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HUMAN PLACENTAL CHOLINE ACETYLTRANSFERASE
NATURE AND MOLECULAR ASPECTS OF THE INHIBITION BY IODO- AND BROMOACETYLCHOLINES

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Abstract—Human placental choline acetyltransferase (ChA) was assayed by the formation of acetyl[^{14}C]choline (ACh) from choline and acetyl[^{14}C]coenzyme A (ACoA). Among the four halogeno-acetylcholines, iodo- and bromoacetylcholines were potent inhibitors (I_{50}: 2×10^{-6} M) of ChA. They were studied in the following experiments: (1) variation of initial velocity as a function of choline concentration (5×10^{-5} to 2×10^{-4} M, ACoA 10^{-5} M) at four levels of iodo- and bromoacetylcholines (5×10^{-7} to 2.5×10^{-6} M); (2) variation of initial velocity as a function of ACoA concentration (6×10^{-6} to 2.5×10^{-5} M, choline 10^{-4} M) at four levels of iodo- and bromoacetylcholines; (3) dialysis of the inhibited ChA; and (4) capacities of iodo- and bromoacetates to inhibit ChA. In (1) and (2), primary plots were noncompetitive and secondary plots of slopes and intercepts were linear. In (3), only 70–80 per cent of the inhibition was reversible. In (4), high levels of halogeno-acetates (10^{-2} M) inhibited ChA by 20–30 per cent. These investigations support the formation of ternary intermediates (CoA ChA IACh or CoA ChA BrACh) during the reaction of placental ChA. The quaternary ammonium head and the positive carboxyl-carbon do seem to contribute to the ChA inhibition by IACh and BrACh. The irreversible component of the inhibition (20–30 per cent) with XACh or iodo- and bromo-acetates can be explained by possible (1) formation of *R*-carboxymethylated ChA (where *R* = —O—, —S— or =N—) which is less active than the original ChA, (2) formation of *S*-carboxymethylated CoA which is slowly dialyzed free from the enzyme surface, or (3) conversion of the part of the enzyme to the —*N*—carboxymethylated form which is inactive.

Halogeno-acetylcholines (XACh, where X = I, Br, Cl or F) were synthesized by Chiou and Sastry [1] for studying similarities and differences of cholinergic sites. The replacement of one of the hydrogens by fluorine, chlorine, bromine and iodine would change the electronic characteristics of carbonyl keto function in a well defined manner. It has been shown that in halogenated acetylcholines, and other choline esters, the electron displacement due to the influence of an α-substituent is the decisive factor for their high affinities at serum cholinesterase and nicotinic receptors [2, 3].

Due to the increase in the atomic size from fluorine to iodine, halogeno-acetylcholines are convenient substances for studying the steric effects of the acyl group at cholinergic sites. Steric hindrance due to halogenation, with or without electronic environment, has been demonstrated in several organic reactions [4, 5]. According to studies of Chiou and Sastry [1, 3, 6–8], steric effects due to halogenation of the acyl group play a significant role in their hydrolysis by acetylcholinesterase and their interaction at muscarinic receptors.

The lability of the C—X bond (where X = halogen) decreases from C—I to C—F [9]. Iodoacetylcholine, BrACh, iodoacetate and bromoacetate alkylate reactive groups (—OH, —NH, and —SH) of proteins or membranes under favorable conditions [9]. Further, there is some evidence that BrACh and IACh react with an —SH group at the nicotinic receptor, if such groups are unmasked by reduction of —S—S groups [3, 10, 11].

Although a number of detailed studies have been described about the interactions of halogeno-acetylcholines at cholinesterases [1, 7] and cholinergic receptors [3, 8], very few detailed investigations about the interactions of halogeno-acetylcholines at choline acetyltransferases (ChA) were reported. Morris and Grewaal [12, 13] have demonstrated that IACh, BrACh and ClACh were potent inhibitors of placental ChA. A coupled kinase system was used with ChA extracted from human placenta. Dialysis of ClACh-inhibited enzyme reversed the inhibition. The only kinetic analysis was done with ClACh, with choline as the variable substrate. These studies indicated uncompetitive inhibition of ChA by ClACh. According to our preliminary investigations [14, 15], all mono-halogeno-acetylcholines except FACh were potent inhibitors of human placental as well as rat brain ChA. Roskoski [16] measured the formation of ACoA and choline from CoA and ACh, and reported that BrACh was a competitive inhibitor of ACh using bovine brain ChA. However, the nature of inhibition caused by all halogeno-acetylcholines using acetyl-CoA (ACoA) and choline as substrates was not reported previously.

Since halogeno-acetylcholines are very similar in structure to ACh, one of the products of the ChA reaction, their ability to inhibit ChA and the nature of their inhibitions are of particular importance. Our

studies with halogenated acetylcholines as inhibitors of human placental ChA are described in the present investigation. Iodo- and bromoacetylcholines were noncompetitive with both variable substrates, ACoA or choline.

MATERIALS AND METHODS

Materials. Halogen-substituted acetylcholines (XACh) were synthesized by the method of Chiou and Sastry [1]. The XACh used were FACh, ClACh, BrACh and IACh, which were obtained by replacing one of the hydrogen atoms in the acetyl group of ACh by F, Cl, Br and I respectively. All were perchlorates except IACh which was an iodide salt.

[^{14}C]ACoA (about 54.4 μCi/m-mole, chemical purity 97.5 per cent) was obtained from New England Nuclear Corp., Boston MA, in 2.5 ml of aqueous solution at pH 4.0 which was diluted to 7.5 ml with phosphate buffer to obtain the stock solution. Unlabeled ACoA (chemical purity, 91–96 per cent) was obtained from Mann Research Laboratories, Inc., Orangeburg, NY and Pierce Biochemicals; Inc., Rockford, IL, as lithium salts. Choline iodide was purchased from Eastman Organic Chemicals, Rochester, NY.

Bio-Rad AG1-X8 anion exchange resin (3.2 m-equiv./g), 200–400 mesh, chloride form, was used for the ChA assay.

