Biochemical Pharmacology, Vol. 21, pp. 787–802. Pergamon Press, 1972. Printed in Great Britain

KINETIC MECHANISMS OF HUMAN PLACENTAL CHOLINE ACETYLTRANSFERASE*

B. V. RAMA SASTRY and G. I. HENDERSON

Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tenn. 37203, U.S.A.

(*Received* 30 *April* 1971; *accepted* 24 *September* 1971)

Abstract—Choline acetyltransferase (ChA) from human placenta was assayed by the formation of [acetyl-^{14}C]choline (ACh) from choline (Ch) and [acetyl-^{14}C]CoA (ACoA). The bisubstrate kinetics of this enzyme (0·15 nmoles ACh/min/mg protein; ACoA, 10^{-5} M; Ch, 5×10^{-4}; pH 7·4, 37°) were studied by the following experiments: (1) variation of the initial velocity as a function of Ch concentration (6×10^{-5} to $2{\cdot}5 \times 10^{-4}$ M) at fixed ACoA concentrations (6×10^{-6} to $2{\cdot}5 \times 10^{-5}$ M); (2) variation of the initial velocity as a function of ACoA concentration (6×10^{-6} to $2{\cdot}5 \times 10^{-5}$ M) at fixed Ch concentrations (6×10^{-5} to $2{\cdot}5 \times 10^{-4}$ M); (3) enzyme inhibition by ACh (10^{-2} M) and CoA ($2{\cdot}5 \times 10^{-5}$, 10^{-4} M) with Ch as the variable substrate and ACoA as the fixed substrate; and (4) enzyme inhibition by ACh and CoA with ACoA as the variable substrate and Ch as the fixed substrate. In experiments (1) and (2), double reciprocal plots intersecting in the third quadrant were obtained. In experiment (3), ACh is a competitive inhibitor and CoA is a noncompetitive inhibitor. In experiment (4), CoA is a competitive inhibitor and ACh a noncompetitive inhibitor. These results suggest that the steady state concentrations of ternary complexes (ACoA . ChA . Ch, CoA . ChA . ACh) are very low. CoA was about 100 times stronger as a product inhibitor than ACh. Therefore, an ordered Theorell–Chance mechanism was tentatively postulated with ACoA as the leading substrate and CoA as the obligatory product. A ping-pong mechanism has been proposed for the human placental ChA in the published literature which was based upon the parallel double reciprocal plots in experiments (1) and (2) at higher substrate concentrations for ACoA and choline than those used by us. At these high substrate concentrations, the experimental points fall within the region of mixed first-order and zero-order kinetics instead of first-order kinetics on the initial velocity–substrate concentration curves. Therefore, the intersecting double reciprocal plots in experiments (1) and (2) were altered into plots which appeared approximately parallel. At high substrate concentrations of ACoA, a significant degree of substrate inhibition and product inhibition due to CoA was observed.

CHOLINE ACETYLTRANSFERASE (ChA) is an enzyme which catalyzes the biosynthesis of acetylcholine (ACh) from acetylcoenzyme A (ACoA) and choline. It occurs in nervous as well as non-nervous tissues.[1] It was prepared and was partially purified from different animal tissues, namely housefly brain,[2] human placenta,[3] squid head ganglia,[4] rat brain,[5,6] and calf caudate nucleus.[7] There were significant quantitative differences in the effects of sodium halides, interaction of iodoacetate with the sulfhydryl groups of the enzyme and the interaction of both substrates with the enzyme. These observations indicate that there are different isozymes of ChA in different tissues.

Three different kinetic mechanisms have been suggested for the action of ChA from different tissues (Fig. 1). These mechanisms are based on the standard nomenclature

* A preliminary report of this investigation was presented at the meeting of the Federations of American Societies for Experimental Biology, Chicago, Ill., April 1971 (*Fedn Proc.* **30**, 621, 1971).

suggested by Cleland[8] for bisubstrate interactions. Schuberth[3] reported parallel double reciprocal plots of initial velocity versus variable substrate concentrations of one substrate at fixed concentrations of the second substrate using human placental ChA. These plots agree with a ping-pong mechanism for ChA reaction (Fig. 1A). Potter *et al.*[5] reported double reciprocal plots which did intersect in the third quadrant using rat brain ChA. They used lower substrate concentrations than those used by Schuberth.[3] Their data suggest an ordered mechanism (Fig. 1B) for ChA reaction. White and Cavalitto[7] studied the product inhibition of ChA from calf caudate nucleus and suggested a Theorell–Chance type of mechanism[9] for ChA (Fig. 1C). Therefore, a question arises as to whether ChA isozymes from different mammalian tissues exhibit different mechanisms of action.

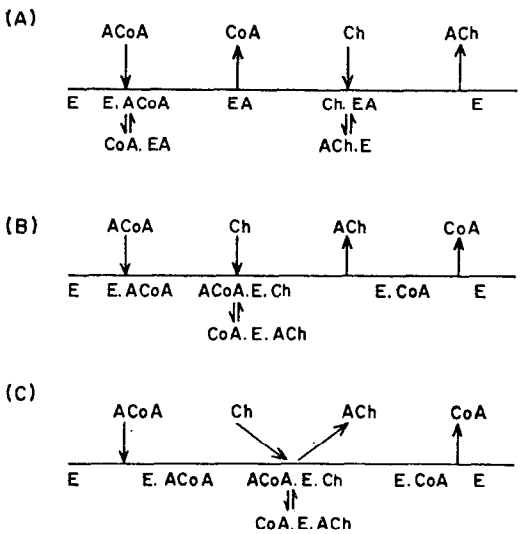

FIG. 1. Suggested kinetic mechanisms for the synthesis of acetylcholine (ACh) from choline (Ch) and acetylcoenzyme A (ACoA) by choline acetyltransferases (ChA) of different tissues. (A) Ping-pong mechanism postulated for human placental ChA; (B) ordered or sequential mechanism postulated for rat brain ChA; (C) Theorell–Chance mechanism postulated for calf caudate nucleus ChA.

Among well known cases, an enzyme from different sources acts by the same mechanism of action. There are differences in the three types of kinetic mechanisms which can be detected if the data on (1) initial velocities at low substrate concentrations of choline and ACoA, (2) product inhibition by ACh and CoA, and (3) substrate inhibition by ACoA and choline were available on the same ChA preparation.[10] Therefore, we have obtained the above information using human placental ChA in all of our studies.

