ON THE MECHANISM OF SYNTHESIS OF ACETYL CHOLINE

II. THE SYNTHESIS OF CITRATE BY BRAIN ENZYMES*

by

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LIPTON AND BARRON[1] reported that a water soluble preparation from acetone-dried brain powder prepared according to NACHMANSOHN AND JOHN[2] was able to synthesize acetyl choline in the presence of citric acid. With semicarbazide as a carbonyl trapping agent, they demonstrated that a keto-acid was formed along with acetyl choline. It was then postulated that synthesis of acetyl choline under those conditions occurred in two steps: I. citrate $\rightleftharpoons$ oxaloacetate + "active" acetate. II. "active" acetate + choline $\rightleftharpoons$ acetyl choline. We present evidence in this paper that the two reactions are catalysed by two different enzymes: citrogenase, which performs reaction I, and choline acetylase, the enzyme discovered by NACHMANSOHN AND MACHADO[3] which performs reaction II. Furthermore, the keto-acid formed during the synthesis of acetyl choline has been identified as oxalacetate.

METHODS

The preparation used in these experiments was a calcium-free RINGER-phosphate (pH 7.4) extract of an acetone-powder of rabbit brain centrifuged at 12 000 r.p.m. for one hour, which will be called brain enzymes. 1 ml of this solution contains 20 mg dry weight, of which 3.62 mg is protein and is equivalent to 100 mg of the acetone powder. The yeast juice was prepared by heating 1 g of ANHEUSER-BUSCH dried beer yeast in 4 ml of water for 10 minutes at 90°; the mixture was cooled, centrifuged, and the supernatant neutralized, and diluted to 2.5 ml. In general the mixture for the synthesis of acetyl choline consisted of: 0.1 ml. 0.9 *M* KCl; 0.1 ml 0.6 *M* NaF; 0.1 ml 0.06 *M* eserine salicylate; 0.1 ml 0.015 *M* choline; 0.2 ml 0.045 *M* adenosine triphosphate (ATP); 0.3 ml of yeast juice; 0.1 ml 0.6 *M* sodium citrate; 1 ml of enzyme solution; and enough calcium-free RINGER-phosphate to give a final volume of 3 ml. The mixture was incubated for one hour at 37° in an evacuated THUNBERG tube unless otherwise stated. For oxalacetic acid determinations, 0.1 ml 0.6 *M* semicarbazide was added as the trapping agent. Whenever substances were added or deleted, the mixture volume was maintained at 3.0 ml by adjusting the RINGER-phosphate volume. The final pH of the solution was adjusted to 7.4. Acetyl choline was determined by the frog rectus method of CHANG AND GADDUM[4]; oxalacetate, by the method of FRIEDEMANN AND HAUGEN[5]; citrate, by the titration method of PUCHER[6]. ATP was determined as inorganic P after seven minutes in *N* HCl at 100°. Acetyl phosphate was determined according to LIPMANN AND TUTTLE[7]; inorganic P by the method of FISKE AND SUBBAROW[8]. ATP was prepared according to the method of DUBOIS *et al*[9]. The sample contained no acetate.

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Separation of the Two Steps in the Synthesis of Acetyl Choline

In order to differentiate the two reactions which take place during the synthesis of acetyl choline by these brain enzymes, the effect of the different components of the system on the formation of oxalacetate (reaction I) and of acetyl choline (reaction II) was studied. The components were withdrawn one by one and the extent of reactions I and II was compared with that obtained by the system containing all the components. Oxalacetate formation did not occur in the absence of yeast juice and eserine. Addition of ATP and K^+ increased oxalacetate formation by 93 and 88% respectively, while fluoride had no effect at all. The presence of fluoride was necessary for maximum activity of reaction II (Table I). The lack of stimulation of acetyl choline synthesis by addition of K^+ must be due to its presence in yeast juice. There was no oxalacetate formation in the absence of citrate.