Phosphate buffer was prepared from KH_2PO_4 and KOH according to the instructions of Dawson *et al.* [17]. Buffer–incubation (B–I) mixture refers to a solution containing a mixture of the salts (see under ChA assay), as well as cold unlabeled substrates; 0.1 ml of the solution yields desired salt and substrate concentrations when diluted to the volume of the final reaction mixture.

Enzyme preparation. Full-term placentae (each about 1.0 kg) were cleared of blood and connective tissue and homogenized in cold acetone (−12°). The resulting homogenate was filtered, and the acetone-insoluble material was washed twice with cold acetone, and dried under vacuum. Approximately 40 g of acetone-insoluble powder was obtained from each placenta.

The method for partial purification of ChA is similar to that used by Morris [18] and Sastry and Henderson [19] and was selected for good yield and stability of the enzyme during the experimental procedure. The acetone-insoluble powder was extracted with cold 0.02 M sodium bicarbonate buffer (pH 7.4, 20 ml/g of powder) which contained 5.0 mM EDTA, 0.3 M NaCl and 20 mM NaCN. The pH of the dark red extract was lowered to pH 5 with concentrated HCl and the resulting precipitate was discarded after centrifugation at 5000 *g* for 15 min. Most of the ChA activity remained in the supernatant to which solid $(NH_4)_2SO_4$ was added to yield a 10% (w/v) solution. The precipitate was then discarded and more solid $(NH_4)_2SO_4$ was added to the filtrate to give a 25% (w/v) solution. The resulting mixture was centrifuged at 5000 *g* for 15 min and the supernatant was discarded. The sediment was drained of the fluid and was subsequently dissolved in cold distilled water. The above solution was immediately frozen and freeze dried. Approximately 1 g of the freeze-dried protein powder was obtained from 32 g of acetone-insoluble powder. The final freeze-dried preparation was a light brown powder and was stable at −12° for an indefinite period of time. This preparation was further purified by gel filtration through Sephadex G-100 or Sephadex G-200. The protein fraction corresponding to molecular weight of about 61,000 was found to be ChA. The reported molecular weight for placental ChA varied between 59,000 and 67,000 [20, 21].

Final placental ChA preparation was free from arylamine *N*-acetyltransferase [22]. It contained traces of acetylcholinesterase and cholinesterase when analyzed by differential radiometric assay described by Henderson and Sastry [23]. Therefore, physostigmine was routinely included for inhibiting cholinesterases in the assay method. The placental ChA preparation contained trace amounts of carnitine acetyltransferase but it did not contain carnitine to interfere in the assay procedure for choline ChA (see assay for ChA). ChA solutions for study were prepared by dissolving given quantities of the freeze-dried powder in 0.05 M phosphate buffer (pH 7.4) containing 20 mM NaCN and 5 mM EDTA.

Assay for choline acetyltransferase. The assay for ChA activity was developed as follows. The total volume of the mixture was 0.3 ml which was composed of three 0.1-ml aliquots added sequentially to a 75 × 10 mm test tube. The first 0.1 ml of the aliquot consisted of 0.1 ml of (B—I) mixture which brought the final concentrations to 1.3 M NaCl, 2×10^{-4} M physostigmine sulfate, 0.02 M $MgSO_4$ and given concentrations of choline and/or labeled ([^{14}C]ACoA) and unlabeled ACoA. The second aliquot of 0.1 ml buffer was reserved for dissolving various compounds, such as activators, inhibitors and additional substrates. The third aliquot of 0.1 ml consisted of the enzyme preparation.

The basic principle of this assay is the conversion of [^{14}C]ACoA to [^{14}C]ACh by ChA in the presence of choline. A fixed variable substrate was dissolved in the first aliquot of B—I solution while the variable compound (substrate or inhibitor) was dissolved in the second aliquot of 0.1 ml buffer. The reaction was started by the addition of the enzyme to 0.2 ml of preincubated (37°) B–I solution. After different intervals (min), the reaction was stopped by placing the tube in an ice bath. The ^{14}C-substrate was separated from the [^{14}C]-product by applying 0.1 ml of the cold incubation medium to a column of anion exchange resin (Bio-Rad, AG1-X8; 1.3 g, dry weight). Immediately after 0.1 ml of the reaction mixture disappeared from the surface of the column, 2.0 ml of distilled water was added in four successive 0.5-ml portions to wash out the [^{14}C]ACh. The eluate was collected in a scintillation vial to which 15 ml of "fluor" (5 g of 2,5-diphenyloazol (PPO) and 100 g naphthalene/liter of *p*-dioxane) was added. The vial was counted and the ^{14}C-yield was correlated to ACh production. At all substrate concentrations, ACh production was linear as a function of time during the first 15 min. Initial linear velocities were calculated from the reaction time of the first 10 min.

Sensitivity of this assay was in the pmole quantities of [^{14}C]ACh synthesized. The system used for

these studies contained about 150,000 dis./min of [^{14}C]ACoA, representing 1.5 to 150 nmoles of total ACoA/0.3 ml of incubation medium. This gives specific activities ranging from 10^3 dis./min/nmole to 10^5 dis./min/nmole of [^{14}C]ACh produced.

The anion exchange resin absorbed over 99.9 per cent of [^{14}C]ACoA added to it and allowed over 99.9 per cent of [^{14}C]ACh to pass through the column. In controls, 0.1 ml of ChA was replaced with 0.1 ml buffer.

The conditions listed above were adequate to stop the reaction at a predetermined time. This is supported by the following observations. First, the initial linear part of the ACh yield–time curve passes through the origin. Second, ChA was inactive at 0–4°. The reaction was stopped by immersion of the tubes in an ice bath. Third the anion exchange resin is strongly acidic and the pH of the eluates was about 4.0 at which ChA was inactive. The cold 0.1-ml sample of the incubation medium was removed from the tube and added to the column in no more than 30 sec. Therefore, the exchange procedure should add no significant error to the true values of [^{14}C]ACh synthesis.

