J. Physiol. (1956) 132, 343–357

MECHANISMS OF ACETYLCHOLINE SYNTHESIS IN THE BLOWFLY

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(*Received* 2 *November* 1955)

The organophosphorous insecticides are strong anticholinesterases, and their insecticidal action is attributed to this property. This hypothesis presupposes the presence in insects of acetylcholine (ACh) and the enzyme systems for its hydrolysis and synthesis, that is, cholinesterase and choline acetylase. Cholinesterase has been demonstrated in the nervous tissue of insects (Richards & Cutcomp, 1945), and recent evidence has established the presence of ACh also (Lewis, 1953; Augustinsson, 1954; Chefurka & Smallman, 1955). However, there is no comparable evidence for the remaining component, choline acetylase, and the enzymes associated with it in the synthesis of ACh.

The present study provides evidence for the presence of choline acetylase, coupled with enzymic acetyl donor systems, in an insect. This evidence completes the basis for the analogy, between insects and mammals, of the lethal effects of cholinesterase inhibitors; for ACh, cholinesterase and choline acetylase are now known to occur in insects.

METHODS

Larvae of the blowfly, *Lucilia sericata*, were reared to pupation on minced liver, and the adults provided with water, sucrose and liver until they were killed at 10–12 days after emergence. Liver was withheld overnight before the flies were killed.

Acetone-dried powders were prepared from the heads. The flies were immobilized in ice, decapitated, and the heads ground in a cold mortar in Analar acetone at $-4°$ C. The volume of acetone used was 50 to 100 times the volume of tissue. It was found possible to separate most of the cuticle from the acetone powder at this stage, because during grinding the cuticle was broken up into relatively large pieces which accumulated on the bottom of the mortar, so that by decanting and repeated washing with fresh acetone, most of the cuticle remained in the mortar. The acetone suspension was filtered in the cold, and the resultant powder was stored over P_2O_5 in an evacuated desiccator at 4° C.

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The powders were extracted with a cysteine-saline solution immediately before use. L-Cysteine hydrochloride to give 6 mg/ml. was added to 0·9% NaCl and quickly neutralized with N/3 NaOH. To this solution the powder was added immediately (10 mg/ml.) and the extraction carried out by stirring in an ice-packed centrifuge tube for 15 min. The preparation was then centrifuged at 3000 rev/min for 10 min, the supernatant fluid decanted, and aliquots added to the incubation flasks containing the other reagents. In some experiments the extraction was carried out in the presence of an anion-exchange resin to remove endogenous CoA and anions such as acetate and citrate (Balfour & Hebb, 1952). The resin, 'De-Acidite E', was prepared by treatment with 10% NaOH followed by 10% HCl and then washed exhaustively with de-ionized water until the pH of the washings was about 4. Freshly prepared cysteine-saline solution was mixed with approximately half its volume of resin, the solution was neutralized with N/3 NaOH, the powder added and the extraction carried out as before.

The extracts were incubated in small flasks shaken in a Warburg respirometer bath at 37° C. Unless otherwise stated, each flask contained in a total volume of 4·7 ml.: extract of 25 mg acetone-dried powder; cysteine, 15 mg; NaF, 2 mg; KCl, 6 mg; $MgCl_2$, 4 mg; eserine sulphate, 0·5 mg; Sørensen's phosphate buffer (pH 7), 0·3 ml.; choline chloride, 3 mg; sodium acetate 0·01 M (3·8 mg) or sodium citrate 0·01 M (16·4 mg); ATP (disodium salt), 4 mg; co-enzyme A (CoA) (Pabst, 75% pure), 100 μg, equivalent to 30 Lipmann units.

At the end of the incubation period, the reaction was stopped by addition of 0·5 ml. of N/3 HCl, the incubate was boiled momentarily, and stored at 4° C until assay. The ACh content of incubates was assayed on the eserinized frog's rectus abdominis muscle, correcting for the presence of sensitizing substances (Feldberg, 1950). All values refer to ACh chloride.

RESULTS

Table 1 shows that the blowfly head contains the enzyme system necessary for the synthesis of ACh. The insect preparation produced ACh at a rate several times greater than the rate of synthesis obtained with an acetone powder of guinea-pig brain, incubated under the same conditions. Moreover, with the insect tissue acetate was more effective than citrate as acetyl donor, whereas with the guinea-pig brain they were equally effective.

