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Choline Acetyltransferase. Evidence for an Acetyl-Enzyme Reaction Intermediate†

Robert Roskoski, Jr.

ABSTRACT: Choline acetyltransferase (EC 2.3.1.6) catalyzes the following reversible reaction: acetyl-coenzyme A + choline ⇌ acetylcholine + coenzyme A. The partially purified bovine brain enzyme catalyzed the [^{14}C]choline–acetylcholine exchange reaction in the absence of detectable coenzyme A thereby providing indirect evidence for a covalent acetyl-enzyme intermediate. When the enzyme was incubated with [^{14}C]acetyl-coenzyme A or [^{14}C]acetyl[^{3}H]choline, and the reaction mixture was passed through a Sephadex G-50 column, ^{14}C label was associated with the protein in the eluent. This [^{14}C]acetyl-enzyme intermediate was chemically competent in that it reacted with choline or coenzyme A to give the corresponding acetyl derivative. The bond between the acetyl group and enzyme was not disrupted by hot trichloroacetic acid nor by 6 M guanidium-Cl treatment. The acetyl-enzyme bond was a thio ester by the following criteria: acid stability, cleavage by dilute alkali (pH 10), cleavage by 3 M salt-free hydroxylamine (pH 5.7) to give acetylhydroxamate and performic acid oxidizability. The choline acetyltransferase active-site sulfhydryl is most likely a cysteine SH since the enzyme did not contain detectable 4′-phosphopantetheine.

Acetylcholine is a neurotransmitter at the vertebrate neuromuscular junction and is a putative, but not proven, neurotransmitter in the mammalian central nervous system (*cf.* Iverson, 1970). Choline *O*-acetyltransferase (EC 2.3.1.6) catalyzes the bioformation of acetylcholine with the stoichiometry given in the following chemical equation: acetyl-coenzyme A + choline ⇌ acetylcholine + coenzyme A. The enzyme is adsorbed to membrane at low ionic strength and is readily desorbed at high ionic strength (Fonnum, 1968). The soluble bovine brain enzyme has an estimated molecular weight of 65,000 daltons determined by Sephadex gel filtration (Glover and Potter, 1971). Kinetic studies suggest a sequential mechanism in which acetyl-coenzyme A is probably the leading substrate (Potter *et al.*, 1968). White and Cavallito (1970) reported that [^{14}C]acetyl-coenzyme A formed a complex with crude bovine brain enzyme isolable by Sephadex G-100 gel filtration. They reported that this complex might represent a covalent [^{14}C]acetyl-enzyme or a noncovalent [^{14}C]acetyl-coenzyme A · enzyme complex.

More extensive mechanistic studies have been carried out with the hepatic arylamine *N*-acetyltransferase (EC 2.3.1.5). The results are consistent with the notion that acetyl-coenzyme A reacts with the enzyme to form an intermediate acetyl-enzyme and coenzyme A. Then the acetyl-enzyme reacts with the arylamine to form the *N*-acetylarylamine and the regenerated enzyme (Weber and Cohen, 1967; Steinberg *et al.*, 1971; Riddle and Jencks, 1971). Moreover, Jencks and coworkers (1972) suggest that the acetyl group forms a covalent thio ester intermediate with the enzyme.

The studies in the present paper support the hypothesis of an acetyl-enzyme intermediate in the choline acetyltransferase reaction. The occurrence of a [^{14}C]choline–acetylcholine exchange reaction provides indirect evidence for this notion. The isolation of an acetyl-enzyme intermediate by Sephadex gel filtration using labeled acetyl-coenzyme A or acetylcholine, and the demonstration of the chemical competence of this intermediate provides more direct evidence in support of the hypothesis. Finally, the acetyl-enzyme link exhibits the properties of a thio ester.

Experimental Section

Materials. [^{14}C]Acetyl-coenzyme A (50–60 Ci/mol), [^{14}C]-acetylcholine (4–5 Ci/mol), [^{3}H]acetylcholine (50 Ci/mol), [^{14}C]choline (6.2 Ci/mol), [^{3}H]choline (500 Ci/mol), and Liquifluor were obtained from New England Nuclear Corp.