Rat brain ChA, prepared according to the methods described under enzyme preparation [14], did not utilize carnitine in the above assay procedure indicating the absence of carnitine acetyltransferase. Further, purified carnitine acetyltransferase from pigeon liver did not utilize choline when tested according to the above procedure. These observations indicate that choline is a specific substrate for ChA and the small amounts of carnitine acetyltransferase present in the placental enzyme preparation did not interfere with the ChA procedure.

Inhibition patterns. Inhibition patterns were evaluated according to the methods described by Cleland [24–26]. Primary plots were constructed for the reciprocal of the variable substrate concentration; at one given concentration of the second substrate and different inhibitor concentrations. Secondary plots were constructed for y intercepts and slopes as functions of inhibitor concentration. The following equations, derived by Cleland, were used for evaluation of three basic types of inhibition.

Linear noncompetitive

$$\frac{1}{v} = \frac{K}{V}\left(1 + \frac{I}{K_{is}}\right)\left(\frac{1}{A}\right) + \frac{1}{V}\left(1 + \frac{I}{K_{ii}}\right) \quad (1)$$

Linear competitive

$$\frac{1}{v} = \frac{K}{V}\left(1 + \frac{I}{K_{is}}\right)\left(\frac{1}{A}\right) + \frac{1}{V} \quad (2)$$

Linear uncompetitive

$$\frac{1}{v} = \frac{K}{V}\left(\frac{1}{A}\right) + \frac{1}{V}\left(1 + \frac{I}{K_{ii}}\right) \quad (3)$$

where v = velocity; V, maximal velocity; K, Michaelis constant; A, variable substrate concentration; I, inhibitor concentration, K_{is}, inhibition constant for slopes; and K_{ii}, inhibition constant for intercepts. In simple linear noncompetitive inhibition, $K_{ii} = K_{is}$ [27]. Equations for secondary plots of slopes and intercepts vs inhibitor concentration were used as discussed by Plowman [27].

Radiometric methods. [^{14}C]-samples were counted in 20-ml scintillation vials on a Packard model 3375 Tricarb scintillation spectrometer. The scintillator fluor contained PPO (5 g) and naphthalene (100 g) in 1 liter of 1,4-dioxane. The above spectrometer was equipped with a standardization scale which gave "automatic external standard ratio (AES)" values. An efficiency curve was constructed using [^{14}C]toluene (10^{-2} μCi) as the internal standard and the reaction medium as the quenching agent. In our experiments, the AES values range between 0.45 and 0.65 which correspond to efficiencies between 65 and 81 per cent. The disintegrations/min for each sample was computed from counts/min and the AES value. The counting error was 2 per cent or less.

Statistical methods. Values are expressed as means and standard errors when possible. Experimental points were subjected to linear multiple regression analysis developed by Kao and Chung-Phillips [28, 29]. The data presented in the figures are based on the unit weights. Results based on different weights (comparison at the same unit and minimizing the percentage error) supported the conclusions reached using unit weights. In situations where regression lines intersected, this result was verified using an auxiliary model statistical t-test [30, 31].

RESULTS

Inhibition of placental ChA by halogeno-acetylcholines. Plots for per cent inhibition as a function of the inhibitor concentration are shown in Fig. 1. The per cent inhibition increased rapidly with increasing concentrations of IACh, BrACh and ClACh in the initial part of the curves. Subsequently, the inhibition remained relatively constant with further increases in the concentrations of these three inhibitors. However, with FACh there was a depression of the per cent inhibition–concentration curve at concentrations higher than 5×10^{-5} M. This was probably due to partial activation of ChA at high inhibitor concentrations. No more than 32 per cent inhibition could be reached.

Chloroacetylcholine was a less potent inhibitor of placental ChA than either IACh or BrACh (Table 1).

Evaluation of the inhibition of placental ChA by IACh. Double reciprocal plots of initial velocity as a function of ACoA concentration, at five IACh levels, gave a family of lines which intersected at a point to the left of the origin (Fig. 2A). All these lines exhibited different slopes and intercepts indicating noncompetitive inhibition by ChA. Secondary plots for intercepts (Fig. 2B) and slopes (Fig. 2C) as functions of the inhibitor concentration were straight lines from which apparent K_{is} and K_{ii} (Table 1) were calculated.

Double reciprocal plots for initial velocity as a function of choline concentration are shown in Fig. 3A. IACh and ChA concentrations were the same as those used in Fig. 2A. ACoA concentration was held at 10^{-5} M. There was variation in the slopes and intercepts of these plots which indicated noncompetitive inhibition by IACh. Secondary plots