TABLE 1. Amounts of ACh synthesized (μg/g powder/hr)

	Acetyl donor	
Source	Citrate	Acetate
Blowfly heads	2400	4000
Guinea-pig brain	920	920

To establish the identity with ACh of the active substance produced by the blowfly preparation, incubates prepared without eserine were assayed simultaneously on the frog's rectus abdominis and the guinea-pig's ileum. Using ACh as standard, the estimates obtained with the two test materials agreed within 10% with citrate, and 4% with acetate. These differences are within the experimental error of the assays, indicating that the pharmacologically active substance produced by the blowfly preparation is identical with ACh.

The requirement for coenzyme A, choline and acetyl donor. Table 2 summarizes the results which show that the synthesizing system in the blowfly head requires CoA, choline and an acetyl donor. Balfour & Hebb (1952) used an

anion exchange resin to remove endogenous CoA from acetone powders of mammalian brain and were able to show by this means that CoA was essential for synthesis by the mammalian preparation. Similarly, an acetone powder of blowfly heads extracted in the presence of the resin failed to synthesize ACh in the absence of CoA, as shown in the first line of Table 2. When CoA was added to such resin-treated extracts, the ability to synthesize ACh was restored and the rate depended on whether acetate or citrate was present also (line 2 of Table 2). Extracts of powders not treated with resin synthesized some ACh without the addition of CoA, and this must be taken as evidence that these powders contain the coenzyme endogenously; the amounts present, however,

TABLE 2. Amounts of ACh synthesized in the presence of CoA, choline and acetyl donor (μg/g powder/hr)

Conditions	Acetyl donor		
	None	Citrate	Acetate
Resin treated powder			
No CoA	—	Nil	Nil
CoA present	340	1450	3600
Untreated powder			
No CoA	—	450	320
No choline	60	—	180
CoA and choline present	600	2250	3400

were insufficient to support a high rate of synthesis (line 3 of Table 2). Relatively small amounts of ACh were synthesized also in the absence of added choline or an acetyl donor, indicating that these substances are also present endogenously. The table shows finally that high rates of synthesis were obtained only when the extracts were fortified with CoA, choline, and an acetyl donor.

The effect of pH

The effect of pH was examined, (*a*) on the extraction of the enzyme system, and (*b*) on its activity during incubation.

Effect of pH *on extraction.* Two portions of an acetone powder of blowfly heads were extracted in cysteine-saline solution, one at pH 5·4 and the other at pH 7·2. After centrifuging, the supernatant fluids were decanted and the residues were both extracted again in cysteine-saline solution at pH 7·2. Each of the four extracts was incubated, either with citrate or with acetate. The pH of all the incubation mixtures was adjusted to 7·2 immediately before beginning the incubation.

The results are given in Table 3, and show that the amounts of ACh synthesized were approximately the same, irrespective of whether the first extraction was carried out at pH 5·4 or 7·2, and this was true with both citrate and acetate. The second extraction at pH 7·2 yielded extracts with low synthesizing activity, which remained the same whether the first extraction

was made at pH 5·4 or 7·2. Thus, in the range from pH 5·4 to 7·2, the enzymes necessary for synthesis were equally and efficiently extracted.

Effect of pH *during incubation.* Aliquots of an extract of blowfly heads were added to incubation mixtures, complete except for the omission of CoA, and the pH of the mixtures adjusted to the desired values between pH 5·6 and 8·0. The CoA, which had been withheld to prevent synthesis during the pH adjustment, was then added and the mixtures incubated as usual.

TABLE 3. Effect of pH of extraction on synthesis of ACh (μg/g/hr)

Extraction	pH	Citrate	Acetate
1st	5·4	2800	4200
2nd	7·2	130	150
1st	7·2	2700	4500
2nd	7·2	120	150

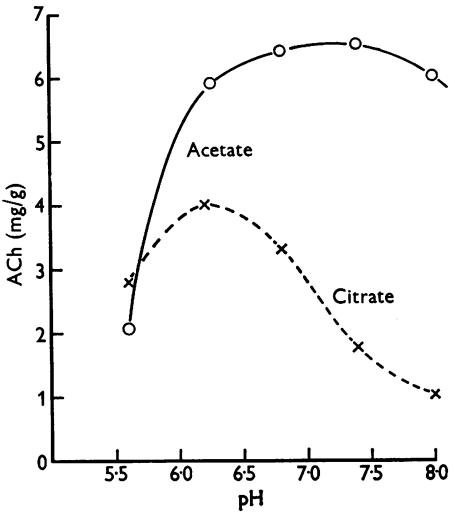

Fig. 1. Effect of pH of the incubation mixture on rate of synthesis of ACh.