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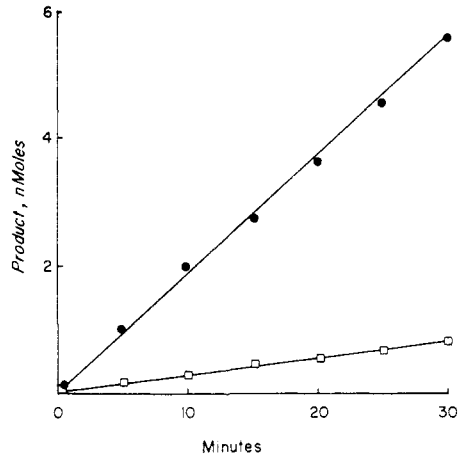

FIGURE 1: Time course of [^{14}C]choline–acetylcholine exchange and [^{14}C]acetyl-coenzyme A formation. For the exchange reaction, the incubation medium contained 2 mM [^{14}C]choline, 1 mM acetylcholine, medium C, and 50 μg of protein extract in a volume of 20 μl. After incubation for the specified time at 37°, the reaction was stopped by the addition of 5 μl of 1 N formic acid. Carrier (5 μl of a solution containing 0.6 M each of choline and acetylcholine) was added; then 10-μl aliquots were applied in 2-cm strips to alumina thin-layer plates. The acetylcholine (R_F 0.5) was resolved from choline (R_F 0.3) with $CHCl_3$–methanol–formic acid–water (65:31:2:2, v/v). The acetylcholine zones were cut out for radioactivity determination. To assay [^{14}C]acetyl-coenzyme A biosynthesis, the incubation medium contained 2 mM [^{14}C]acetylcholine, 100 μM coenzyme A, medium C, and 50 μg of protein extract in a volume of 20 μl. The reaction was stopped with 5 μl of 1 N formic acid and carrier (5 μl of 0.1 M acetyl-coenzyme A) was added. Then 5 μl of solution was applied to PEI-cellulose thin layers in 2.5-cm strips and chromatographed with a solution of 10 mM potassium phosphate (pH 7.0) and 1.3 M KCl. Acetyl-coenzyme A (and coenzyme A) had an R_F 0.6 and acetylcholine (and choline) moved at the front. The uv-absorbing acetyl-coenzyme A zone was cut out for radioactivity determination. (●) [^{14}C]Acetyl-coenzyme A synthesis and (□) [^{14}C]acetylcholine formed in the exchange reaction.

Chromatographically pure coenzyme A and acetyl-coenzyme A were a product of P-L Biochemicals. The chromatographic developing apparatus and the alumina and silica gel thin-layer plates were products of Eastman Kodak. Polygram Cel 300 PEI plates were purchased from Brinkmann.

Acetyl[^{3}H]choline was prepared by incubating 10 μmol of [^{3}H]choline (250 Ci/mol), 20 μl of acetic anhydride (200 μmol) in 100 μl of pyridine for 90 min at 100° in a sealed vial. The reaction mixture was applied to a Dowex 50-X8 (H^+) column (0.5 × 2 cm) made of a Pasteur pipet, washed with 100 ml of water, and eluted with 1 N HCl. The fractions containing the radioactive product (usually the first 6 ml) were combined and concentrated on a Büchler Roto-Evap apparatus at 40° under vacuum. The product, which was resolved from choline, comigrated with acetylcholine on paper electrophoresis carried out as described below.

Radioactivity Determinations. Radioactivity on paper or on thin-layer plates was determined by liquid scintillation counting in vials containing 5 ml of toluene with 0.42% Liquifluor. Radioactivity in aqueous solutions was measured similarly using 10 ml of Bray's (1960) solution. Labeled standards were counted in parallel to measure the efficiency.

Assay of [^{14}C]Acetylcholine Biosynthesis. The method of Potter (1971), with minor modifications, was used for the choline acetyltransferase assays. The final incubation mixture contained 100 μM [^{14}C]acetyl-coenzyme A, 2 mM choline chloride, and enzyme, supplemented with the following medium (C). In medium C the components were present to give the following concentrations in the assay mixtures: 10 mM potassium phosphate, 0.1 mM EDTA, 0.1 mM eserine sulfate, 50 mM KCl, and 1% butanol (v/v), all adjusted to pH 7.4. Incubations were carried out in 20 μl at 37°. Reactions were stopped with 10 μl of 0.5 N formic acid–100 mM acetylcholine. To resolve [^{14}C]acetylcholine from labeled precursor 10-μl aliquots were subjected to low-voltage (30 V/cm) paper electrophoresis (Whatman No. 1) in 1 M formic acid–1 M acetic acid solution for 20 min in a Gelman electrophoresis apparatus. After drying for 15 min at 100° to remove volatile acid and developing the acetylcholine in an I_2 chamber, the marker zones were cut out for radioactivity measurement.