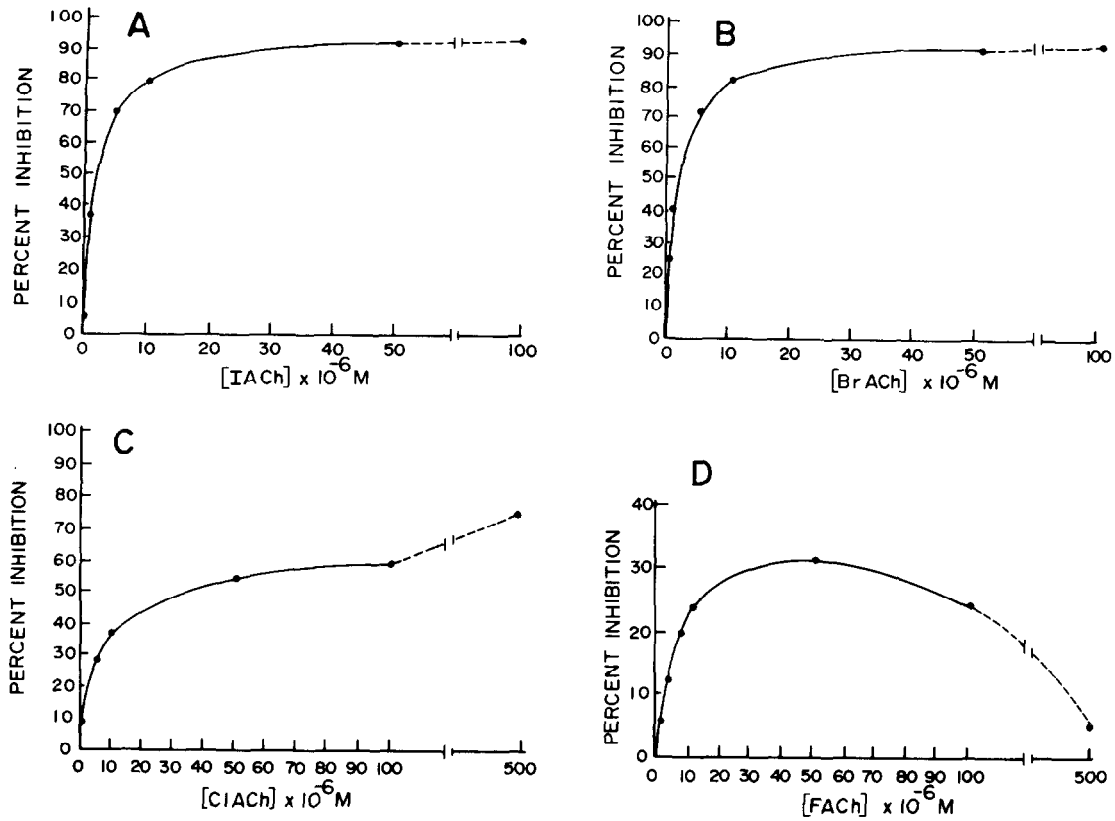

Fig. 1. Per cent inhibition of choline acetyltransferase (ChA) plotted as a function of the concentration of halogeno-acetylcholine (XACh, where X = halogen). Choline concentration is 10^{-4} M, acetylcoenzyme A (ACoA) is 10^{-5} M, and ChA is 0.1 mg/ml of the incubation medium. Panels A, B, C and D represent inhibitions by IACh, BrACh, ClACh and FACh respectively. Each point is the mean of three to five values.

for intercepts (Fig. 3B) and slopes (Fig. 3C) were straight lines. Values for apparent K_{is} and K_{ii} are shown in Table 1.

Evaluation of the inhibition of placental ChA by BrACh. Double reciprocal plots for inhibition by BrACh with ACoA as the variable substrate are shown in Fig. 4A. The resulting set of five lines varied both in slopes and intercepts, thereby indicating a noncompetitive inhibition by BrACh. Secondary plots for intercepts (Fig. 4B) and slopes (Fig. 4C), from which apparant K_{ii} and K_{is} were calculated, were linear (Table 1).

With choline as the variable substrate, the double reciprocal plots (Fig. 5A) were a family of lines with different intercepts and slopes, which indicated noncompetitive inhibition by BrACh. Secondary

Table 1. Inhibition of placental choline acetyltransferase by halogeno-acetylcholines*

Parameter (unit)	Iodoacetylcholine	Bromoacetylcholine
Inhibition by 10^{-6} M (%)‡	41.9 ± 0.3†	33.9 ± 0.3†
I_{50} (M)§	1.9×10^{-6}	2.2×10^{-6}
K_{ii} with choline as variable substrate (M)	5.4×10^{-7}	3.8×10^{-7}
K_{is} with choline as variable substrate (M)	2.1×10^{-6}	3.1×10^{-6}
K_{ii} with acetylcoenzyme A as variable substrate (M)	3.8×10^{-7}	1.4×10^{-7}
K_{is} with acetylcoenzyme A as variable substrate (M)	1.7×10^{-7}	2.1×10^{-7}

* Each value is the mean of three to five observations.

† Mean ± S.E.

‡ Per cent inhibited by chloracetylcholine and fluoracetylcholine at 10^{-6} M was 10.8 ± 0.8 and 1.0 ± 0.9 respectively.

§ The I_{50} value for chloracetylcholine was 3.3×10^{-5} M. Fluoroacetylcholine was a weak inhibitor.

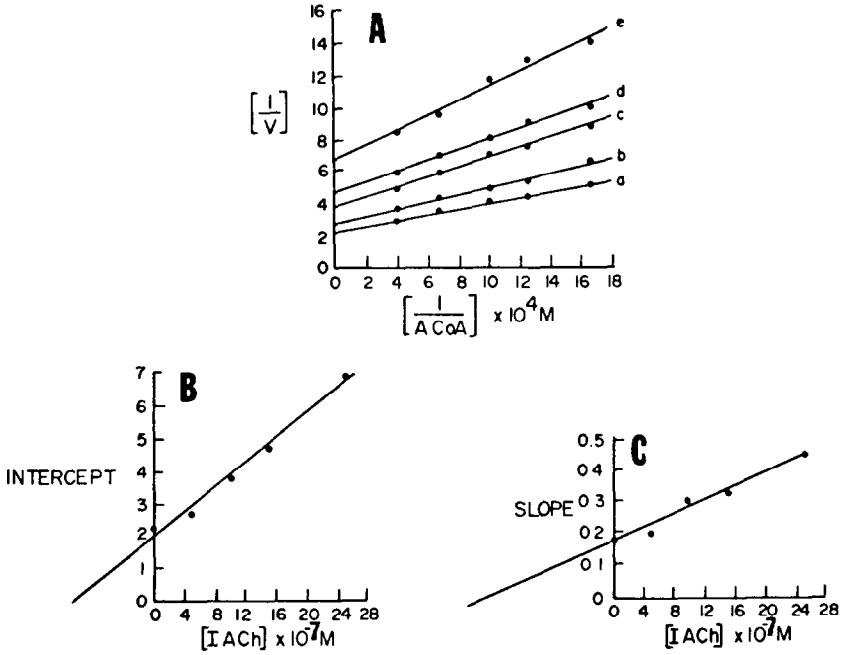

Fig. 2. Inhibition of placental choline acetyltransferase (ChA) by iodoacetyl-choline (IACh) with acetylcoenzyme A (ACoA) as the variable substrate. Panel A: initial velocity is plotted as a function of the reciprocal of ACoA concentration with choline at 10^{-4} M, ChA at 1.0 mg/ml of the incubation medium, and five concentrations of IACh. (a) 0, (b) 5×10^{-7} M, (c) 10^{-6} M, (d) 1.5×10^{-6} M and (e) 2.5×10^{-6} M. Each point is the mean of three to five values. Panel B: intercepts in panel A are plotted as a function of IACh concentration. Panel C: Slopes in panel A are plotted as a function of IACh concentration.