The existence of an ACh–ChA–acetylcholinesterase (AChE) system in the human placenta has been demonstrated by a number of workers.[1] Since the placentae of man and animals lack innervation, a question arises as to whether the ACh–ChA–AChE system of non-nervous origin is identical to or similar to that of the brain AChE.[11,12] The synthesis of ACh in the nervous tissue as well as in the placenta was catalyzed by ChA's.[1,13] However, there is no unequivocal evidence to indicate that the nature of

placental ChA is similar to that of brain ChA. Therefore, we are interested in the similarities (or differences) in the kinetic mechanisms of placental and brain ChA's. This study indicated a Theorell–Chance type of mechanism[9] for human placental ChA. A similar mechanism was postulated for ChA of calf caudate nucleus.[7]

MATERIALS AND METHODS

Enzyme preparation. Full-term human placentae were used as the source of the enzyme. Each placenta (about 1·0 kg) was placed in a plastic bag immediately after delivery and was cooled in an ice bath. It was processed immediately as described below in a cold room (4°) or was frozen at −12° for future processing. It was sliced, cleared free from blood and connective tissue as much as possible, and homogenized in cold acetone (−12°). The resulting homogenate was filtered, and the acetone-insoluble material was washed twice with cold acetone and dried in vacuum. Each placenta of about 1·0 kg yields about 40 g of acetone-insoluble powder. There were no significant differences in ChA activities of unwashed acetone-insoluble powder and the acetone-insoluble powder washed twice with cold acetone.

The method for partial purification of ChA is similar to that of Morris.[14] The acetone-insoluble powder was extracted with 0·02 M $NaHCO_3$ buffer (pH 7·4, 20 ml/g powder) which contained 0·5 mM EDTA, 0·3 M NaCl and 0·02 M NaCN. The presence of NaCN in the extracts was found to prevent the loss of ChA activity.[3] The pH of the extract was adjusted to 5·0 with HCl and the resulting precipitate was discarded. The filtrate (F-1) contained most of the ChA activity of the acetone-insoluble powder. Solid $(NH_4)_2SO_4$ was added to the filtrate F-1 to bring its concentration to 15% (w/v) and the resulting mixture was centrifuged for 15 min at 5000 *g*. The sediment contained little ChA. Further quantities of $(NH_4)_2SO_4$ were added to the supernatant to bring its concentration to 25% (w/v) and the mixture was centrifuged at 5000 *g* for 15 min. The sediment contained ChA (specific activity, 3·47 nmoles ACh formed/mg protein/min) which was freeze-dried for storage. About 1·0 g of this active protein was obtained from 32 g of acetone-insoluble powder. For enzyme solutions, 6·7 mg of this protein was dissolved in 10 ml of 0·05 M phosphate buffer containing 20 mM NaCN and 5 mM EDTA.

Substrates. [^{14}C]acetyl coenzyme A (0·68 mg, 48·8 mc/m-mole) was supplied by New England Nuclear Corp., Boston, Mass., in 0·5 ml of aqueous solution at pH 4·0. The radiochemical purity of this sample was greater than 97·7 per cent when assayed by paper (Whatman No. 3) chromatography in isopropanol–pyridine–water (1:1:1). Its chemical purity was 97·5 per cent when assayed by the technique of ultraviolet ratios in 0·1 M KH_2PO_4 buffer (pH 7·0). No free sulfhydryl groups were detected by the nitro-prusside test. The above sample of [^{14}C]ACoA was diluted to 7·5 ml with phosphate buffer to give the stock solution of [^{14}C]ACoA. Unlabeled ACoA was purchased from Mann Research Laboratories, Inc., New York. The purity of this sample was about 96 per cent when assayed by ultraviolet ratios, and only a trace of CoA was detected in this sample. Choline iodide was supplied by Eastman Organic Chemicals, Rochester, N.Y.

Solutions and reagents. All ChA assays were carried out in 0·05 M phosphate buffer (pH 7·4) prepared from KH_2PO_4 and KOH according to the instructions of Dawson *et al.*[15]

The buffer–substrate reagent contained 0·05 M phosphate buffer, 0·3 M NaCl, 2×10^{-2} M $MgCl_2$, 2×10^{-4} M physostigmine sulfate, and known concentrations of choline iodide and unlabeled ACoA, which were varied according to the requirements.

The buffer–substrate incubation medium contained nine parts of buffer–substrate reagent and one part [^{14}C]ACoA stock solution.

Kinetics of the enzymatic synthesis of acetylcholine. The amount of [^{14}C]acetylcholine ([^{14}C]ACh) formed during the enzymatic synthesis was estimated by a radiometric method. One-tenth ml of the buffer–substrate incubation medium and 0·1 ml buffer were mixed and preincubated for 5 min in test tubes (75 × 10 mm) at 37°. Then 0·1 ml of the enzyme solution was added to each test tube and the incubation continued for 10 min. At the end of the incubation period, each test tube was placed in an ice bath to stop the reaction. Then 0·1 ml of the cold incubate was placed on a column (length, 12 cm; volume, 3 ml) of Bio-Rad AG-1-x8 anion-exchange resin (200–400 mesh, chloride form). Each column was washed with 4 × 0·5 ml of distilled water. Effluent was collected in scintillation vials to which 15 ml fluor was added. The samples were counted for radioactivity, which gave estimates of [^{14}C]ACh formed during the enzymatic synthesis.

In the above procedure, cooling the reaction mixture in the ice bath and its exposure to the anion-exchange resin were adequate means for arresting the reaction. Potter *et al.*,[5] and Glover and Potter[16] reported that cooling in an ice bath was a suitable method for inactivation of the enzyme. We found that no [^{14}C]ACh was formed when the temperature of the reaction medium was kept at 0°. Exposure to the anion-exchange resin lowered the pH of the reaction medium to 4·0. At pH 4·0, the enzyme was inactivated irreversibly in our experiments and studies reported by other investigators.[14] The total duration of time for the addition of 0·1 ml of the reaction medium to the anion-exchange resin and its adsorption by the column was less than 15 sec. No significant differences were found in the amount of [^{14}C]ACh formed even when this period was increased to 1 min. Fifteen sec was considerably smaller than the reaction time of 10 min. All of the above experiments indicated that insignificant amounts of [^{14}C]ACh were synthesized during 15 sec prior to addition to the column.