TABLE I: Resolution of Choline and Carnitine Acetyltransferase Activity by Ethanol Fractionation.[a]

Ethanol Fraction (%)	Product Formed (pmoles)	
	Acetylcholine	Acetylcarnitine
0–25	0.1	15
25–50	48	0.1

[a] To the concentrated, dialyzed CM-Sephadex enzyme fraction (5 mg of protein/ml) at 0°, 95% ethanol was added to give 25% (v/v) concentration. After 20 min, the suspension was centrifuged at 14,000*g* for 10 min. Then 95% ethanol was added to the supernatant, and the 25–50% ethanol fraction was obtained in a similar manner. The precipitates were taken up in a small volume of buffer A without dialysis. The choline acetyltransferase was assayed as described in the Experimental Section. The carnitine acetyltransferase assay was carried out similarly except that carnitine (2 mM) was substituted for choline in the incubation medium and acetylcarnitine carrier was used during the electrophoresis. The assays were 5 min at 37° using 10 μg of protein. Nearly 100% of each enzyme activity was recovered.

Enzyme Purification. Choline acetyltransferase was prepared from fresh, whole bovine brain by the procedure of Glover and Potter (1971) through the CM-Sephadex step. The combined active fractions were concentrated by pressure dialysis. To remove the salt used to elute the enzyme from the column, the concentrated protein solution was dialyzed against 250 volumes of 5 mM potassium phosphate (pH 7.4) and 0.1 mM EDTA (buffer A) twice. The enzyme was stored at 0–4° and was stable for months.

Trichloroacetic Acid Precipitation of Protein in the Sephadex Filtrate. Carrier bovine serum albumin (0.2 ml of 0.5% solution/ml of final solution) and enough 50% trichloroacetic acid (w/v) to give a final acid concentration of 10% were added to the designated protein fractions. After 20 min at 0°, the suspension was centrifuged at 14,000*g* for 10 min. The precipitate was resuspended in 2 ml of 10% trichloroacetic acid and recentrifuged. The precipitate was similarly washed in 10% trichloroacetic acid at ambient temperature, twice in 2 ml of ethanol–ether (72:25, v/v) and twice in 2 ml of ether. The precipitate was dissolved in 1.0 ml of hydroxide of Hyamine at 80° and then placed in 5 ml of toluene and 0.42% Liquifluor, and the radioactivity was measured by liquid scintillation spectrometry. The coenzyme A and 4′-phosphopantetheine content was determined by the procedure described in the following section.

Microbiological Assay for Coenzyme A and 4′-Phosphopantetheine. These cofactors were converted to pantothenic acid and assayed microbiologically by *Lactobacillus plantarum*