The results are shown in Fig. 1. Both with acetate and with citrate, the amount of ACh synthesized was dependent on pH. However, the pH had different effects on the relative activities of the acetate-utilizing system and of the system utilizing citrate. At pH 5·6 the synthesis with acetate was somewhat less than with citrate, whereas at higher pH the synthesis with acetate was always greater, and at pH 8 it was about six times greater than with citrate. Moreover, the pH optima of the two systems were different: with citrate the optimum was at pH 6·2–6·4, and with acetate it extended from about pH 7·0 to 7·5. Thus, with acetate as acetyl donor, the synthesis of ACh by the blowfly preparation was most effective in a neutral or slightly alkaline medium; with citrate as acetyl donor, synthesis was optimal in an acid medium.

The effect of coenzyme A concentration

Incubation mixtures were made up to contain various concentrations of CoA from 0 to 48 Lipmann units/ml. Before adding the CoA the pH of the incubates was adjusted to 6·6 because of the finding that at this pH the synthesis was nearly optimal with either acetate or citrate.

The relation between CoA concentration and synthesizing activity is illustrated in Fig. 2, which shows that with either acetate or citrate, the amount of ACh synthesized is dependent on the CoA concentration. In the absence of added CoA small amounts of ACh were synthesized, owing to the presence of

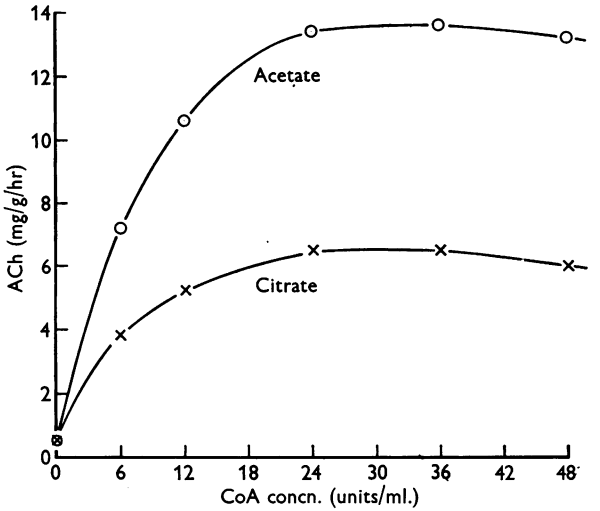

Fig. 2. Effect of CoA concentration on rate of synthesis of ACh.

endogenous CoA; acetate and citrate were equally effective. With the addition of low concentrations of CoA, the synthesis with both acetate and citrate was accelerated, and this acceleration continued until the concentration of CoA reached 36 units per ml. A further increase in the concentration of CoA resulted in some inhibition with acetate as well as with citrate.

Comparison of the two curves in Fig. 2 shows that the synthesis with acetate increased more rapidly than that with citrate as the CoA concentration increased. For instance, at a CoA concentration of 6 units per ml., 3·8 mg ACh/g powder/hr was synthesized with citrate, and 7·2 mg with acetate; the corresponding values at 36 units CoA per ml. were 6·5 and 13·6 mg. Similar results were obtained in another experiment in which the synthesis of ACh (mg/g/hr) with citrate and acetate respectively was: 0·32 and 0·45 without added CoA; 2·9 and 6·7 at 6 units CoA per ml.; 4·2 and 10·6 at 12 units CoA per ml.

The rate-limiting effect of acetyl coenzyme A formation

CoA itself does not enter directly into the reaction producing ACh; it is first acetylated, and in this form serves as the source of acetyl groups which are then transferred to choline by the terminal enzyme, choline acetylase (Korkes, del Campillo, Korey, Stern, Nachmansohn & Ochoa, 1952). The findings that the rate of synthesis with the blowfly preparation depended on the CoA concentration, and that at all CoA concentrations used acetate was more effective than citrate, suggest that the synthesis is limited by the formation of acetyl CoA, and that the acetylation of CoA proceeds more readily with acetate than with citrate.