TABLE I

ACETYL CHOLINE AND OXALACETATE FORMATION FROM BRAIN ENZYMES

Effect of the different components of the system: the figures give micromoles of substance formed per gram protein per hour.

Component	Oxalacetate		Acetyl choline	
	Without component	With all components	Without component	With all components
Yeast juice	1.0	67.8	1.8	13.5
ATP	14.9	65.1	5.5	13.5
Fluoride	63.0	64.0	5.4	13.5
Choline	48.4	67.2	7.1	13.5
Eserine	1.9	67.2	3.5	13.5
K^+	35.8	67.2	12.3	13.5
Enzyme	0.0	67.2	0.0	13.5

The Effect of Yeast Juice

An effort was made to elucidate the factors in yeast juice responsible for the striking stimulation of acetyl choline and oxalacetate formation. The juice was ashed and the inorganic residue was tested. Oxalacetate formation was increased by this residue, which was completely replaceable by Mg^{++}; the increase was greater when yeast juice was added to Mg^{++} (Table II). Mg^{++} alone, which was shown by FELDBERG AND HEBB[10] to

TABLE II

OXALACETATE FORMATION BY BRAIN ENZYMES

Effect of yeast juice and Mg^{++}. Figures give oxalacetate formation per g protein per hour.

Cofactor	Oxalacetate micromoles
None	13.6
0.3 ml yeast juice	33.7
Ash of 0.3 ml yeast juice	26.8
0.02 *M* $MgSO_4$	28.5
0.02 *M* $MgSO_4$ + ash of yeast juice	29.0
0.02 *M* $MgSO_4$ + 0.3 ml yeast juice	52.4

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stimulate synthesis of acetyl choline, did not replace yeast juice in its effect on this synthesis. The yeast juice factor may be similar to that reported by LIPMANN AND KAPLAN[11] as essential for the acetylation of sulfanilamide and choline. Undoubtedly Mg^{++} and some organic substances present in yeast are necessary for the decomposition of citrate into oxalacetate and acetate, and for the acetylation of choline.

The Role of Sulfhydryl Compounds

NACHMANSOHN and his coworkers[2, 3, 12] have given evidence that choline acetylase is a –SH enzyme. They reported that when cysteine was added to preparations of acetone-dried brain, not only is there an increase of acetyl choline synthesis but acetate becomes an effective precursor of acetyl choline. Addition of cysteine (0.02 *M*) to undialysed solutions of brain increased acetyl choline synthesis whether in the absence of citrate and acetate or in the presence of these substances. After 24 hours dialysis, the effect of cysteine was more marked. The cysteine effect seems to be due exclusively to reactivation of –SH groups present in the protein moiety of the acetylation enzyme, for similar increases were obtained with glutathione and with 2.3-dimercaptopropanol. 2.3-Dimercaptopropanol showed the best stimulating effect for the synthesis of acetyl choline (Table III).

TABLE III

EFFECT OF SULFHYDRYL COMPOUNDS ON ACETYL CHOLINE SYNTHESIS

Cysteine and glutathione, 0.02 *M*; 2.3-dimercaptopropanol, 0.01 *M*. Figures give micromoles acetyl choline formation per gram protein per hour.

Enzyme	Substrate	Acetyl choline formation			
		Control	Cysteine	Glutathione	Dimercaptopropanol
Undialysed					
	None	6.6	14.3	14.0	25.5
	Citrate	13.2	24.6	23.6	32.2
	Acetate	9.4	13.0	13.5	24.7
Dialysed					
	None	3.9	15.4		
	Citrate	6.6	27.5		
	Acetate	3.9	22.8		