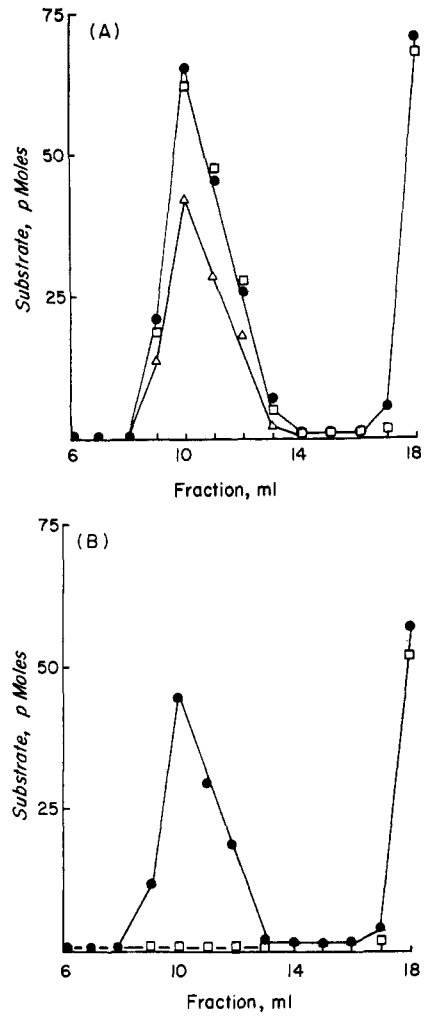

FIGURE 2: Formation of enzyme–substrate complexes from choline acetyltransferase and [^{14}C]acetyl-coenzyme A. The reaction mixture contained 0.5 mg of protein, medium C, and 100 μM [^{14}C]acetyl-coenzyme A in 200 μl. The mixture was incubated at 37° for 15 min, chilled on ice, and applied to a Sephadex G-50 (fine) column (1 × 20 cm) which was equilibrated with 5 mM phosphate buffer (pH 5.9) and 0.1 mM EDTA. The column was eluted with the same buffer. Fractions of 1 ml were collected and aliquots were taken for radioactivity determination, precipitation in trichloroacetic acid, and coenzyme A determination as described in the Experimental Section. (A) Phosphate buffer elution: (●) ^{14}C label; (□) coenzyme A; (△) hot trichloroacetic acid insoluble ^{14}C label. (B) Guanidium-Cl elution: the reaction, which was carried out as in part A was stopped by adding 200 μl of reaction mixture. The column was eluted with 6 M guanidium-Cl and 5 mM potassium phosphate (pH 5.9). The fractions were dialyzed twice against 250 volumes of buffer A for 2 hr to remove the denaturant prior to the measurements. (●) ^{14}C label; (□) coenzyme A.

(ATCC 8014). To hydrolyze the pyrophosphate bond in coenzyme A, the sample (0.5 ml) was heated to 90° for 30 min in 3 N HCl in a sealed ampoule. After cooling to ambient temperature, the solution was neutralized with 6 N KOH. Subsequent steps in the conversion of coenzyme A and 4′-phosphopantetheine to pantothenic acid include alkaline hydrolysis and alkaline phosphatase treatment as described by Pugh and Wakil (1965). Recoveries of standard coenzyme A and 4′-phosphopantetheine were greater than 85% with a sensitivity of 5 pmol/assay.

Results

General Characteristics of the Enzyme Reaction. The time course of the reaction was linear until about one-third of the substrate [^{14}C]acetyl-coenzyme A was depleted. Therefore, routine assays were carried out under conditions in which less than 5% of this substrate was converted to product. The partially purified enzyme after CM-Sephadex chromatography was composed of nine bands on 6.5% polyacrylamide gel electrophoresis (pH 8.6). The enzyme activity comigrated with one of the bands, and the enzyme was estimated to be 5–10% pure. The specific activity of contaminant carnitine *O*-acetyltransferase ranged from 5 to 30% that of the choline acetyltransferase in the several preparations used in the experiments to be reported. These two enzymes can be resolved by ethanol fractionation (Table I). The contamination of one by the other was less than 0.2%, indicating that the synthesis of the two products is catalyzed by distinct enzymes. There was no detectable acetylcholine esterase (EC 3.1.1.7) activity in the CM-Sephadex fraction used in the present experiments.

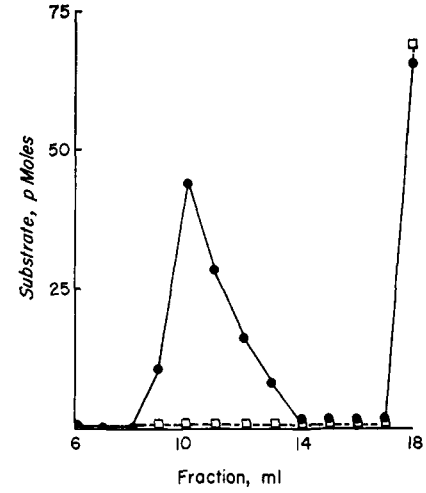

FIGURE 3: Formation of [^{14}C]acetyl-enzyme from choline acetyltransferase and labeled precursor. The experiment was performed as described in Figure 2 except that 2 mM total [^{14}C]acetylcholine and acetyl[^{3}H]choline (^{14}C, 5 Ci/mol and ^{3}H, 25 Ci/mol) were used as substrate. The same results were obtained with guanidium-Cl and phosphate buffer elution. (●) Total and trichloroacetic acid precipitable ^{14}C label; (□) ^{3}H label.