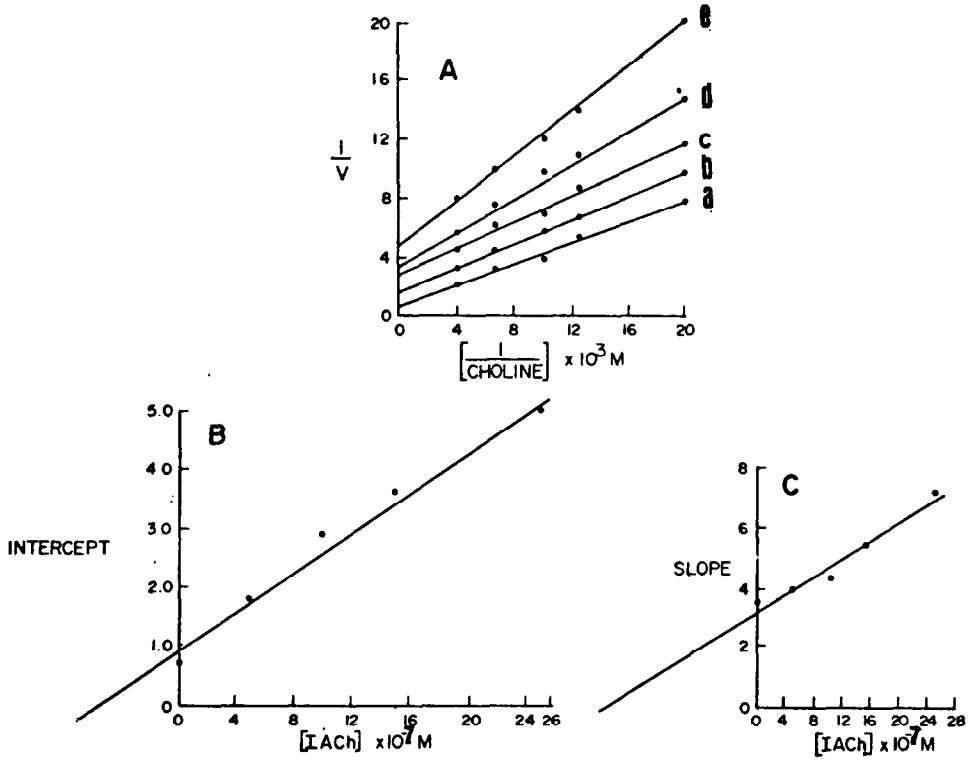

Fig. 3. Inhibition of the placental choline acetyltransferase (ChA) by iodo-acetylcholine (IACh) with choline as the variable substrate. Panel A: initial velocity is plotted as a function of the reciprocal of choline concentration with acetylcoenzyme A (ACoA) at 10^{-5} M, ChA at 1.0 mg/ml of the incubation medium, and five concentrations of IACh: (a) 0, (b) 5×10^{-7} M, (c) 10^{-6} M, (d) 1.5×10^{-6} M and (e) 2.5×10^{-6} M. Each point is the mean of three to five values. Panel B: vertical intercepts in panel A are plotted as a function of IACh concentration. Panel C: Slopes in panel A are plotted as a function of IACh concentration.

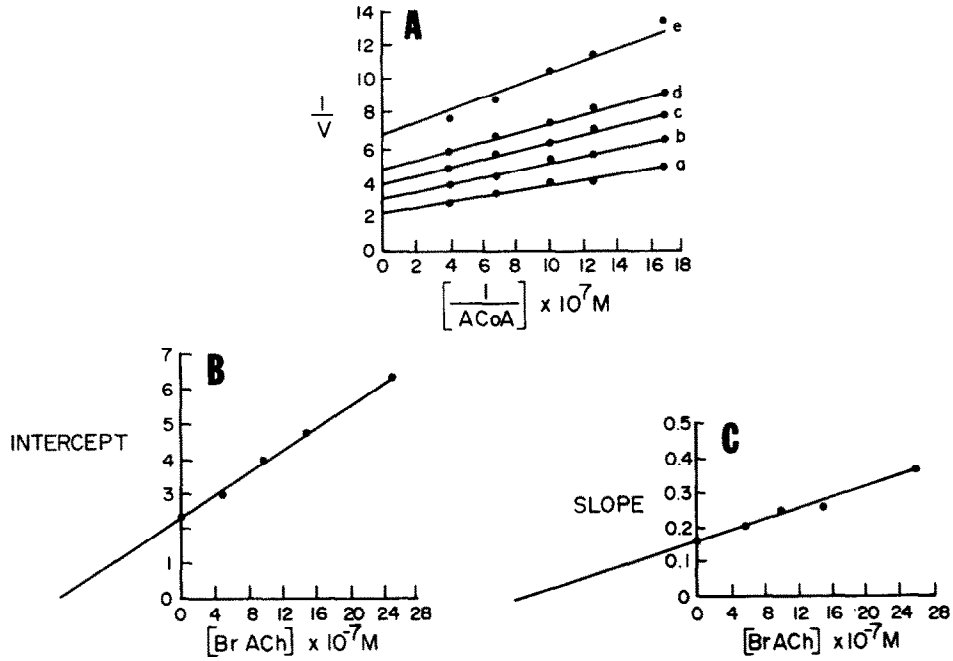

Fig. 4. Inhibition of placental choline acetyltransferase (ChA) by bromo-acetylcholine (BrACh) with acetylcoenzyme A (ACoA) as the variable substrate. Panel A: initial velocity is plotted as a function of the reciprocal of ACoA concentration with choline at 10^{-6} M, ChA at 1.0 mg/ml of the incubation medium, and five concentrations of BrACh: (a) 0, (b) 5×10^{-7} M, (c) 10^{-6} M, (d) 1.5×10^{-6} M and (e) 2.5×10^{-6} M. Each point is the mean of three to five values. Panel B: vertical intercepts in panel A are plotted as a function of BrACh concentrations. Panel C: slopes in panel A are plotted as a function of BrACh concentration.