Effect of Some Inhibitors on the Formation of Oxalacetate and of Acetyl Choline

Separation of individual enzymes taking part in a series of reactions can sometimes be effected with the help of inhibitors which attack some group essential for the activity of the enzyme and of inhibitors which prevent formation of the substrate—protein complex. Heating the enzyme solutions for half an hour at 50° inhibited oxalacetate formation by 84%, and acetyl choline formation completely. *p*-Chloromercuric benzoic acid (a sulfhydryl enzyme inhibitor) inhibited the synthesis of acetyl choline while it produced an increase in the formation of oxalacetate. Methyl-bis (β-chloroethyl) amine (nitrogen mustard) inhibited the synthesis of acetyl choline, but had no effect on the formation of oxalacetate. Finally, fluoroacetate had no effect on the formation of oxalacetate, and very little effect on the synthesis of acetyl choline (10% inhibition) (Table IV). It can be seen from these experiments that a separation of the two reactions,

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(1) formation of oxalacetate and acetate and (2) formation of acetyl choline, can be obtained with the aid of *p*-chloromercuric benzoic acid which stimulates the first reaction and inhibits the second; and with methyl-bis (β-chloroethyl) amine, which has no effect on oxalacetate formation and inhibits acetyl choline synthesis.

TABLE IV

EFFECT OF SOME INHIBITORS ON OXALACETATE AND ON ACETYL CHOLINE FORMATION

Figures give micromoles per gram protein per hour.

Inhibitor	Concentration *M*	Oxalacetic Acid	Inhibition Per cent	Acetyl Choline	Inhibition Per cent
None Heat, 50°, 30 min		13.6 2.1	84.5	34.5 0	complete
None pCl-Hg benzoic acid	0.0001	6.9 10.3	Increase	58.8 16.2	72
None pCl-Hg benzoic acid	0.001	10.5 24.3	Increase	43.5 1.2	97
Control Methyl-bis-(β-chloroethyl) amino	0.015	18.8 18.2	None	52 15.1	71
Control Fluoroacetic acid	0.01	13.6 13.0	None	34.5 30.8	10

Identification of the Keto-acid

The classic reaction postulated by LIPTON AND BARRON[1] was based on the formation of a semicarbazone of a keto-acid, which was not more fully identified. It is possible to demonstrate the identity of the keto-acid by the technique of FRIEDEMANN AND HAUGEN[8]. On determining the light absorption at 4200 Å and 5200 Å they found that the alkaline hydrazones of different keto-acids gave ratios $\frac{E_{4200\text{ Å}}}{E_{5200\text{ Å}}}$ which could be used not only to identify them but also to determine the relative quantity of each. The ratios of the light absorption of the 2.4-dinitrophenylhydrazone at 4200 and 5200 Å differentiates α-ketoglutaric acid from oxalacetic and pyruvic acids. Oxalacetic acid is distinguished from pyruvic acid by the fact that less colour development occurs when its hydrazone is extracted with toluene than when it is extracted with ethyl acetate. By applying these two tests to the yeast juice and to the products of the enzyme reaction mixture it was found that oxalacetic acid was formed by the enzyme system and that pyruvic acid was present in yeast juice (Table V).

LIPMANN AND TUTTLE[7] have used hydroxylamine as a trapping agent for acetyl phosphate, with which it forms a hydroxamic acid. The brain enzymes gave no evidence of acetyl phosphate formation in the presence of this reagent.

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TABLE V

THE NATURE OF THE KETO-ACID FORMED

Material extracted	$\frac{E_{4200\ Å}}{E_{5200\ Å}}$	$\left(\frac{E_{Ethyl\ acetate}}{E_{Toluene}}\right)$ 4200 Å
α-Keto-glutaric acid	2.00	0.37
Oxalacetic acid	1.29	0.20
Pyruvic acid	1.29	1.15
1.0 ml yeast juice	1.31	1.08*
3.0 ml enzyme system	1.30	0.30

* Contains 0.15 micromole pyruvic acid.