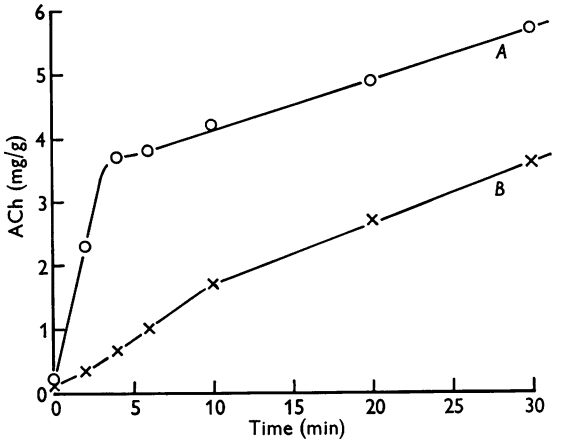

Fig. 3. The rate-limiting effect of acetyl CoA formation with acetate as acetyl donor. Curve *A*: the rate of ACh production after a 30 min induction period with all components present except choline; choline added at zero time. Curve *B*: the same, except that CoA and choline were withheld during the induction period and added together at zero time.

To test whether the formation of acetyl CoA was indeed limiting on the synthesis of acetylcholine, the formation of acetyl CoA was separated from the acetylation of choline by a preliminary incubation period without choline. Extracts with all components present except choline were incubated for 30 min. At the end of this period choline was added, and during the next 30 min samples were withdrawn at short intervals and assayed for ACh. For comparison, an aliquot of the same extract was incubated in the same way except that CoA was also withheld during the first 30 min and then added together with the choline. CoA was provided at the optimum concentration of 30 units/ml. and the pH of all incubates was adjusted to 6·6.

Acetyl coenzyme A formation with acetate. A comparison of the curves *A* and *B* of Fig. 3 shows that with acetate as acetyl donor, the formation of acetyl CoA is limiting on the synthesis of ACh. When acetyl CoA was allowed

to form and accumulate during the 30 min induction period, 2·1 mg/g ACh was produced in the first 2 min after addition of choline (curve *A*), whereas only 0·23 mg/g ACh was formed when CoA and choline were added together after the induction period without CoA, so that no pre-formed acetyl CoA was available at zero time (curve *B*). Curve *A* shows that with pre-formed acetyl CoA available, the initial rate of formation of ACh was rapid, but that after about 4 min it slowed abruptly, presumably because the acetyl CoA accumulated during the induction period had been exhausted. Thereafter the synthesis of ACh appeared to be dependent on the coincident formation of acetyl CoA as shown by the nearly identical rate of synthesis when no pre-formed acetyl CoA was available (curve *B*). The vertical distance between the parallel portions of the two curves from 10 to 30 min provides an estimate of the amount of acetyl CoA formed during the induction period, and indicates that acetyl CoA equivalent to about 2·2 mg/g ACh was formed from CoA and acetate during the 30 min period before the addition of choline.

In a similar experiment in which the CoA concentration was only 6 units per ml. instead of 30, the two curves conformed to the same pattern but the separation between their parallel portions was less and indicated that acetyl CoA equivalent to 0·55 mg/g ACh was formed during the induction period. Thus a five-fold increase in CoA concentration, from 6 to 30 units/ml., resulted in a four-fold increase in the amount of acetyl CoA available to the choline acetylase at the end of the induction period. The concentration of CoA therefore affects the rate of synthesis of ACh by its effect on the formation of acetyl CoA, and thus accounts for the increase in synthesis with increasing concentrations of CoA as illustrated in Fig. 2.

Acetyl coenzyme formation with citrate. Fig. 4 shows the results obtained with citrate as acetyl donor. When CoA (30 units/ml.) was present during the 30 min induction period (curve *A*), the initial rate of ACh synthesis was greater than when CoA was withheld during this period and added together with choline at zero time (curve *B*). Thus the production of ACh followed the same pattern as with acetate. However, the amount of acetyl CoA formed during the induction period was much less than in the corresponding experiment with acetate (Fig. 3) as shown by the lower initial rate of ACh production and the small separation between parallel portions of the curves *A* and *B*. Using this separation to estimate the amount of acetyl CoA formed during the induction period, acetyl CoA equivalent to about 0·3 mg/g ACh was formed with citrate, as compared to 2·2 mg/g with acetate.

These estimates of the formation of acetyl CoA may be used to calculate the proportion of the added CoA which was acetylated during the induction period. This has been done in Table 4, using the data from the three experiments described in this section.

