-NaCl. (a) For choline at different acetyl-CoA concentrations: 22.9 μM (○), 11.6 μM (●), 8.1 μM (△) and 4.0 μM (▲). (b) For acetyl-CoA at different choline concentrations: 68 μM (○), 40.8 μM (●), 30.6 μM (△) and 21 μM (▲).

Table 1. *Effect of sodium chloride concentration on K_m values for substrates*

Values for the apparent K_m (K) in the presence of one substrate were obtained from the points of interception of the primary reciprocal plots. Values of K_m (K') when both substrates were saturating were obtained from secondary plots of intercepts on the ordinate against the reciprocal of the second substrate concentration.

Substrate	Concn. of NaCl ...	300 mM		500 mM	
		K (μM)	K' (μM)	K (μM)	K' (μM)
Choline		42	526	93	197
Acetyl-CoA		5.7	72	9.5	23

Fig. 2. Secondary intercept plot derived from Fig. 1(a). The intercepts on the ordinate of Fig. 1(a) are plotted against the reciprocal of the concentration of acetyl-CoA used.

Inhibition of the forward reaction by acetylcholine was more difficult to study, but by using di-isopropyl phosphorofluoridate to inhibit irreversibly the cholinesterase activity apparently still present in the purified enzyme (see the Materials and Methods section) and by making allowance for the small amount (0.1%) of choline present in the acetylcholine iodide used, clear-cut results were obtained. Inhibition by acetylcholine was competitive with respect to choline at both a non-saturating (Fig. 4) and a saturating concentration of acetyl-CoA. Acetylcholine inhibited non-competitively with respect to acetyl-CoA at a fixed non-saturating concentration of choline (Fig. 5). There was no inhibition by acetylcholine at several acetyl-CoA concentrations when choline was present at a saturating concentration (Table 2).

DISCUSSION

The convergent primary double-reciprocal plots obtained with both the placental and rabbit brain enzymes are characteristic of a sequential mechanism in which both substrates must combine with

the enzyme before the release of any products (Cleland, 1963). This is in marked contrast with the parallel plots obtained by Schuberth (1966) with the placental enzyme and is in accord with the results of Potter *et al.* (1968) obtained with rat brain enzyme and with those of White & Cavallito (1970). Glover & Potter (1971) have reported convergent plots intersecting on the abscissa with partially purified choline acetyltransferase from ox brain striate nuclei. It is difficult to reconcile the fundamental difference between the present results and those of Schuberth (1966). The use of different assay systems, however, may be responsible. In the present work acetylcholine formation has been measured directly whereas Schuberth (1966) measured the rate of thiol release from acetyl-CoA.

Choline acetyltransferase is usually slightly more active in 500 mM-sodium chloride than in 300 mM-sodium chloride, but the kinetics were examined at both concentrations as the experiments of Schuberth (1966) were done in 300 mM-sodium chloride. Table 1 shows that the higher concentration of sodium chloride had the effect of increasing the apparent K_m of both substrates in the absence of the second substrate. This could possibly be caused by competition between Na^+ and the substrates for some anionic site on the enzyme. The effect, however, seems to be reversed with respect to changes in the K_m values at saturating concentrations of the second substrates. The interaction of sodium chloride with the enzyme is evidently complex but the basic sequential nature of the mechanism was nevertheless unaltered by these changes in sodium chloride concentration.

Kinetic plots of the type discussed above can only distinguish between Ping Pong and sequential mechanisms. The latter include (Cleland, 1963) Ordered Bi Bi, Theorell-Chance, Random Bi Bi and Rapid Equilibrium Random Bi Bi. Product-inhibition studies, however, can serve to distinguish between these sequential mechanisms. Table 3 (adapted from Cleland, 1963) shows the product inhibition patterns to be expected for the various mechanisms. The present findings are underlined,

Fig. 3. Inhibition of the forward reaction by CoA. (a) Reciprocal plots for acetyl-CoA with constant choline (86.7 μM) at different CoA concentrations: none (○), 41.8 μM (●), 167 μM (Δ) and 418 μM (▲). (b) Reciprocal plots for choline with constant acetyl-CoA (6.4 μM) at different CoA concentrations: none (○), 5 μM (●), 10 μM (Δ) and 25 μM (▲).

Fig. 4. Inhibition of the forward reaction by acetylcholine. Reciprocal plots for choline with constant (unsaturating) acetyl-CoA (75 μM) at different acetylcholine concentrations: none (○), 10 mM (●), 20 mM (Δ) and 30 mM (▲).

Fig. 5. Inhibition of the forward reaction by acetylcholine. Reciprocal plots for acetyl-CoA with constant choline (100 μM) at different acetylcholine concentrations: none (○), 15 mM (●) and 30 mM (▲).

Table 2. Effect of a saturating choline concentration on product inhibition by acetylcholine

Synthesis of [1- ^{14}C]acetylcholine was measured (for details see the Materials and Methods section) at three different acetylcholine concentrations over a range of concentrations of acetyl-CoA. The concentration of choline was fixed and saturating at 12.5 mM.