[^{14}C]Choline–Acetylcholine Exchange. The partially purified choline acetyltransferase catalyzes the [^{14}C]choline–acetylcholine exchange reaction. The amount of coenzyme A present in the partially purified enzyme measured by bioassay was less than 0.2% that of the enzyme (on a molar basis) estimated by active-site labeling (see below). The rate of this exchange is about 10% of the rate of [^{14}C]acetyl-coenzyme A synthesis from [^{14}C]acetylcholine and coenzyme A (Figure 1).

These data support the notion that the acetylcholine reacts with the enzyme to form acetyl-enzyme and choline. The acetyl-enzyme, with retention of group transfer potential, reacts with [^{14}C]choline forming [^{14}C]acetylcholine and free enzyme, thereby accounting for the exchange reaction. Further experiments were undertaken to test for the participation of a covalently linked acetyl-enzyme intermediate in the choline acetyltransferase reaction.

Isolation of Enzyme·Substrate and Enzyme–Intermediate Complexes by Sephadex Gel Filtration. When [^{14}C]acetyl-coenzyme A or [^{14}C]acetylcholine is incubated with the partially purified choline acetyltransferase, and the reaction mixture is filtered through a Sephadex G-50 column, radiolabel is associated with the protein in the eluent (Figures 2 and 3). That a covalent [^{14}C]acetyl-enzyme intermediate is formed from the enzyme and labeled acetylcholine was shown in two

TABLE II: Chemical Competence of the [^{14}C]Acetyl-Enzyme.[a]

Donor for [^{14}C]Acetyl-Enzyme Formation	Product (pmoles)	
	Acetyl-coenzyme A	Acetylcholine
[^{14}C]Acetyl-coenzyme A	36	42
[^{14}C]Acetylcholine	44	43

[a] The [^{14}C]acetyl-enzyme was prepared by Sephadex gel filtration as described in Figures 2 and 3. Aliquots (0.8 ml) containing 60 pmol of acetyl-enzyme were incubated in medium C with either 1 mM choline, 100 μM coenzyme A, or no acceptor for 15 min at 37°. The reaction was stopped with 40 μl of 10 N formic acid. The solutions were concentrated on a Büchler Roto-Evap apparatus at 40° under vacuum. After addition of carrier, acetylcholine was resolved from other components by paper electrophoresis and acetyl-coenzyme A, by PEI-cellulose chromatography. Radioactivity was measured as described in the Experimental Section. The amount of label comigrating with carrier when the incubation was carried out in the absence of acceptor substrate was usually less than 0.5%. With [^{14}C]acetyl-coenzyme A as substrate, followed by Sephadex gel filtration, about 5% of the label comigrated with acetyl-coenzyme A in the absence of added acceptor.

additional experiments. First, when the enzyme was incubated with [^{14}C]acetylcholine and acetyl[^{3}H]choline, followed by gel filtration, only ^{14}C label was associated with the protein (Figure 3). This result excludes the isolation of a noncovalent acetylcholine·enzyme complex. Second, when this reaction mixture was passed through a Sephadex column equilibrated with 6 M guanidium-Cl, the radiolabel was not dissociated from the protein. Using [^{14}C]acetyl-coenzyme A as substrate, and quantitating the acetyl group content by radioactivity and coenzyme A content by microbiological assay, analysis of the protein eluent from the Sephadex filtrate gave the following results (Figure 2). First, using phosphate buffer as eluent (nondenaturing conditions), the coenzyme A content equaled the acetyl group content. When this protein was precipitated with $(NH_4)_2SO_4$, both the acetyl and coenzyme A content of the precipitate remained the same. However, when the protein was precipitated with trichloroacetic acid, two-thirds of the [^{14}C]acetate remained associated with protein but the coenzyme A was completely discharged. Furthermore, when the incubation mixture was filtered through Sephadex equilibrated with 6 M guanidinium-Cl, radiolabel was associated with the protein, but coenzyme A was not. The amount of radiolabel associated with the protein under these denaturing conditions was about 67% that under nondenaturing conditions. These experiments provide evidence for the formation of the following complexes in approximately a 2:1 ratio: [^{14}C]acetyl-enzyme·coenzyme A and [^{14}C]acetyl-coenzyme A·enzyme, where (·) denotes a noncovalent and (–) denotes a covalent bond.