The Bio-Rad anion-exchange resin has a capacity of 3·2 m-equiv./g. In control experiments, when buffer–substrate incubation medium (0·3 ml) which contained [^{14}C]ACoA was added to the column, an insignificant amount of radioactivity (0·034 per cent of the total radioactivity added to the column) was detected in the eluates. When the buffer–substrate incubation medium in which [^{14}C]ACoA was substituted by an equivalent amount of sodium [^{14}C]acetate was added to the column, no significant radioactivity was detected in the eluates. When [^{14}C]ACoA was substituted by [^{14}C]ACh in the buffer–substrate incubation medium, 99·9 per cent of the total radioactivity was recovered in the eluates. These experiments indicated that [^{14}C]ACoA and [^{14}C]acetate (if present as an impurity in trace amounts) were adsorbed by the anion-exchange column and [^{14}C]ACh formed by the enzymatic synthesis was recovered quantitatively in the eluates.

The above procedures were used to study the bisubstrate kinetics (see under kinetic methods) of ChA by the following experiments: (1) variation of the initial velocity as a function of choline concentration (6×10^{-5} to $2{\cdot}5 \times 10^{-4}$ M) at fixed ACoA concentrations (6×10^{-6} to $2{\cdot}5 \times 10^{-5}$ M); (2) variation of the initial velocity as a

function of ACoA concentration (6×10^{-6} to $2{\cdot}5 \times 10^{-5}$ M) at fixed choline concentrations (6×10^{-5} to $2{\cdot}5 \times 10^{-4}$ M); (3) enzyme inhibition by ACh (10^{-2} M) and CoA ($2{\cdot}5 \times 10^{-5}$ and 10^{-4} M) with choline as the variable substrate and ACoA as the fixed substrate; (4) enzyme inhibition by ACh and CoA with ACoA as the variable substrate and choline as the fixed substrate; and (5) the substrate inhibition, if any, with ACoA and choline.

Radiometric methods. All samples were counted in 20-ml vials using a Packard model 3375 Tri-Carb scintillation spectrometer. Scintillation fluor contained 2,5-diphenyloxazole (PPO, 0·5%) and naphthalene (10%) in 1,4-dioxane. The scintillation 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} μc) as the internal standard and the reaction medium as the quenching agent. The efficiency was 75 per cent when the AES value was 0·55. In most of our experiments, the AES values range between 0·45 and 0·65, which corresponded to efficiencies between 65 and 81 per cent. The disintegrations per minute for each sample were computed from counts per minute and the AES value. The counting error was less than 2 per cent.

Kinetic methods. In the reaction of ChA, ACoA and choline are participants as substrates and ACh and CoA are formed as products. The kinetic theories for bi-substrate reactions, which were developed by a number of investigators,[10] were applied to study the ChA reaction in the forward direction. The following kinetic equations were used in our studies for the graphical analysis of the data.

Variable concentrations of substrate A, fixed concentrations of substrate B:

$$\frac{1}{v_1} = \frac{K_a}{V_1 a}\left(1 + \frac{\bar{K}_a K_b}{K_a b}\right) + \frac{1}{V_1}\left(1 + \frac{K_b}{b}\right) \tag{1}$$

where a, b = concentrations of substrates A and B; K_a = the Michaelis constant of substrate A; $\bar{K}_a$ = the dissociation constant for A, the leading substrate; V_1 = the maximal velocity; v_1 = initial velocity.

Variable concentrations of substrate B, fixed concentrations of substrate A:

$$\frac{1}{v_1} = \frac{K_b}{V_1 b}\left(1 + \frac{\bar{K}_a K_b}{K_b a}\right) + \frac{1}{V_1}\left(1 + \frac{K_a}{a}\right) \tag{2}$$

where K_b = Michaelis constant of substrate B.

The details for the determination of initial velocities (v_1) at various concentrations of A and B, evaluation of the primary plots which were represented by equations (1) and (2), calculation of the slopes and intercepts from the primary plots, replots for the slopes and intercepts of the primary plots (secondary plots) and calculation of K_a, K_b, $\bar{K}_a$ and V_1 from the primary and the secondary plots were described by Mahler and Cordes.[10] The methods for the identification of the type of inhibition (i.e. competitive, uncompetitive or noncompetitive) in the case of bisubstrate enzymes were described by Mahler and Cordes[10] and Cleland.[17]

Statistical methods. The experimental data were computed in an Olivetti-Underwood 101 programmable calculator. Linear regression line analysis by the method of least squares was used to fit the experimental points by straight lines in the primary plots and the secondary plots. Experimental values were expressed as mean $\pm$ S. E. wherever possible.

RESULTS

Relation between ChA concentration and the rate of ACh synthesis. The rate of ACh production with increasing concentrations of the enzyme is plotted in Fig. 2. With initial concentrations of 4×10^{-5} M for ACoA and 4×10^{-4} M for choline, the rate of ACh synthesis was linear until the concentration of the enzyme protein was increased to 0·325 mg/0·3 ml of incubation medium. The depression of the curve at high enzyme concentrations was possibly due to the utilization of the substrate as well as the accumulation of the products, especially CoA, which is a strong inhibitor (see Fig. 6).

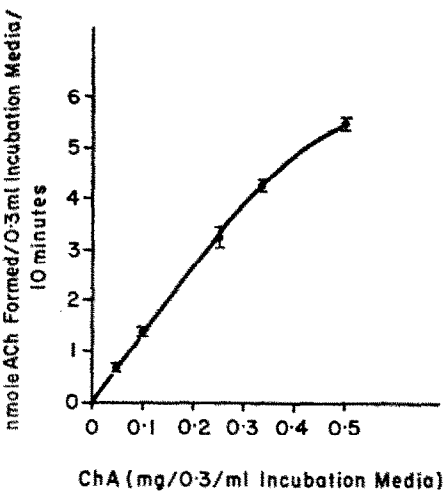

FIG. 2. Relation between ChA concentration and ACh synthesis. Choline concentration, 5×10^{-4} M; ACoA concentration, 5×10^{-5} M. Each point is a mean from three observations.

Rate of enzymatic synthesis of ACh as a function of time. The rate of ACh formation was linear for the first 10 min when the enzyme concentration was 0·49 mg protein/0·3 ml of incubation medium (Fig. 3). At lower enzyme concentrations, the rate of formation was linear for longer periods. With an enzyme concentration of 0·28 mg protein/0·3 ml, the rate of ACh formation was linear for a period longer than 10 min. The enzyme concentrations used in the following kinetic studies were within 0·15–0·3 mg protein/0·3 ml.

Variation of the initial velocity as a function of ACoA concentration at fixed choline concentrations. Double reciprocal plots for bisubstrate reactions were plotted with ACoA as the variable substrate (6×10^{-6} M to $2{\cdot}5 \times 10^{-5}$ M) and choline as the fixed substrate (6×10^{-5} to $2{\cdot}5 \times 10^{-4}$ M) in Fig. 4A. Initial velocities were determined from ACh formation during the first 10 min of reaction. The amounts of CoA and ACh formed during the 10 min of reaction were too low to cause any product inhibition. Choline did not exhibit significant substrate inhibition. Therefore, the data are accurate for interpretation by kinetic methods.