In the first two columns of Table 4, the amounts of CoA and the difference

in ACh production with and without pre-formed acetyl CoA are given in μg per incubate containing 25 mg acetone powder. From the amounts of CoA used, the theoretical yield of acetyl CoA, if all the CoA (mol. wt. 767) were acetylated, is calculated as micromoles and given in column 3. In column 4, the difference in ACh production is converted to micromoles to give an estimate of the microequivalents of pre-formed acetyl CoA actually available after the induction period. From a comparison of columns 3 and 4, an estimate of the percentage of CoA which was acetylated during the induction period is obtained.

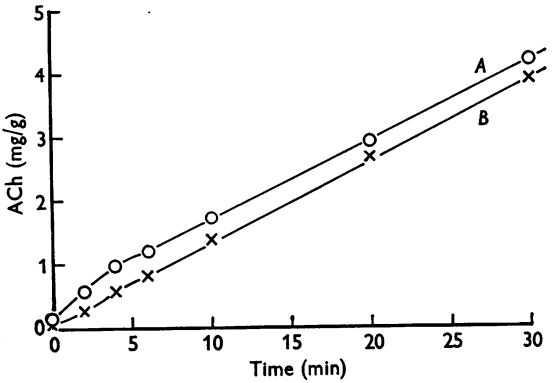

Fig. 4. The rate-limiting effect of acetyl CoA formation with citrate as acetyl donor. Details for curves *A* and *B* are the same as in Fig. 3.

TABLE 4. Amounts of acetyl CoA formed with acetate and citrate

Acetyl donor	CoA* (μg/25 mg powder)	ΔACh† (μg/25 mg powder)	Acetyl CoA (μmole) Calculated for full acetylation of CoA	Acetyl CoA (μmole) Estimated from ΔACh	Percentage of CoA acetylated
Acetate	75	13·7	0·09	0·07	78
Acetate	375	55·0	0·49	0·30	61
Citrate	375	7·5	0·49	0·04	8

* Pabst CoA corrected for 25% impurities.

† The difference in ACh production with and without pre-formed acetyl CoA, e.g. the difference between the parallel portions of curves *A* and *B* in Fig. 3.

The results show that the estimated formation of acetyl CoA with acetate corresponds to the acetylation of 78% of the available CoA at the lower concentration, and 61% at the higher concentration of CoA. With citrate, however, only 8% of the CoA appears to have been available in the acetylated form at the end of the induction period. Thus, the formation of acetyl CoA with citrate as substrate proceeds less readily than with acetate, and this difference may well account for the lower rates of ACh synthesis obtained when the blowfly preparation was incubated with citrate as acetyl donor.

The activity of choline acetylase and the presence of two enzymes for the acetylation of coenzyme A

With a supply of acetyl CoA, accumulated during the induction period, the initial rate of ACh synthesis after the addition of choline should provide a measure of the activity of choline acetylase independent of the formation of acetyl CoA. Such a measure is in fact available from Fig. 3 (curve *A*), which shows that 2·1 mg ACh/g powder were synthesized in the initial 2 min of the reaction, indicating a rate of about 1 mg/g/min. However, since the rate decreased to 0·7 mg/g/min during the subsequent 2 min, some decline may have occurred already during the initial 2 min and the optimal rate may be higher than 1 mg/g/min. It seemed necessary, therefore, to measure the rate of synthesis at intervals shorter than 2 min, and this has been done in the following experiment.

Aliquots of an extract were incubated with all components present except choline which was added after 30 min. One sample was withdrawn immediately before choline was added at zero time: after this samples were withdrawn at 1 min intervals during the subsequent 4 min.

Fig. 5, curve *A*, shows that with acetate 1·3 mg ACh/g powder was synthesized in the first minute of the reaction, after allowing for the small amount present at zero time. During the same period, only 0·3 mg/g was synthesized with citrate (curve *B*). The choline acetylase was therefore capable of synthesis at the rate of 1·3 mg/g/min. This rate may still be less than optimal because it was not sustained after the first minute.

Since both acetate and citrate can act as substrates for the production of acetyl CoA, it seemed possible that two different enzymes for the acetylation of CoA are present. In that case, the provision of both acetate and citrate together should further increase the rate of ACh synthesis, provided that the choline acetylase can still accept the additional acetyl CoA. An increase was in fact found, for when in the experiment illustrated in Fig. 5, an aliquot of the same extract was provided with both acetate and citrate, 1·7 mg/g was synthesized during the first minute (curve *C*). Furthermore, this rate was the sum of the rates with acetate and citrate provided separately (acetate alone, 1·3; citrate alone, 0·3; acetate and citrate, 1·7). This additive synthesis was maintained throughout the period of the experiment. For instance, at 4 min after the start of the reaction the values were: 3·1 mg with acetate, 0·8 mg with citrate, and 4·2 mg with acetate and citrate together. This additive effect and the absence of interaction between acetate and citrate provide evidence that the acetate and citrate utilizing systems are separate enzyme systems providing acetyl CoA for the choline acetylase.