Bioreactions of the [^{14}C]Acetyl-Enzyme. If the postulated acetyl-enzyme is a *bona fide* intermediate in the enzymic reaction, it ought to react with the acceptor substrates to form the reaction products. The [^{14}C]acetyl-enzyme, prepared from [^{14}C]acetyl-coenzyme A or [^{14}C]acetylcholine, was isolated by Sephadex gel filtration. The [^{14}C]acetyl-enzyme prepared from either acetyl donor reacts with either acceptor to form [^{14}C]acetylcholine or [^{14}C]acetyl-coenzyme A (Table II). About 67% of the acetyl groups was transferred from enzyme to the acceptor under the conditions of the experiment. On the one hand, the transfer from the acetyl-enzyme prepared from [^{14}C]acetyl-coenzyme A does not exclude a concerted reaction between acetyl-coenzyme A and choline since both the acetyl and coenzyme A moieties are associated with the protein in the Sephadex filtrate (Figure 2). On the other hand, transfer from the acetyl-enzyme formed from acetylcholine argues against a concerted reaction between acetylcholine and coenzyme A in the present experiment since the intermediate acetyl-enzyme complex does not contain choline (Figure 3). Further experiments are required to show that this is the mechanism under conditions of turnover. Even though the acetyl-enzyme formed from [^{14}C]acetyl-coenzyme A still contained stoichiometric amounts of coenzyme A, curiously, only 5% of the radioactivity was released from the protein in the absence of exogenous acceptor substrate.

Properties of the Acetyl-Enzyme Link. Using [^{14}C]acetyl-coenzyme A or [^{14}C]acetylcholine as substrate, the radioactivity associated with the protein in the Sephadex G-50 eluent is precipitated by 10% trichloroacetic acid (Figure 2). Moreover, the labeled acetate is not dissociated from the protein by hot trichloroacetic acid (Table III) nor by 6 M guanidium-Cl (Figure 3). These results indicate that the acetyl group is covalently linked to the protein. Treatment of the acetyl-enzyme with neutral, salt-free 3 M hydroxylamine (15 min at 50°) liberates the acetate as the corresponding acetylhydroxamate. Furthermore, dilute alkali (pH 10) discharges the acetyl group as acetate. These results are consistent with the notion that the acetyl group is bound to the enzyme as thio ester. To substantiate this hypothesis, the acetyl-enzyme was subjected to performic acid oxidation. Thioesters are cleaved by this treatment, but oxygen esters are not (Harris *et al.*, 1963). All the protein-bound labeled acetate is liberated by performic acid oxidation. In control experiments, virtually 100% of the acetate was liberated from acetyl-coenzyme A and none was liberated from acetylcholine. Although not quantitatively recovered because of volitility, the liberated product comigrates with acetate on silica gel thin layer chromatograms. Table III summarizes these experiments.

Assay of the Partially Purified Choline Acetyltransferase for 4′-Phosphopantetheine. Because of the involvement of 4′-phosphopantetheine in acyl-transfer reactions in fatty acid biosynthesis (Pugh and Wakil, 1965; Vagelos *et al.*, 1966), peptide antibiotic synthesis (Kleinkauf *et al.*, 1970) and *Klebsiella aerogenes* citrate lyase (Srere *et al.*, 1972), the enzyme fraction catalyzing acetylcholine biosynthesis was assayed for this cofactor. The choline acetyltransferase content was estimated by the amount of trichloroacetic acid precipitable [^{14}C]acetyl-protein using [^{14}C]acetyl-coenzyme A or [^{14}C]acetylcholine as substrate. The values for enzyme concentration obtained with these two precursors varied about 20%. A 5-mg sample of protein extract which bound 1150 pmol of [^{14}C]acetate contained less than 5 pmol of 4′-phosphopantetheine as measured microbiologically. Thus, there is less than 0.5 mol of pantetheine/100 mol of enzyme.