All plots did intersect in the third quadrant (Fig. 4A). From the projection of the point of intersection on the abscissa, $\bar{K}_a$ (dissociation constant for ACoA) was calculated (Table 1). The secondary plots were drawn for: (1) the intercepts in Fig. 4A

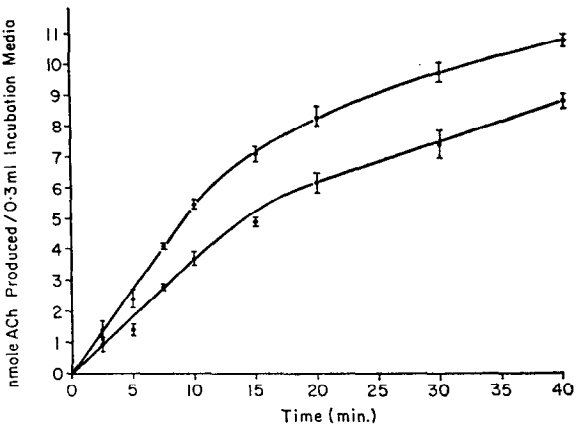

FIG. 3. Rate of enzymatic synthesis as a function of time. Enzyme concentrations in milligrams of protein/0·3 ml of incubation medium; top curve, 0·49; lower curve, 0·28. Choline concentration, 10^{-3} M; ACoA concentration, 10^{-4} M. Each point is a mean from three observations.

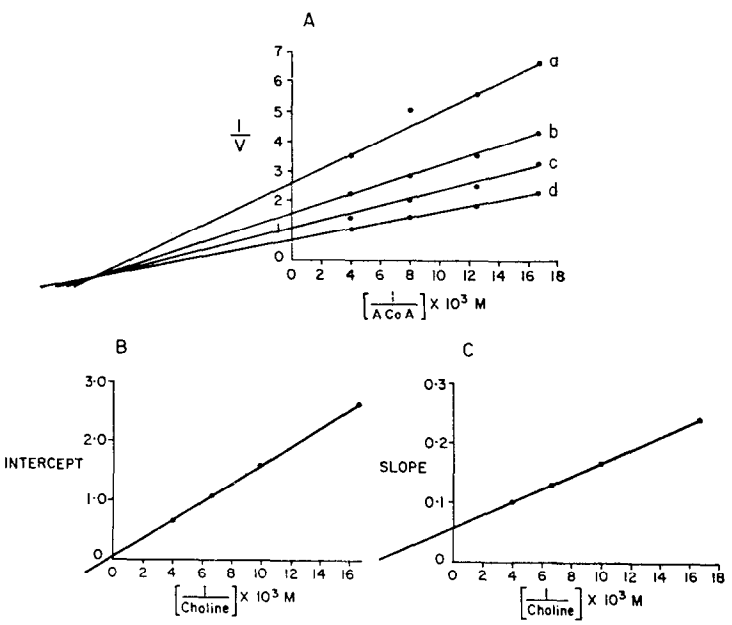

FIG. 4. (A) Primary plots: double reciprocal plots for 1/v vs. 1/s with ACoA as the variable substrate and choline as the fixed substrate. V was expressed as nanomoles per milliliter of enzyme per minute. Choline concentrations (M): 6×10^{-5} (plot a), 10^{-4} (plot b), $1{\cdot}5 \times 10^{-4}$ (plot c), $2{\cdot}5 \times 10^{-4}$ (plot d). ChA, 0·3 mg protein/0·3 ml incubation medium. Each point is a mean from three observations. (B) A secondary plot for the intercepts in Fig. 4A vs. the reciprocals of choline concentrations. (C) A secondary plot for the slopes in Fig. 4A vs. the reciprocals of choline concentrations.

versus the reciprocals of choline concentrations (Fig. 4B), and (2) the slopes in Fig. 4A versus the reciprocals of choline concentrations (Fig. 4C). From the above set of primary and secondary plots, and four kinetic parameters, V_1, K_a (for ACoA), K_b (for choline) and $\bar{K}_a$ (for ACoA), were calculated (Table 1).

TABLE 1. KINETIC PARAMETERS FOR HUMAN PLACENTAL CHOLINEACETYLASE

Parameter	Variable substrate	
	ACoA	Choline
Maximal velocity (V_1):	20·44 nmoles ACh/ml/min	33·23
Dissociation constant* of the leading substrate ($\bar{K}_a$ for ACoA)	$7{\cdot}4 \times 10^{-6}$ M	$8{\cdot}0 \times 10^{-6}$ M
K_a for ACoA	$1{\cdot}13 \times 10^{-4}$ M	$1{\cdot}48 \times 10^{-4}$ M
$\bar{K}_b$ for choline	$3{\cdot}12 \times 10^{-3}$ M	$5{\cdot}07 \times 10^{-3}$ M

* It was assumed that ACoA is the leading substrate. Further experimental evidence is necessary before ACoA is established as the leading substrate.

Variation of the initial velocity as a function of choline concentration at fixed ACoA concentrations. Double reciprocal plots for choline as the variable substrate (6×10^{-5} to $2{\cdot}5 \times 10^{-4}$ M) and ACoA as the fixed substrate (6×10^{-6} to $2{\cdot}5 \times 10^{-5}$ M) are shown in Fig. 5A. Under the conditions of this experiment, the product inhibition and