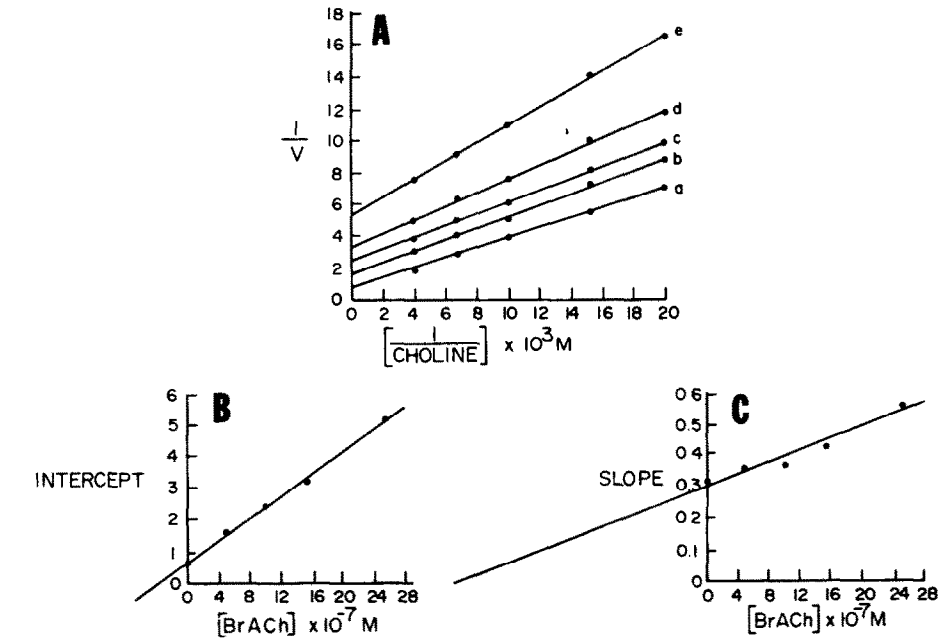

Fig. 5. Inhibition of placental choline acetyltransferase (ChA) by bromoacetylcholine (BrACh) with choline as the variable substrate. Panel A: initial velocity is plotted as a function of the reciprocal of choline concentration with acetylcoenzyme A (ACoA) at 10^{-5} M, ChA at 1.0 mg/ml of the incubation medium, and five concentrations of BrACh: (a) 0, (b) 5×10^{-7} M, (c) 10^{-6} M, (d) 1.5×10^{-6} M and (e) 2.5×10^{-6} M. Each point is the mean of three to five values. Panel B: vertical intercepts in panel A are plotted as a function of BrACh concentration. Panel C: slopes in panel A are plotted as a function of BrACh concentration.

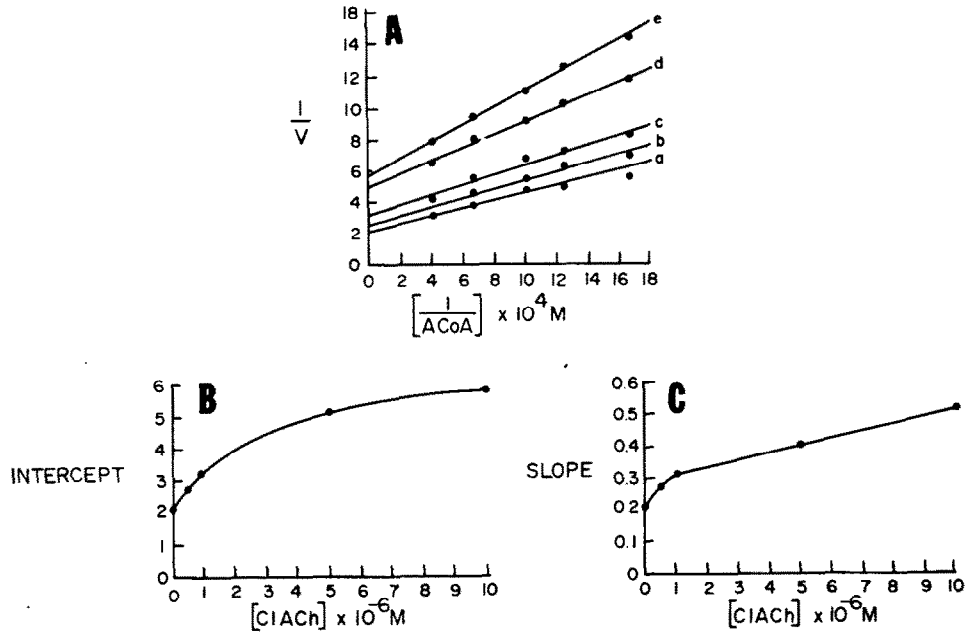

Fig. 6. Inhibition of placental choline acetyltransferase (ChA) by choloroacetylcholine (ClACh) with acetylcoenzyme A (ACoA) as the variable substrate. Panel A: initial velocity is plotted as a function of the reciprocal of ACoA concentration with choline at 10^{-4} M, ChA at 1.0 mg/ml of the incubation medium, and five concentrations of ClACh: (a) 0, (b) 5×10^{-6} M, (c) 10^{-5} M, (d) 5×10^{-5} M and (e) 10^{-4} M. Each point is the mean of three to five values. Panel B: Vertical intercepts in panel A are plotted as a function of ClACh concentration. Panel C: slopes in panel A are plotted as a function of ClACh concentration.

plots for intercepts (Fig. 5B) and for slopes (Fig. 5C), from which K_{ii} and K_{is} were calculated, were linear (Table 1).