Further evidence for the independence of the acetate and citrate systems is apparent from Fig. 5 which shows that, after an induction period with acetate,

the rate of synthesis remains about the same whether or not citrate is added at the beginning of the reaction (points plotted on curve *A*).

The three curves *A*, *B* and *C* in Fig. 5, all decrease in slope with time, indicating that the choline acetylase is not fully saturated with respect to acetyl CoA. Thus, when both the citrate and acetate systems are operative, the additional CoA made available is accepted and results in an equivalent increase in ACh synthesis. The resultant initial rate indicated in curve *C* provides the best estimate of the activity of the choline acetylase from blowfly heads, and shows that the enzyme is capable of synthesizing ACh at the rate of at least 100 mg/g powder/hr.

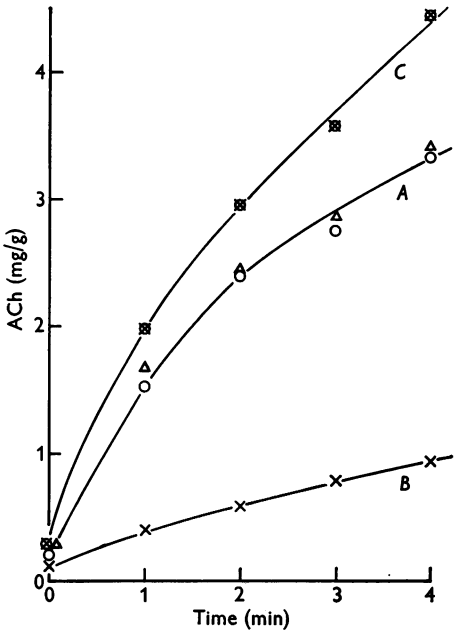

Fig. 5. The activity of choline acetylase, and the additive effect of acetate and citrate for the formation of acetyl CoA. The three curves show the rate of ACh synthesis when choline was added to incubates at zero time after a 30 min induction period during which the acetyl donor was: acetate (curve *A*, circles); citrate (curve *B*); acetate and citrate (curve *C*); acetate during the induction period, and citrate added with choline at zero time (curve *A*, triangles). In each case the concentration of acetate or citrate was 0·01 M.

DISCUSSION

Despite the recent interest in ACh and cholinesterase in insects, little attention has been given to the system required for the synthesis of ACh. Tobias, Kollros & Savit (1946), when studying the mode of action of DDT, reported an experiment in which homogenates of the nerve cords of cockroaches produced 47 μg ACh/g tissue when incubated anaerobically for 30 min with choline,

ATP, and yeast juice; an acetyl donor was apparently not added. Lewis (1953) found that the ACh content of blowfly homogenates increased from 7·5 μg/g tissue to 35 μg/g during 5 hr incubation with eserine but without other additions. Until now these findings provided the only evidence for the ACh synthesizing system in insects, but they afforded little basis for evaluating its activity or defining its components and mechanisms.

In the present experiments the ACh synthesizing system has been demonstrated in the head of the blowfly and shown to be highly active. The essential components of the system are choline acetylase coupled with enzymic acetyl donor systems, one of which utilizes acetate, the other citrate.

In the light of current knowledge, the relative utilization of acetate and citrate provides an insight into the mechanism of ACh synthesis in the blowfly. Mechanisms for the utilization of acetate and citrate in the synthesis of ACh have been proposed. Both compounds may act as acetyl donors for the formation of acetyl CoA. With acetate as substrate, acetyl CoA is formed by a complex reaction involving ATP and CoA catalysed by the acetate-activating enzyme of animal tissues and yeast (Jones, Black, Flynn & Lipmann, 1953). With citrate as substrate, acetyl CoA is formed in the presence of CoA by reversal of the reaction, acetyl CoA + oxalacetate $\rightleftharpoons$ citrate, catalysed by the condensing enzyme (Stern, Shapiro, Stadtman & Ochoa, 1951). Both these reactions yielding acetyl CoA may then be coupled with choline acetylase in the presence of choline to yield ACh (Korkes *et al.* 1952).