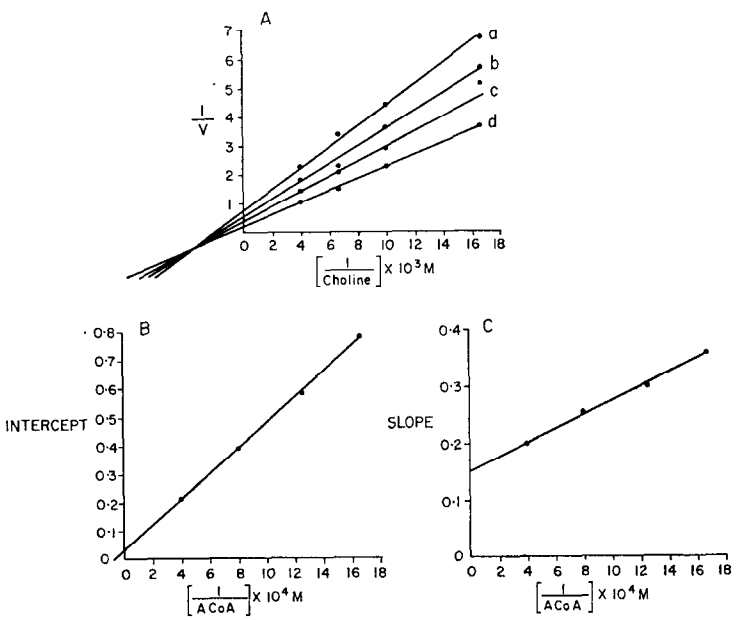

FIG. 5. (A) Primary double reciprocal plots for 1/v vs. 1/s with choline as the variable substrate and ACoA as the fixed substrate. ACoA concentrations (M): 6×10^{-6} (plot a), 8×10^{-6} (plot b), $1{\cdot}2 \times 10^{-5}$ (plot c), $2{\cdot}5 \times 10^{-5}$ (plot d). (B) A secondary plot for the intercepts in Fig. 5A vs. the reciprocals of ACoA concentrations. (C) A secondary plot for the slopes in Fig. 5A and the reciprocals of ACoA concentrations.

the substrate inhibition were nonsignificant. All double reciprocal plots did intersect in the third quadrant. Two secondary plots were drawn for the intercepts in Fig. 5A versus the reciprocals of ACoA concentrations (Fig. 5C). From the above sets of primary and secondary plots, the four kinetic parameters, V_1, K_a, K_b and $\bar{K}_a$ were calculated (Table 1).

The commercial sample of ACh, which was recrystallized twice from isopropanol–ethyl acetate, contained 0·3 per cent choline as an impurity. This was detected by incubating ACh with ChA and [^{14}C]ACoA and estimating the [^{14}C]ACh formed. The plot in Fig. 5A was corrected for choline contamination.

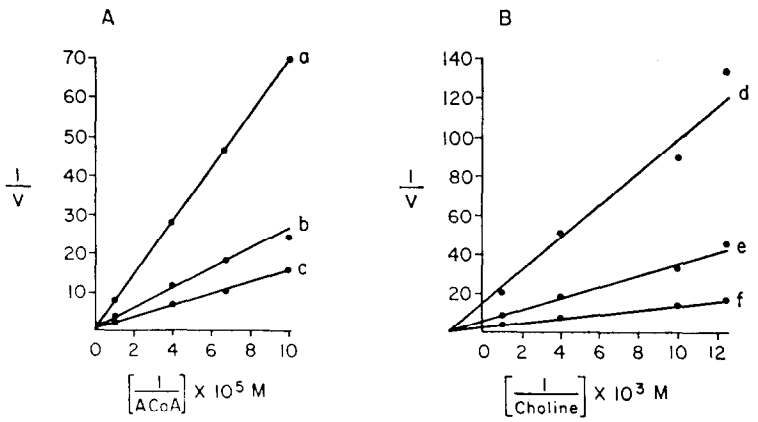

FIG. 6. (A) Double reciprocal plots for 1/v vs. 1/s for CoA inhibition of ACoA. CoA concentrations (M): $2·5 \times 10^{-5}$ (plot a), 10^{-4} (plot b). Plot c represents a control plot without CoA in the medium. Choline concentration, 10^{-4} M; ChA 0·3 mg protein/0·3 ml incubation medium. Each point is a mean from three observations. (B) Double reciprocal plots for 1/v vs. 1/s for CoA inhibition of choline. CoA concentrations (M): $2·5 \times 10^{-5}$ (plot d), 10^{-4} (plot e). Plot f represents a control plot without CoA in the medium. ACoA concentration, 7×10^{-6} M, ChA, 0·3 mg protein/0·3 ml incubation medium. Each point is a mean of three observations.

Inhibition of ChA by CoA. In order to select suitable conditions to study the kinetic mechanisms, the inhibition of the enzyme by CoA, one of the products of the enzyme reaction, was studied at various concentrations of CoA. CoA inhibited the enzyme with an I_{50} of $3·34 \times 10^{-5}$ M at the choline concentration of 10^{-4} M. With higher choline concentrations, the I_{50} for CoA increased. At the choline concentration of 5×10^{-4} M, the I_{50} for CoA was 6×10^{-5} M. When the CoA concentration was 10^{-6} M, the inhibition of the enzyme was about 9 and 5 per cent at choline concentrations of 10^{-4} M and 5×10^{-4} M respectively. The double reciprocal plots for the inhibition of ChA by CoA with ACoA as the variable substrate are shown in Fig. 6A. Increasing concentrations of CoA increased the slopes without affecting the intercepts. Although the intercepts were small, all three lines crossed the ordinate at the same point above the abscissa. To make sure that this observation was true, the graph shown in Fig. 6A was replotted on 8×16 in. graph paper and the lines were drawn by regression line analysis (see Statistical methods). Even then, all three lines crossed the ordinate at the same point above the abscissa. The intercepts were significant and were equal, although they were small. Therefore, the inhibition by CoA was competitive.

The double reciprocal plots for the inhibition of ChA by CoA with choline as the variable substrate and ACoA as the fixed substrate are shown in Fig. 6B. Inhibition by CoA affected both slopes and intercepts. All plots intersected on the abscissa. Therefore, the inhibition was noncompetitive.

Inhibition of ChA by ACh. ACh, one of the products of ChA reaction, was a weak inhibitor of placental ChA. A concentration of 10^{-2} M ACh caused about 28 per cent inhibition. Concentrations of 10^{-5} M or lower did not inhibit the enzyme significantly. The double reciprocal plots for the inhibition of ChA by ACh with ACoA as the variable substrate had different slopes and intercepts (Fig. 7B). They converged to a point to the left of the vertical axis (not shown in Fig. 7B) and below the abscissa.

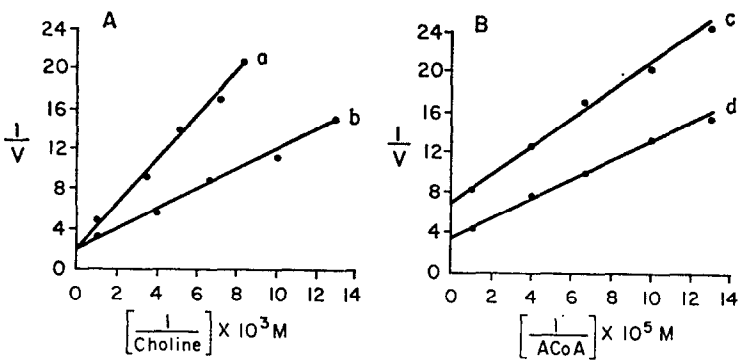

FIG. 7. (A) Double reciprocal plots for 1/v vs. 1/s for ACh inhibition of choline. ACh concentration was 10^{-2} M for obtaining plot a. Plot b represents a control plot without ACh in the medium. ACoA concentration, 7×10^{-6} M; ChA, 0·3 mg protein/0·3 ml incubation medium. Each point is a mean of three observations. (B) Double reciprocal plots for 1/v vs. 1/s for ACh inhibition of ACoA; choline, 5×10^{-4} M for obtaining plot c. Plot d represents a control plot without ACh in the medium. ChA was 0·3 mg protein/0·3 ml incubation medium. Each point is a mean of three observations.