Discussion

Choline *O*-acetyltransferase catalyzes the reversible transfer of the acetyl group from acetyl-coenzyme A to choline. The experiments in the present paper support the notion of a covalent acetyl-thioenzyme reaction intermediate. The occurrence of a [^{14}C]choline–acetylcholine exchange reaction in the absence of detectable coenzyme A is consistent with the idea

of a covalent acetyl-enzyme intermediate. Measuring the concentration of coenzyme A by the sensitive microbiological assay, and the concentration of enzyme by the binding of acetate, the molar ratio of coenzyme A to choline acetyltransferase was less than 0.5%. The rate of the exchange reaction was 10% that of acetyl-coenzyme A biosynthesis from acetylcholine and coenzyme A. This slow rate of exchange may represent another example of substrate synergism (Bridger *et al.*, 1968). The reversibility of the choline acetyltransferase reaction has permitted the preparation of an acetyl-thioenzyme from both acetylcholine and acetyl-coenzyme A. Using [^{14}C]acetyl[^{3}H]choline as substrate, only the [^{14}C]acetyl group was associated with the enzyme. Using [^{14}C]acetyl-coenzyme A, approximately equal amounts of the acetyl moiety and coenzyme A were associated with the eluant protein. Trichloroacetic acid precipitation discharged all of the coenzyme A and about one-third of the acetate. These results suggest that two types of complexes exist: [^{14}C]acetyl-enzyme·coenzyme A (67%) and [^{14}C]acetyl-coenzyme A·enzyme (33%). Denaturation by trichloroacetic acid or guanidium-Cl would be expected to discharge all the coenzyme A and one-third of the acetate. It seems less likely that all the complex would be in the form of a noncovalent [^{14}C]acetyl-coenzyme A·enzyme complex, which would form [^{14}C]acetyl-enzyme upon denaturation.

The evidence that the acetyl-enzyme link is a thio ester includes: acid stability, cleavage by dilute alkali, and cleavage by neutral salt-free hydroxylamine. Perhaps the most convincing evidence is the conversion of a substrate which is completely resistant to performic acid oxidation, namely acetylcholine, to a trichloroacetic acid precipitable form which is readily cleaved. The enzymic SH donor is not 4′-phosphopantetheine and therefore is most probably a polypeptidic cysteine sulfhydryl. Additional experiments are required to establish this point.

The high affinity of the acetyl-enzyme for coenzyme A may explain, in part, the finding of enzyme kinetics consistent with a sequential mechanism and not parallel line or "Ping-Pong" kinetics with the neural enzyme (Potter *et al.*, 1968; White and Cavallito, 1970; Glover and Potter, 1971; White and Wu, 1973). The latter type of enzyme kinetics occurs when product is released prior to reaction with a subsequent substrate (Cleland, 1963). If the product release were kinetically slow, that is if the coenzyme A release from the active site of the choline acetyltransferase were slow, changes in both slope and intercept might be seen in double-reciprocal plots at different concentrations of the fixed substrate coenzyme A (Cleland, 1970) in agreement with the kinetic studies previously mentioned. Although the adherence of an enzyme to parallel line kinetics points to a two-stage chemical mechanism (Arion and Nordlie, 1964), such a mechanism is not excluded when the kinetics are not of this type. For example, *E. coli* succinate thiokinase does not exhibit Ping-Pong kinetics. Rather, the kinetic studies are consistent with a sequential mechanism in which all three substrates combine with enzyme before the release of product (Moffet and Bridger, 1970), even though the reaction involves a kinetically competent phosphoenzyme intermediate (Bridger *et al.*, 1968).

Finally, it must be mentioned that the postulated acetyl-thioenzyme intermediate in the choline acetyltransferase reaction might be an adventitious product. Further experiments are required to demonstrate that the rate constants for the formation and further reaction of this alleged intermediate are adequate to account for the observed rate of the enzymic reaction.

TABLE III: Identification of the Acetyl-Protein Link as Thio Ester.[a]

Treatment	Product
1. Hot trichloroacetic acid	Protein-bound acetate
2. Alkali, pH 10	Acetate
3. 3 M Hydroxylamine, pH 5.7	Acetylhydroxamate
4. Performic acid oxidation	Acetate