Our evidence shows that acetate and citrate are in fact brought into reaction by two separate enzyme systems in the blowfly preparation. The rates of ACh synthesis with acetate and citrate are widely different, the pH-activity relationships differ markedly, and classical evidence for the presence of two enzymes is afforded by the demonstration that the rate of synthesis with acetate and citrate together is the sum of the rates with each separately.

In the blowfly preparation, in contrast to mammalian brain, the acetate-utilizing system for the synthesis of ACh is more effective than that using citrate. Early work with mammalian brain demonstrated that synthesis was greatly potentiated by citrate, whereas acetate had little or no effect (Nachmansohn & John, 1945; Feldberg & Mann, 1946). Later work showed that under appropriate conditions (i.e. high cysteine concentration and the use of purified CoA), acetate as well as citrate could act as acetyl donor; although, with crude brain extracts, the activity with acetate was still less than with citrate (Balfour & Hebb, 1952; Persky & Barron, 1950). On the other hand, the activity of an extract from squid head ganglia was strongly potentiated by acetate and actually inhibited by citrate (Nachmansohn & Weiss, 1948). The blowfly preparation is therefore similar to the squid preparation in that both are more strongly potentiated by acetate than by citrate, suggesting that these invertebrate tissues contain a higher concentration of the acetate

activating enzyme than does mammalian brain. An alternative suggestion is that preferential utilization of acetate is associated with more highly concentrated enzyme preparations. Both the squid and the blowfly preparations are considerably more active than mammalian brain, and moreover, Hebb (1955) has observed that high concentrations of mammalian brain extracts favour acetate utilization, whereas lower concentrations favour citrate.

The relatively low rate of ACh synthesis with citrate was shown in our experiments to result from the low rate of acetyl CoA formation. Recent studies on the mechanism of acetyl CoA formation with citrate indicate that a low rate of formation in our system would, in fact, be expected. The formation of acetyl CoA from citrate by reversal of the condensing enzyme reaction yields oxalacetate as a reaction product (Persky & Barron, 1950; Stern *et al.* 1951). The equilibrium of this reaction is strongly in favour of citrate, but removal of oxalacetate tends to favour the formation of acetyl CoA (Stern *et al.* 1951). Indeed, it has been shown that the synthesis of ACh with citrate may be increased appreciably when oxalacetate is removed by trapping with semicarbazide (Lipton & Barron, 1946) or by reduction to L-malate in the presence of reduced diphosphopyridine nucleotide and malic dehydrogenase (Korkes *et al.* 1952). Under the conditions of our experiments, the formation of oxalacetate in the absence of a trapping mechanism would thus reduce the formation of acetyl CoA, and hence the synthesis of ACh.

Other anomalies with citrate may possibly be resolved in the same way. For instance, when mammalian extracts were used in high concentration, the synthesis of ACh with acetate increased proportionately, but the synthesis with citrate was reduced (Balfour & Hebb, 1952). This reduced synthesis may result from the more rapid formation of oxalacetate in these concentrated extracts and an equilibrium less favourable to acetyl CoA formation. Similarly, highly active preparations obtained from the squid and the blowfly may show reduced synthesis with citrate, compared to acetate, because of the rapid formation of oxalacetate. These considerations recall the early observation by Feldberg & Mann (1946) that oxalacetate strongly inhibited the synthesis of ACh by mammalian brain extracts.

The concept, first suggested by Lipton & Barron (1946), that two steps are involved in the synthesis of ACh, is illustrated by experiments with the blowfly preparation in which the first step, the formation of acetyl CoA, was separated from the second, the acetylation of choline. In these experiments the substrates required for acetyl CoA formation were provided during an induction period without choline; on the subsequent addition of choline, the initial rate of ACh synthesis was very much higher than when all components were added together. In the latter case, in which the formation of acetyl CoA was coincident with the acetylation of choline, the rate of ACh synthesis was clearly limited by the rate of acetyl CoA formation.

The rate-limiting effect of acetyl CoA formation probably applies also in many other experiments on ACh synthesis in which the usual procedure is to add all components of the system together. Thus experiments in which the rate of ACh synthesis is estimated at some interval after the simultaneous addition of all components, seem actually to provide a measure of the rate at which acetyl CoA is made available to the terminal enzyme, choline acetylase.

To measure the activity of choline acetylase, therefore, the enzyme must be provided with sufficient acetyl CoA to saturate it. In our experiments a measure of the activity of choline acetylase, independent of the formation of acetyl CoA, was obtained by allowing the blowfly preparation itself to produce acetyl CoA during an induction period. Initial rates as high as 100 mg/g/hr were obtained, and the enzyme was apparently still incompletely saturated with respect to acetyl CoA.