In his discussion of steady state kinetics, Cleland[17] reported that in noncompetitive inhibition the double reciprocal plots might cross above, below, or on the horizontal axis, or they might not all cross at the same point. According to Cleland,[17] there was no good theoretical reason for assuming that the double reciprocal plots should cross on the abscissa, and any inhibition where both slopes and intercepts were affected could be considered as noncompetitive. Therefore, the inhibition of ChA by ACh with ACoA as the variable substrate, in which both slopes and intercepts were affected, was classified as noncompetitive.

Double reciprocal plots for ChA synthesis of ACh at high substrate concentrations. Schuberth[3] reported double reciprocal plots which appeared to be parallel and postulated a ping-pong mechanism based on these plots. He used concentrations of ACoA ($1{\cdot}4 \times 10^{-5}$ to 10^{-3} M) about 100 times higher than those used by us in Fig. 4. Therefore, we repeated our experiments at high substrate concentrations of ACoA ($2{\cdot}5 \times 10^{-5}$ to 5×10^{-4} M). At these high substrate concentrations, the double reciprocal plots did tend to become parallel as shown in Fig. 8A. The plot e in Fig. 8A was constructed according to the conditions reported by Schuberth.[3]

In Fig. 8A, each double reciprocal plot exhibited a trough. This indicated that ACoA exhibited a significant degree of substrate inhibition at high substrate concen-

trations. Similarly, Potter *et al.*[5] reported substrate inhibition of rat brain ChA by high concentrations of ACoA. The slope of the ascending limb, which was adjacent to the ordinate, decreased from the plot a to the plot e. Choline concentrations increased from the plot a to the plot e. In the plot e, the ascending limb adjacent to the ordinate was not significant and was not easily perceptible. Therefore, at choline concentrations used in the plot e or higher, substrate inhibition was not perceptible. Schuberth[3] used choline concentrations equivalent to those in the plot e or higher and did not observe the substrate inhibition.

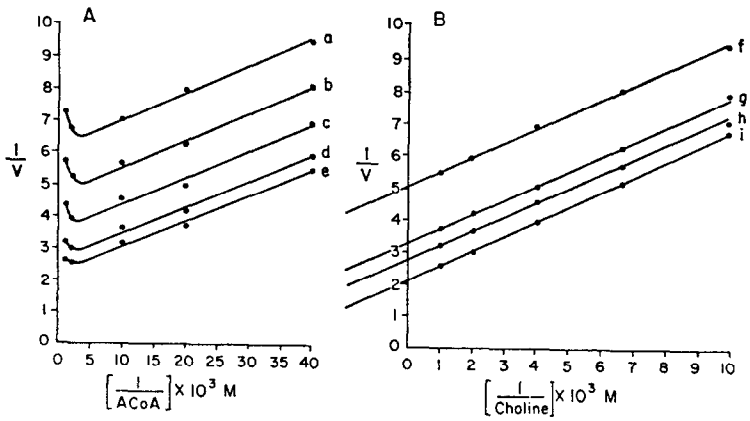

FIG. 8. (A) Double reciprocal plots for 1/v vs. 1/s at high substrate concentrations with ACoA as variable substrate. Choline concentrations (M): 10^{-4} (plot a), $1{\cdot}5 \times 10^{-4}$ (plot b), $2{\cdot}5 \times 10^{-4}$ (plot c), 10^{-4} (plot d), and 10^{-3} (plot e). Concentration of ChA was 0·3 mg protein/0·3 ml of incubation medium. Each point is a mean of three observations. (B) Double reciprocal plots for 1/v vs. 1/s at high substrate concentrations with choline as variable substrate. ACoA concentrations (M): $2{\cdot}5 \times 10^{-5}$ (plot f), 5×10^{-5} (plot g), 10^{-4} (plot h), $2{\cdot}5 \times 10^{-4}$ (plot i).

In Fig. 8B, choline did not exhibit substrate inhibition. No ascending limb adjacent to the ordinate was observed in any of the plots from f to i. Schuberth[3] did not report substrate inhibition with choline.

Plots of initial velocity versus substrate concentration. In order to evaluate the significance of plots in Figs. 4 and 5 as well as those in Fig. 8, the relationships between the initial velocity and a wide range of substrate concentrations were reproduced in Fig. 9.

The initial velocity as a function of choline concentration with ACoA as the fixed substrate was plotted in Fig. 9A. As concentrations of ACoA increased from $2{\cdot}5 \times 10^{-5}$ M to $2{\cdot}5 \times 10^{-4}$ M, the velocities increased. The velocities were lower with an ACoA concentration of 5×10^{-4} M than those with an ACoA concentration of $2{\cdot}5 \times 10^{-4}$ M. This was due to the substrate inhibition caused by ACoA at the concentration of 5×10^{-4} M.

The curves in Fig. 9A were divided approximately into three regions: R_1, R_2 and R_3. The concentrations of ACoA or choline in Fig. 4 were selected from within R_1 region in which velocity increased in proportion to the increase in choline concentration at a fixed concentration of ACoA. According to Segel,[18] one can approach the first-order kinetics by selection of experimental conditions from within this region.