Citrate Synthesis by Brain Enzymes

Further evidence of the validity of equation I was obtained by studying the reaction from right to left, *i.e.*, citric acid synthesis by undialysed and dialysed enzyme solutions. The formation of citric acid by this enzyme with oxalacetate alone was nil. On addition of yeast there was definite synthesis. Oxalacetate and acetate or acetyl phosphate produced only small amounts in the absence of yeast and Mg^{++}. The largest amounts of citric acid were obtained with oxalacetate, acetate, yeast, Mg^{++}, and ATP. Replacement of acetate by acetyl phosphate did not increase citrate synthesis (Table VI).

TABLE VI

SYNTHESIS OF CITRIC ACID BY BRAIN ENZYMES

All experiments contained 0.02 *M* oxalacetate. Na acetate, acetyl phosphate, $MgSO_4$ (Mg^{++}), 0.02 *M*; adenosine triphosphate, 0.003 *M*. Figures give citric acid formation per gram protein per hour.

Additions	Citric acid synthesis	
	Undialyzed enzyme micromoles	Dialyzed micromoles
Yeast	148	178
Acetate	22.6	
Acetate + Mg^{++}	—	43
Acetate + yeast	120	—
Acetate + yeast + Mg^{++}	—	195
Acetate + yeast + Mg^{++} + ATP	—	287
Acetyl Phosphate	29.7	
Acetyl Phosphate + yeast	92	
Acetyl Phosphate + Mg^{++}	56	59
Acetyl Phosphate + Mg^{++} + yeast	148	182
Acetyl Phosphate + Mg^{++} + yeast + ATP	—	215

The striking difference that dialysis has on the activity of acetyl choline synthesis and citric acid synthesis must be pointed out. Dialysis diminished the rate of acetyl choline synthesis, due probably to slow oxidation of the –SH groups of choline acetylase; it increased the synthesis of citric acid due either to the presence of a dialysable inhibitor in the enzyme system or to the oxidation of the –SH groups. Studies on the properties of this enzyme and its purification are being continued.

Whether citric acid is the first product of the condensation reaction or is produced subsequent to the formation of a citrate precursor is not known. The brain enzymes

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contained aconitase, which could be readily measured, as seen in the experiments in Table VII.

TABLE VII

ACONITASE IN BRAIN ENZYMES

2.5 ml enzyme (9 mg protein) + 2.5 ml. 0.1 *M* phosphate, pH 7.4 + 2.5 ml water + 2.5 ml *cis*-aconitate (containing 110 mgs *cis*-aconitic acid) 37.5°. 3 ml samples were withdrawn and were precipitated with 2 cc. of 20 per cent CCl_3COOH.

Time of Incubation minutes	Citric Acid micromoles
0	0.68
30	7.96
60	11.71

Acetyl Phosphate and the Synthesis of Acetyl Choline

Since the discovery by LIPMANN[13] that acetyl phosphate was found in certain bacteria during the oxidation of pyruvic acid, there has been a tendency to extrapolate this finding to other cells. The repeated failures to demonstrate acetyl phosphate in animal tissues has been attributed to the presence of a phosphatase able to hydrolyse acetyl phosphate rapidly[14]. The water-soluble preparation from acetone-dried rabbit brain contained this enzyme. The rate of hydrolysis at 38°, with 0.015 *M* acetyl phosphate, was such that half-reaction occurred in 22.5 min. The presence of 0.02 *M* fluoride had no influence on this reaction. The water soluble rabbit brain enzymes did not hydrolyse adenosine triphosphate appreciably. The enzymes extracted from dog brain contained both acetyl phosphatase and ATP phosphatases (Table VIII).

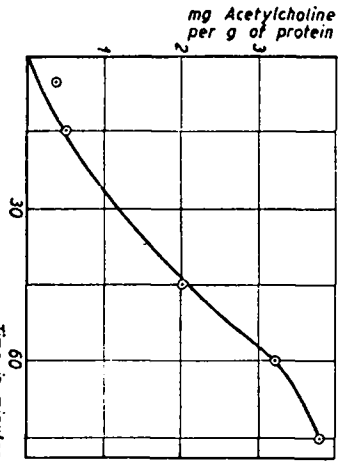

Fig. 1. Acetyl Choline Synthesis by the Brain Preparation. Abscissa, time in minutes. Ordinate, mg of acetyl choline bromide per gram of protein.