Instead of using the tissue extract itself to increase the acetyl CoA concentration, extracts may be supplemented with the enzyme systems for the formation of acetyl CoA derived from another source, or provided with chemically produced acetyl CoA. The first method has been used by Hebb (1955), and the second by Berman, Wilson & Nachmansohn (1953).

Hebb added extracts of pigeon liver, which contain the enzymes for the acetylation of CoA but not choline acetylase, to extracts of nervous tissue, and found that the synthesis of ACh was greatly increased. Since the purpose of her experiments was to increase the sensitivity of the method for demonstrating choline acetylase, the question whether the augmented production of acetyl CoA was sufficient to saturate the choline acetylase was not investigated. Berman *et al.* used acetyl CoA itself as substrate, with choline, for a partially purified preparation of choline acetylase from squid head ganglia. With this method the initial high rate of ACh synthesis was maintained nearly linear with time at low enzyme concentrations. Direct provision of acetyl CoA thus offers a decided advantage for the study of the properties of choline acetylase itself, but the method developed in the present study seems more appropriate to experiments in which interest centres also on the acetyl donor systems to which the choline acetylase is coupled.

It is interesting to compare the activity of the choline acetylase obtained by Berman *et al.* (1953) after a ten-fold purification of squid head ganglia, with the activity of our extract of acetone powders from blowfly heads. With the purified squid enzyme the initial rate of ACh synthesis was about 1·3 μmole/100 μg enzyme/hr. With the preparation from blowfly heads an initial rate of 100 mg ACh/g powder/hr, corresponding to 0·06 μmole/100 μg/hr, was obtained. Thus the activity of the crude extract of blowfly heads was about one-twentieth that of the purified squid ganglion preparation.

To afford a better basis for this comparison, it seems reasonable to assume that of the various tissues of the blowfly head (muscle, glands, cuticle), only

the nervous tissue contains choline acetylase. According to an estimate by Lowne (1893), the fresh weight of the brain of a blowfly is about 0·2 mg. The yield of acetone powder from fresh mammalian brain is one-fifth to one-sixth the weight of the fresh tissue (Feldberg & Mann, 1945), so that on this basis, one blowfly head should yield about 0·04 mg acetone powder of brain tissue. Since the yield of acetone powder from whole fly heads averaged 0·4 mg/head, a large proportion of this acetone powder must have been derived from tissues other than nervous tissue. On these assumptions, the activity of the choline acetylase of the blowfly brain would be several fold greater than that observed with acetone powder of whole heads, and it could be as high as 0·6 μmole ACh/hr/100 μg acetone powder of brain tissue. In that case, the activity of the crude extract of blowfly brain would be approximately one-half the activity of the purified preparation from squid head ganglia. In any case it seems clear that the nervous tissue of the head of the blowfly is one of the richest sources of choline acetylase yet reported.

SUMMARY

1. Extracts of acetone powders of blowfly heads were found to synthesize large amounts of ACh when incubated with ATP, CoA, choline, and acetate or citrate.

2. CoA was essential for the synthesis of ACh, and the rate of synthesis was dependent on its concentration.

3. The rate of synthesis was greater with acetate than with citrate as acetyl donor.

4. The synthesis of ACh was shown to proceed by a two-step process. In the first step, an intermediate conforming with the properties of acetyl CoA is formed and contributes acetyl groups for the second step, the acetylation of choline, catalysed by choline acetylase.

5. For the formation of acetyl CoA, two separate enzyme systems were demonstrated, one utilizing acetate, the other citrate as acetyl donor. The rate of formation of acetyl CoA with citrate was lower than with acetate. This difference probably accounts for the lower rates of ACh synthesis obtained with citrate.

6. The rate of formation of acetyl CoA was limiting on the rate of ACh synthesis by the terminal enzyme, choline acetylase. This was demonstrated with both acetate and citrate.

7. When the activity of choline acetylase was measured under conditions in which the formation of acetyl CoA was no longer limiting, initial rates of synthesis as high as 100 mg ACh/g acetone powder/hr were obtained.

I wish to express my gratitude to Dr W. S. Feldberg for his constant interest in this work and for some of the most fertile ideas for its development. Dr Catherine Hebb kindly provided the estimate of the CoA content of the Pabst CoA preparation.

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