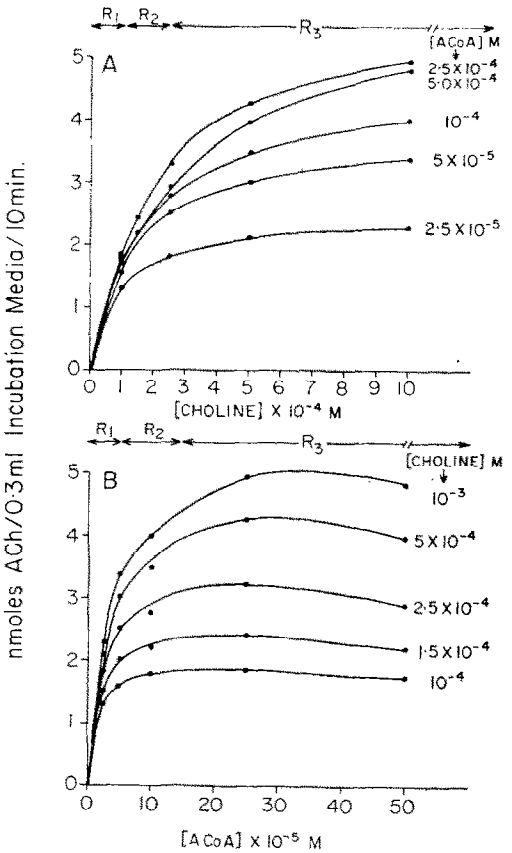

FIG. 9. Plots of initial velocities vs. a wide range of substrate concentrations. (A) Plots of initial velocities vs. choline concentration at fixed ACoA concentrations. (B) Plots of initial velocities vs. ACoA concentrations at fixed choline concentrations. The values on the abscissa were divided approximately into three ranges, R_1, R_2 and R_3 (see the text for explanation). The experimental values in R_1 could not be shown in this figure due to the conglomeration of points. The experimental points in Figs. 4 and 5 fall within the range R_1.

R_2 and R_3 are regions of first-order and zero-order kinetics. To the right of region R_3, conditions approach zero-order kinetics in which velocity remains essentially constant with increasing substrate concentrations, provided there is no substrate inhibition. The experimental conditions in Fig. 8A and the experimental conditions selected by Schuberth[3] fall within region 3. In zero-order kinetics, the double reciprocal plots will be parallel to the abscissa. In the conditions representing the first-order kinetics, we obtained intersecting lines which crossed the abscissa. Therefore, one would expect to obtain the double reciprocal plots which appear parallel and will slope toward the horizontal axis under conditions of region R_3. Schuberth[3] and we obtained the double reciprocal plots (Fig. 8A) which appeared parallel and sloped toward the horizontal axis. However, one could not exclude the possibility that the shape of the double reciprocal plots in Fig. 8A was not influenced partially by the substrate inhibition by ACoA and the product inhibition by CoA.

The initial velocities versus ACoA concentrations with fixed choline concentrations were plotted in a family of curves in Fig. 9B. The initial slopes of the curves increased with increasing choline concentrations. At high ACoA concentrations, the curves were slightly depressed.

The conditions for experiment in Fig. 5 were selected from region R_1, the region of first-order kinetics. The conditions for the experiment in Fig. 8B, and possibly in Schuberth's[3] experiments, were selected from the region R_3, the region of mixed first-order and zero-order kinetics. Due to the reasons already discussed in relation to Fig. 9A, we obtained lines which appeared parallel and sloped toward the abscissa in Fig. 8B.

Schuberth[3] proposed a ping-pong mechanism for human placental ChA based on data which were similar to that shown in Fig. 8A and B. The mechanism should be proposed based upon the data obtained under the condition of first-order kinetics (Figs. 4 and 5). Therefore, the ping-pong mechanism proposed for the human placental ChA should be modified in view of the intersecting lines in Figs. 4A and 5A, which were obtained under conditions of first-order kinetics.

DISCUSSION

Consideration should be given to different aspects in the evaluation of the kinetic mechanism for ChA synthesis of ACh such as: (1) the type of mechanism—random, ordered or ping-pong; (2) the leading substrate; (3) the formation of ternary or binary intermediates; and (4) the nature of the product inhibition.

One of the distinguishing experimental features of the three types of mechanisms is the two sets of 1/v versus 1/s plots.[8,10,17] These plots may meet on, below, or above the abscissa for ordered mechanisms and random mechanisms, and they run parallel for ping-pong mechanisms.[17] With placental ChA, they meet below the abscissa, indicating an ordered mechanism or a random mechanism.

A second characteristic of ordered and random mechanisms is the occurrence of ternary complexes (ACoA . E . Ch $\rightleftharpoons$ CoA . E . ACh) as intermediates (Fig. 1B). In ping-pong mechanisms (Fig. 1A), binary complexes (E . ACoA $\rightleftharpoons$ CoA . EA, Ch . EA $\rightleftharpoons$ ACh . E) occur as intermediates. Morris and Grewaal[19] presented evidence, by isotope exchange methods and human placental enzyme, that a ternary intermediate CoA . E . ACh was formed from CoA, the enzyme (E) and ACh. Therefore, human placental ChA does not act by a ping-pong mechanism in which ternary complexes are not formed; it may act by a random or an ordered mechanism in which ternary complexes are formed.

A third distinguishing feature for these three types of mechanisms is the nature of product inhibition. Among random mechanisms, both products (ACh, CoA) should give competitive patterns with either one of the substrates (ACoA, choline). This is not true in the case of human placental ChA. Therefore, human placental ChA does not act by random mechanisms. Among ordered mechanisms, only the leading substrate should be inhibited competitively by its product and noncompetitively by both products. This is not true in case of human placental ChA. Among ping-pong mechanisms, the leading substrate should be inhibited competitively by one of the products and the second substrate should be inhibited competitively by the second product. CoA was a competitive inhibitor of ACoA and ACh was a competitive inhibitor

of choline. Therefore, the inhibitory patterns by products suggest a ping-pong mechanism for human placental ChA.

In summary, the primary plots (Figs. 4 and 5) and the formation of ternary intermediates suggest an ordered mechanism and the product inhibitory patterns suggest a ping-pong mechanism for human placental ChA. These are the requirements for a special case of an ordered mechanism called the Theorell–Chance mechanism.[9] When steady state concentrations of ternary complexes are very low, the first product (P) appears to be formed directly from the second substrate (B) by interaction with the enzyme–first substrate (EA) complex in Theorell–Chance mechanism.[9,10] Experimentally, the distinguishing features for this mechanism are: (1) that the reciprocal plots show the pattern of an ordered mechanism, and (2) competitive inhibition of the first substrate (A) by the second product (not first product) and the second substrate by the first product (not second product). In our experiments, CoA was a competitive inhibitor of ACoA and a noncompetitive inhibitor of choline. Further, ACh was a competitive inhibitor of choline and a noncompetitive inhibitor of ACoA. These observations indicate that the placental ChA may act by the Theorell–Chance mechanism.