The rate of acetyl choline synthesis under the conditions described previously is shown in Fig. 1. There was formation of 3.35 mg of acetyl choline (measured as the bromide salt) per hour per gram of protein. It can be seen from this curve that readily measurable acetyl choline formation occurred in

TABLE VIII

HYDROLYSIS OF ACETYL PHOSPHATE AND OF ATP BY WATER-SOLUBLE PREPARATIONS OF ACETONE-DRIED BRAIN POWDER

Buffer, phosphate, 0.05 *M*, pH, 7.4. Acetyl phosphate concentration, 0.015 *M*; ATP, 0.003 *M*. Temp. 38°. Duration of experiments, 30 min. Figures give millimoles P per g protein.

Species	Acetyl phosphate Disappearance	Inorganic phosphorus Appearance	ATP labile P. Disappearance
Rabbit	4.3	5.3	0.8
Dog	1.2	2.5	1.3

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22 min. Tests were performed with citrate and acetyl phosphate as substrates for acetylation, and acetyl choline was determined 10 and 20 min after incubation. Acetyl phosphate did not increase acetyl choline formation. Furthermore, there was absolutely no relation between the hydrolysis of acetyl phosphate and synthesis of acetyl choline. It must, however, be stated that there are other sources of "active" acetate for acetylation of choline.

SUMMARY

The experiments presented in this paper give further evidence for the validity of LIPTON AND BARRON's contention that in the synthesis of acetyl choline by water soluble preparations from acetone-dried rabbit brain in the presence of citrate, yeast juice, choline, Mg^{++}, K^{+}, and ATP there are two distinct enzymatic reactions:

I: citrate $\rightleftharpoons$ oxalacetate + "active" acetate;
II: "active" acetate + choline $\rightleftharpoons$ acetyl choline

The identity of oxalacetate was demonstrated by the use of the technique of FRIEDEMANN AND HAUGEN. Furthermore, the reversibility of reaction I was shown by the synthesis of citric acid, which occurred in the presence of enzyme, oxalacetate, acetate, yeast, Mg^{++}, and ATP. It may be concluded from these experiments that the enzyme which catalyses reaction I is citrogenase. Mg^{++} is probably the prosthetic group of the protein. The role of yeast and of ATP is under investigation.

Separation of these two reactions was obtained with fluoride, *p*-chloro-mercuric benzoic acid and methyl-bis (*β*-chloroethyl) amine. Fluoride, necessary for the synthesis of acetyl choline had no effect on the formation of oxalacetate from citrate. *p*-Chloromercuric benzoic acid and methyl-bis (*β*-chloroethyl) amine inhibited the synthesis of acetyl choline while they had no effect on the formation of oxalacetate.

The water soluble enzymes from rabbit brain contained also aconitase and phosphatase. This brain phosphatase hydrolysed acetyl phosphate rapidly in the presence of fluoride. It did not hydrolyse adenosine triphosphate appreciably. The enzymes from dog brain contained both, acetyl phosphate and adenosine triphosphate phosphatases.

RÉSUMÉ

Les expériences présentées dans ce mémoire fournissent une preuve supplémentaire pour l'hypothèse de LIPTON ET BARRON. D'après ces auteurs il y aurait deux réactions enzymatiques distinctes dans la synthèse de l'acétylcholine à partir de préparations solubles dans l'eau de cerveau de lapin desseché à l'acétone et en présence de citrate, d'extrait de levure, de Mg^{++}, de K^{+} et d'ATP. C'est à dire:

I. Citrate $\rightleftharpoons$ oxalacétate + acetate "actif";
II. Acétate "actif" + choline $\rightleftharpoons$ acétylcholine.