		[1- ¹⁴ C]Acetylcholine synthesized (nmol/10 min)				
Acetyl-CoA (μM)	Acetylcholine added (mM)	...	0	10	20	30
6.7			0.43	0.44	0.46	0.43
10			0.71	0.59	0.65	0.64
20			1.16	1.12	1.11	1.19
40			2.11	1.99	2.03	2.12

Table 3. *Product-inhibition patterns for Bi Bi mechanisms*

Abbreviations: C, competitive; UC, uncompetitive; NC, non-competitive; O, no inhibition; unsatd., unsaturated; satd., saturated. The results obtained with placental choline acetyltransferase are underlined to show their compliance with only one mechanism. See Cleland (1963) for details of mechanisms.

Mechanism	Inhibitor	Variable substrate ... Fixed substrate ...		Acetyl-CoA Choline		Choline Acetyl-CoA	
				Unsatd.	Satd.	Unsatd.	Satd.
Ordered Bi Bi	Acetylcholine			NC	UC	NC	NC
	CoA			C	C	NC	O
Theorell-Chance	Acetylcholine			<u>NC</u>	<u>O</u>	C	C
	CoA			<u>C</u>	<u>C</u>	<u>NC</u>	<u>O</u>
Rapid Equilibrium Random Bi Bi	Acetylcholine or CoA			C	O	C	O
Random Bi Bi	Acetylcholine or CoA			NC	NC	NC	NC

showing their compliance with only one of these mechanisms, namely that of Theorell-Chance. The latter mechanism has been described by Cleland (1963) as a limiting case of the Ordered Bi Bi mechanism in which the steady-state concentration of the central ternary complexes is not kinetically significant. Both random mechanisms are eliminated by the product-inhibition patterns obtained with respect to one substrate at unsaturating concentrations of the second substrate. Discrimination between the Ordered Bi Bi and Theorell-Chance mechanisms depends entirely on the nature of the inhibition patterns given by acetylcholine if, as seems likely (see below), acetyl-CoA is the leading substrate. The Theorell-Chance mechanism requires that inhibition by acetylcholine should be competitive with respect to choline at all concentrations of acetyl-CoA; this requirement has been met. The other distinctive feature of the Theorell-Chance mechanism is that there should be no inhibition by acetylcholine with respect to acetyl-CoA at saturating concentrations of choline. This experiment was performed several times with the same result (Table 2), namely, no inhibition of acetylcholine synthesis.

In a limited study with a crude preparation of rat brain choline acetyltransferase, Kaita & Goldberg (1969) found that acetylcholine inhibited competitively with respect to choline and non-competitively with respect to acetyl-CoA, in agreement with the present work. White & Cavallito (1970), who used partially purified calf caudate nucleus enzyme with only one concentration of product inhibitor, also postulate a Theorell-Chance mechanism. While the present paper was being prepared for publication a brief report appeared (Henderson & Sastry, 1971) in agreement with our results for the placental enzyme. By contrast with these findings Glover & Potter (1971) found that acetylcholine only slightly inhibited the

forward reaction (45% when present in a 200mm concentration) and that the inhibition was non-competitive with respect to choline and uncompetitive with respect to acetyl-CoA. From these results they concluded that the Ordered Bi Bi mechanism was operating. Unless a true species difference in mechanisms exists it is difficult to reconcile these results with the present work and with the other studies mentioned above. Glover & Potter (1971) do, however, say that their results do not exclude the possibility of a Theorell-Chance mechanism in which choline is the leading substrate. The reaction order cannot be decided by product-inhibition patterns and requires either binding studies or isotopic-exchange measurements at equilibrium (Silverstein & Boyer, 1964). White & Cavallito (1970) have obtained some evidence from binding studies with radioactive acetyl-CoA that this is the leading substrate. If this is so then acetyl-CoA and CoA are the outer substrate-product pair, with choline and acetylcholine forming the inner pair.

The problem of the regulation of acetylcholine synthesis in the nervous system is of great interest and product inhibition studies *in vitro* may be relevant to an understanding of this process. The present work shows that acetylcholine inhibits its own synthesis competitively with respect to choline. It can be calculated from the results in Fig. 4 that at a non-saturating concentration of acetyl-CoA and at a concentration of choline (67 μ M) rather more than that likely to be present in nerve endings (Bligh, 1952; Glover & Potter, 1971) 30mm-acetylcholine inhibits its own synthesis by nearly 60%. Since the concentration of acetylcholine in synaptic vesicles has been estimated to be 100–200mm (Whittaker & Sheridan, 1965) it is possible that the concentration of unbound acetylcholine in equilibrium with the choline acetyltransferase in the nerve endings could approach

30mM and thus regulate its own synthesis by feedback inhibition. Other possibilities for control, of course, exist and may include ionic changes related to nerve depolarization, substrate availability or, as has been suggested by Glover & Potter (1971), mass action.

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