Evaluation of the inhibition of placental ChA by ClACh. A double reciprocal plot of ClACh inhibition with ACoA as the variable substrate gave a noncompetitive inhibition pattern as with IACh and BrACh (Fig. 6A). Secondary plots of intercept (Fig. 6B) and slope (Fig. 6C) were hyperbolic rather than linear.

When the variable substrate was choline, the double reciprocal primary plots were a family of apparently parallel lines (Fig. 7A). Secondary plots (Fig. 7B) for intercepts were apparently convex or

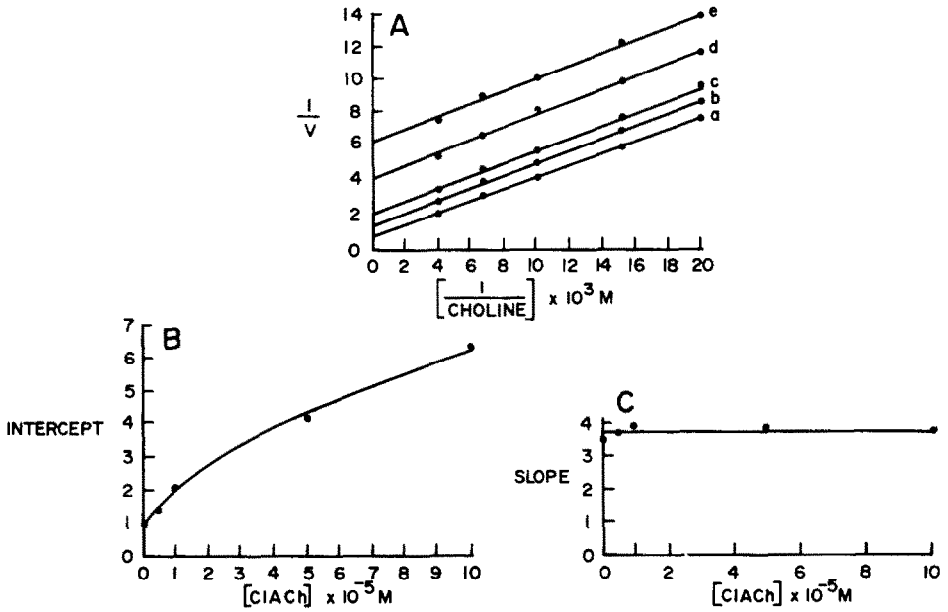

Fig. 7. Inhibition of placental choline acetyltransferase (ChA) by chloroacetylcholine (ClACh) with choline as the variable substrate. Panel A: initial velocity is plotted as a function of the reciprocal of choline concentration with ACoA at 10^{-5} M, ChA at 1.0 mg/ml of the incubation medium and five concentrations of ClACh: (a) 0, (b) 5×10^{-6} M, (c) 10^{-5} M, (d) 5×10^{-5} M and (e) 10^{-4} M. Each point is the mean of three to five values. Panel B: vertical intercepts in panel A are plotted as a function of ClACh concentration. Panel C: slopes in panel A are plotted as a function of ClACh concentration.

hyperbolic. The secondary plot for slopes is parallel to the abscissa except at very low inhibitor concentrations (Fig. 7C). These observations are similar to those reported by Morris and Grewaal [12, 13].

Reversibility of the inhibition of placental ChA by iodo- and bromoacetylcholines. The two effective ChA inhibitors (IACh and BrACh, 10^{-4} M) were studied with regard to the reversibility of their inhibition of placental ChA using equilibrium dialysis. About 70–80 per cent of inhibition was reversible during the first 2 hr of dialysis.

Inhibition of placental ChA by sodium iodoacetate and bromoacetate. Sodium iodoacetate and bromoacetate were weak inhibitors of ChA. A concentration of 10^{-3} M of iodo- or bromoacetates caused only about 20–30 per cent inhibition.

DISCUSSION

Consideration should be given to different factors in the evaluation of the inhibition of ChA by halogeno-acetylcholines such as: (1) the quaternary nitrogen atom, (2) the electronic characteristics of carboxyl carbon, (3) the size of the acyl tail, (4) the lability of the halogen atom and the nonspecific interactions of halogeno-compounds with the enzyme proteins, (5) the nature of ChA inhibition caused by halogeno-acetylcholines, and (6) reversibility of inhibition caused by halogeno-acetylcholines.

The quaternary ammonium head in the halogeno-acetylcholines is essential because the tertiary analogs were less potent for inhibiting ChA than their corresponding halogeno-acetylcholines [32]. The ether analogs of halogeno-acetylcholines in which the —CO— group was replaced by —CH_2— group were poor inhibitors of ChA [33–35]. This indicates that the —CO— group is essential for ChA inhibition caused by halogeno-acetylcholines. The ester bond in halogeno-acetylcholines is not essential for inhibiting ChA because, the keto-analogs of halogeno-acetylcholines are good inhibitors of ChA [36, 37]. It seems to be the positive carbon in the carbonyl group which is required for ChA inhibition. Other compounds in which any positive carbon is located in the position of a carbonyl-carbon in relation to a quaternary ammonium group (e.g. acryloylcholine [38]; (naphthylvinyl)pridines [39, 40]; stilbazoles [41, 42]; and 2-benzoylethyltrimethylammonium [35, 43] were potent inhibitors of ChA. These observations indicate that the positive quaternary ammonium group and positive carbonyl-carbon group are both essential in halogeno-acetylcholines for inhibiting ChA. The carboxyl-carbons in halogeno-acetylcholines are more electrophilic than those of ACh, and, therefore, they are better inhibitors of ChA than ACh.