L'oxalacétate a été identifié par le technique de FRIEDEMANN ET HAUGEN. De plus, la réversibilité de la réaction I a été démontrée par la synthèse de l'acide citrique, synthèse qui avait lieu en présence d'enzyme, d'oxalacétate, d'acétate, de levure, de Mg^{++} et d'ATP. L'on peut conclure de ces expériences que l'enzyme qui catalyse la réaction I est de la citrogénase, Mg^{++} représente propabablement le groupe prosthétique de la protéine. Le rôle de la levure et de l'ATP est examiné.

Nous avons pu séparer ces deux réactions par le fluorure, le mercure II et la méthyl-bis-(*β*-chloroéthyl) amine. Le fluorure qui est nécessaire pour la synthèse de l'acétylcholine, n'avait pas d'effet sur la formation d'oxalacétate à partir de citrate. Les deux autres réactifs empêchaient la synthèse de l'acétylcholine et n'avaient pas d'effet non plus sur la formation d'oxalacétate.

Les enzymes solubles dans l'eau du cerveau de lapin contenaient aussi de l'aconitase et de la phosphatase. Cette phosphatase de cerveau hydrolysait rapidement l'acétylphosphate en présence de fluorure. Elle n'hydrolysait pas l'adénosine triphosphate de façon appréciable. Les enzymes du cerceau de chien contenaient aussi bien des phosphatases d'acétylphosphate que d'ATP.

ZUSAMMENFASSUNG

Die hier dargelegten Versuchsergebnisse erbringen einen weiteren Beweis für die Gültigkeit der Behauptung von LIPTON UND BARRON, wonach bei der Acetyl-Cholinsynthese aus wasserlöslichen

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Präparaten von mit Aceton getrockneten Kaninchenhirn in Gegenwart von Citrat, Hefesaft, Cholin, Mg^{++}, K^{+} und ATP, zwei verschiedene enzymatische Reaktionen stattfinden.

I. Citrat $\rightleftharpoons$ Oxalacetat + "aktives" Acetat;
II. "aktives" Acetat + Cholin $\rightleftharpoons$ Acetylcholin.

Das Oxalacetat wurde nach der Arbeitstechnik von FRIEDEMANN UND HAUGEN identifiziert.

Weiters wurde die Umkehrbarkeit der Reaktion I durch die in Gegenwart von Enzym, Oxalacetat, Acetat, Hefe, Mg^{++} und ATP stattfindende Synthese der Zitronensäure nachgewiesen. Aus diesen Versuchen kann geschlossen werden, dass das Enzym, welches Reaktion I katalysiert, Citrogenase ist. Mg^{++} ist wahrscheinlich die prosthetische Gruppe des Eiweisstoffes. Die Rolle, welche Hefe und ATP spielen wird untersucht.

Eine Trennung dieser beiden Reaktionen wurde mit Fluorid, Quecksilber (II)-*p*-chlorobenzoat und Methyl-bis-(β-chloroäthyl)-amin erreicht. Fluorid, welches fur die Acetylcholinsynthese notwendig ist, hatte keine Wirkung auf die Bildung von Oxalacetat aus Citrat. Die beiden anderen Reagentien hemmten die Acetylcholinsynthese und hatten ebenfalls keine Wirkung auf die Oxalacetatbildung.

Die wasserlöslichen Enzyme aus Kaninchenhirn enthielten ebenfalls Aconitase und Phosphatase. Diese Gehirnphosphatase hydrolysiert rasch Acetylphosphat in Gegenwart von Fluorid. Adenosintriphosphat wurde dagegen nicht wesentlich hydrolysiert. Das Enzym aus Hundehirn enthielt sowohl Acetylphosphat — wie Adenosintriphosphatphosphatasen.

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