There are several reasons for suggesting that ACoA is the leading substrate: (1) ACoA is a coenzyme and coenzymes are usually the leading substrates in the reaction;[10] (2) the K_m of ACoA was more than 30 times lower than that of choline,

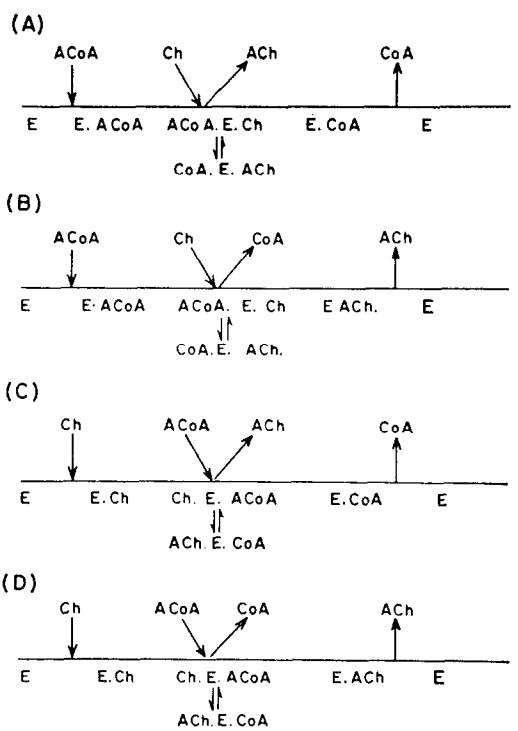

FIG. 10. Possible variations of Theorell–Chance mechanisms for ChA. In (A) and (B) ACoA is the leading substrate. In (C) and (D), choline is the leading substrate. Our data suggest the mechanism 12A for ChA.

suggesting that ACoA probably binds more strongly than choline; and (3) CoA was a hundred times stronger than ACh to inhibit ChA, indicating that CoA was the last product to dissociate from the enzyme. Furthermore, CoA was the competitive inhibitor of ACoA, suggesting that ACoA was the first substrate.

From the above observations, we tentatively suggest an ordered Theorell–Chance mechanism for placental ChA with ACoA as the leading substrate and CoA as the obligatory product (Fig. 10A). There are three other possible orders (Fig. 10B, C, D) for the association of substrates with ChA and the dissociation of the products. These three orders are not valid for ChA due to the following experimental observations. (1) According to orders 10B and 10C, ACh is a competitive inhibitor of ACoA and CoA is a competitive inhibitor of choline. We found that ACh was a competitive inhibitor of choline. (2) According to order 10D, one expects that ACh is a better product inhibitor of ChA than CoA. CoA was about a hundred times stronger as a product inhibitor than ACh.

Besides for human placental ChA, a Theorell–Chance mechanism was tentatively postulated for ChA of calf caudate nucleus.[7]

Available information indicates that the ACh–ChA–AChE system is of importance in the placental function during pregnancy.[13] This function may be related to the transport of materials across the placental barrier.[13,20] According to present indications, ChA is located in the chorionic villi, in the cell layers separating maternal and fetal blood compartments.[13] There are two components for ACh-like substances in the chorionic villi, a bound form (0·17–0·22 μg/g of wet tissue) and a free form (0·01 μg/g of wet tissue).[20] Assuming uniform distribution, ACh concentration reaches $2{\cdot}2 \times 10^{-4}$ M in the chorionic villi. There is some experimental evidence that the bound ACh is located in "granules", which are liberated from the tropoblast epithelium, especially the syncytium, of the chorionic villi.[21] Based on our knowledge on the concentrations of ACh in the nervous tissue and the synaptic vesicles,[22] one would expect that the concentration of ACh in the granules should be at least 100 times higher than $2{\cdot}2 \times 10^{-4}$ M. Therefore, ACh concentration reaches $2{\cdot}2 \times 10^{-2}$ M or higher in the granules of the placenta. According to our results, $2{\cdot}2 \times 10^{-2}$ M ACh inhibits ChA significantly. The above information is suggestive that, besides the general metabolic state of the chorionic villi, substrate availability and mass action kinetics, product accumulation may contribute to the control *in vivo* of ACh synthesis in the placenta.

Acknowledgements—The work presented in this communication was supported by United States Public Health Service Research Grant No. NS-04699. Mr. G. I. Henderson was supported by a predoctoral fellowship from the United States Public Health Service (Training Grant No. GM-00058-12).

REFERENCES

1. C. A. Hebb, *Physiol. Rev.* **37**, 196 (1957).
2. K. N. Mehrotra, *J. Insect Physiol.* **6**, 215 (1961).
3. J. Schuberth, *Biochim. biophys. Acta* **122**, 470 (1966).
4. A. K. Prince, *Proc. natn. Acad. Sci. U.S.A.* **57**, 1117 (1967).
5. L. T. Potter, V. A. S. Glover and J. K. Saelens, *J. biol. Chem.* **243**, 3864 (1968).
6. F. de Balbian Verster, V. B. Haarsted and E. C. White, *Pharmacologist* **11**, 291 (1969).
7. H. L. White and C. J. Cavallito, *Biochim. biophys. Acta* **206**, 343 (1970).
8. W. W. Cleland, *Biochim. biophys. Acta* **67**, 104 (1963).
9. H. Theorell and B. Chance, *Acta chem. scand.* **5**, 1127 (1951).

10. H. R. MAHLER and E. G. CORDES, in *Biological Chemistry*, pp. 285–299. Harper & Row, New York (1971).
11. M. G. ORD and R. H. S. THOMPSON, *Nature, Lond.* **165,** 927 (1950).
12. S. I. ZACKS and G. B. WISLOCKI, *Proc. Soc. exp. Biol. Med.* **84,** 438 (1953).
13. G. BULL, C. HEBB and D. RATKOVIC, *Nature, Lond.* **190,** 1202 (1961).
14. D. MORRIS, *Biochem. J.* **98,** 754 (1966).
15. R. M. C. DAWSON, D. C. ELLIOT, W. H. ELLIOT and K. M. JONES, *Data for Biochemical Research*, p. 200. Oxford University Press, London (1969).
16. V. A. S. GLOVER and L. T. POTTER, *J. Neurochem.* **18,** 571 (1971).
17. W. W. CLELAND, in *The Enzymes—Kinetics and Mechanism* (Ed. P. D. BOYER), Vol. 11, pp. 1–61. Academic Press, New York (1970).
18. I. H. SEGEL, *Biochemical Calculations*, pp. 366–371. John Wiley, New York (1968).
19. D. MORRIS and D. S. GREWAAL, *Biochem. J.* **114,** p 85 (1969).
20. H. C. CHANG, *Chin. J. Physiol.* **13,** 145 (1938).
21. I. C. WEN, H. C. CHANG and A. WONG, *Chin. J. Physiol.* **10,** 559 (1936).
22. A. A. KAITA and A. M. GOLDBERG, *J. Neurochem.* **16,** 1185 (1969).