The steric effects of halogenations did not seem to play an important role in ChA inhibition caused by halogeno-acetyl cholines. The iodine atom is the largest of the halogen atoms and IACh has the largest Van der Waals' radius for the C—R bond (where R = halogen or H). Iodo- and bromoacetylcholines were more potent inhibitors of ChA than ACh which has the smallest Van der Waals' radius for the C—R bond (where R = H). The increase in the size of the halogen atom increases the Van der Waals' force between the enzyme and the substrate. It is possible that Van der Waals' forces might have contributed for ChA inhibition caused by halogeno-acetylcholines. However, further investigations are necessary for evaluating the role of Van der Waals' forces for ChA inhibition by halogeno-acetylcholine because the differences in the degree of ChA inhibition caused by IACh, BrACh and ChACh were small.

Of the four XACh available, FACh, ClACh, BrACh and IACh, the last three were used to study the inhibitory effects on placental ChA. With IACh and BrACh, all primary plots are non-competitive and all secondary plots are linear. That is, all inhibitory patterns for IACh and BrACh are linear-noncompetitive. The fact that the inhibitory patterns are linear with IACh and BrACh indicates that the inhibitors are acting at only one site on the enzyme. Since both plots are noncompetitive, it appears that the two XACh are acting on enzyme forms (E'). In other words, the inhibitors are acting at sites on the enzyme which are not directly involved in substrate binding, but which are involved in the product synthesis. In view of the structural similarities between XACh and ACh, one can reasonably predict that XACh may interact with sites from which the product, ACh, will be released. In other words, IACh (and BrACh) form the ternary complexes CoA · E' · IACh [CoA · E' $ICH_2COOCH_2CH_2N(CH_3)_3$]. If IACh and BrACh were to bind at the site on ChA from which ACh was released, they should be competitive inhibitors of ACh in a backward ChA reaction. Further, inhibition of exchange reaction was decreased by IACh if the latter was preincubated with the enzyme in the presence of ACh, suggesting a common binding site. Bromoacetylcholine was found to be a competitive inhibitor of ACh [16].

ClACh shows an inhibitory pattern different from those of BrACh and IACh. It is a noncompetitive inhibitor of placental ChA with ACoA as the variable substrate and an apparently uncompetitive inhibitor with choline as the variable substrate. Inhibition of placental ChA by ClACh is hyperbolic (convex) noncompetitive for ACoA and hyperbolic (convex) uncompetitive for choline. The hyperbolic or convex secondary plots indicate a low degree of activation in addition to inhibition. The most likely source of this activation is the presence of choline as a product of autohydrolysis of ClACh. The concentration of ClACh in the kinetic studies was about ten times higher than that of IACh or BrACh. It was necessary to raise ClACh levels again by a factor of 10 (up to 10^{-4} M) to inhibit significantly placental ChA. This gave a maximum ClACh concentration equal to choline and above that for ACoA. Due to the above problems, valid kinetic constants with the present experimental conditions are not possible. Therefore, the data with ClACh should be considered inconclusive.

Morris and Grewaal [12, 13] reported that, using human placental ChA, and choline as the variable substrate, a primary plot for ClACh inhibition was "very nearly parallel in nature." They concluded that inhibition was uncompetitive. No secondary

plot was presented; however, a secondary plot constructed from their primary plot data appeared to be hyperbolic (convex). Inhibition was reported to be maximal at short incubation times, declining when preincubation was prolonged. The authors stated that this activation correlated with the rate of autohydrolysis. Under proper experimental conditions, it may be possible to show that mechanisms for the inhibition of ChA by ClACh are similar to those of IACh and BrACh.

Activation of the ChA reaction was observed (Fig. 1D) easily at high concentrations of FACh. This activation cannot be explained by choline formed from FACh in the reaction. The rate of autohydrolysis of FACh was not much different from that of ClACh [3]. No activation was observed with ClACh. Therefore, FACh or fluoracetate may have some activating influence on ChA.

Due to the lability of the iodine atom, a reactive group (—SH, —OH, —NH—) of one of the components in the reaction mixture may be alkylated. The likely group may be a —SH or —NH— of the enzyme (E') from which the products are released or —SH of CoA.

There is some indirect evidence for the alkylation of the enzyme by IACh and BrACh: (a) The order of inhibitory potencies of the XACh was IACh > BrACh > ClACh > FACh. The C—I and C—Br bonds are more labile than C—Cl and C—Br bonds and more likely to alkylate the enzyme, while the last two compounds are the less stable, less likely to alkylate, and are the less effective inhibitors. (b) The enzyme activity was not completely recovered upon dialysis. (c) Halogenated acetates are poor alkylating agents. They were poor inhibitors of ChA. Iodoacetate is a well known carboxymethylating agent of some enzymes. (d) The conditions of the reaction are favorable for alkylation [44], but not for thiol-ester formation [21] by BrACh or IACh. (e) Alkylation by iodoacetate or IACh is temperature dependent. Alkylation of ChA (or CoA) was not observed when preincubated at 4° or 25° [13], but it was significant at 37°. (f) Oxidation of thiol groups in ChA with 5,5'-dithobis-(2-nitrobenzoic acid) decreased the enzyme activity by 50 per cent [45]. (g) Alkylation of —SH groups by IACh was observed when they were formed by the reduction of disulfide bridges in the plasma membranes [11, 12]. All of the above observations suggest that possibly an —SH group was involved in the residual inhibition by IACh or BrACh. Esterification of choline with a halogeno-acetate may facilitate the binding of the halogeno-acetyl moiety with the active site of E'. It is possible that the carboxymethylated E' (E'–CH_2COOH) was about 70–80 per cent as active as ChA (E). It is further possible that the ester bond of E'–$CH_2COOH_2CH_2N(CH_3)_3$ was hydrolyzed during the 7–8 hr of dialysis and the carboxymethylated enzyme was recovered. Alternately, IACh (or BrACh) alkylated the —SH group of CoA with the formation of the *S*-carboxymethylated derivative of CoA, which is slowly dissociated from CoA. Recently the presence of an histidine nucleus in the active site of ChA has been postulated [46]. It is possible that part of the enzyme is inactivated irreversibly under the present experimental conditions by the alkylation of the —NH— group of histidine. More work will be required to distinguish these possibilities.

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