[a] These experiments were carried out in parallel with acetyl-enzyme prepared from [^{14}C]acetyl-coenzyme A and [^{3}H]-acetylcholine as described in Figures 2 and 3. Trichloroacetic acid precipitation was carried out as described in the Experimental Section. To test for hot trichloroacetic acid stability, the precipitate, suspended in 10% acid, was heated to 90° for 20 min, then washed with ethanol–ether as described in the Experimental Section. Alkaline liberation was carried out as follows: 20 μl of water and 2 μl of 1 N KOH were added to ether-washed precipitates. The precipitates were macerated with a steel spatula; the tubes were stoppered, and placed in a 50° water bath for 15 min. After cooling to ambient temperature, 2 μl of 1 N acetic acid carrier was added and 10 μl of resulting solution was applied to a 2-cm strip on a silica gel thin-layer plate. The chromatogram was developed in petroleum ether–pyridine (2:1, v/v) for 60 min. After drying, the chromatogram was sprayed with Bromocresol Green (0.1 g/100 ml of 95% ethanol). The acetate zone, which had an R_F 0.6, was cut out for radioactivity measurement. For hydroxylamine liberation, 20 μl of 3 M salt-free hydroxylamine (pH 5.7) was added to the ether-washed precipitate. After heating 15 min at 50°, 2 μl of 0.1 M acetylhydroxamate and 2 μl of 1 M acetic acid were added. Then 5 μl was applied to a 2-cm strip on a PEI-cellulose thin layer and the chromatogram was developed with 50 mM potassium phosphate (pH 7.4). The hydroxamate was located with 1% $FeCl_3$–0.5 N HCl spray at the front and the acetic acid was located with Bromocresol Green with a R_F 0.4. These zones were removed for radioactivity determination. For performic acid oxidation at ambient temperature, the ether-washed precipitate was dissolved in 15 μl of formic acid (97%) to which 0.5 μl of 30% H_2O_2 was added. After 30 min a second aliquot of 0.5 μl of H_2O_2 was added. At 60 min the reaction was stopped by addition of 100 μl of H_2O and 30 μl of glacial acetic acid carrier. Then 5-μl aliquots were chromatographed on a silica gel thin layer as described above. As a control, the acetyl-enzyme was dissolved in 15 μl of formic acid, but the H_2O_2 additions were omitted. In the former case the liberated material comigrated with acetate and in the latter case the radiolabel remained at the origin, presumably bound to protein.

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Preparation and Properties of Chromium(III)–Nucleotide Complexes for Use in the Study of Enzyme Mechanisms†

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ABSTRACT: Stable complexes of Cr^{3+} with various nucleotides have been prepared by heating the nucleotide with hexaaquochromium at 80° and pH 3 for 12 min and purified by ion exchange methods. Compounds prepared were CrATP, CrCTP, CrGTP, CrUTP, CrXTP, CrITP, CrADP, CrCDP, CrGDP, and CrUDP. The resulting complexes are stable at acid pH, but are hydrolyzed above pH 7. In CrATP and the other triphosphates all three phosphates appear coordinated to chromium, and the pK of the secondary hydroxyl of the γ-phosphate appears to be 2.2. In CrADP and other diphosphates both phosphates are coordinated and the secondary phosphate pK is also low. In these and other compounds mentioned below, the remaining coordination positions are presumably taken by water. CrATP shows a magnetic moment of 3.83 BM, as expected for a mononuclear chromium complex, and an axial-looking electron paramagnetic resonance spectrum with $g_{\perp} = 1.97$ and $g_{\parallel} = 4.7$. By heating chromium complexes containing ammonia with ATP or ADP, $Cr(NH_3)_2ATP$, $Cr(NH_3)_3ATP$, $Cr(NH_3)_2ADP$, $Cr(NH_3)_3ADP$, $Cr(NH_3)_4ADP$, and $Cr(NH_3)_4(ADP)_2$ have been prepared. Other chromium complexes prepared include Cr(formate)ATP (which appears to have been the "CrADP" described by Foster, D. M., and Mildvan, A. S. (1972), *Bioinorg. Chem. 1*, 133), $Cr(oxalate)_2ADP$, Cr(PPP), Cr(PP), and $Cr(PP)_2$. Since chromium nucleotides strongly and specifically inhibit a number of enzymes which have MgATP or other nucleotides as substrates, these compounds are proving very useful in binding studies and as inhibitors for kinetic analysis of enzyme mechanisms.

It is now generally accepted that for most enzymes that have nucleotides as substrates the Mg^{2+} complex rather than the free nucleotide is the active form of the substrate. For kinetic studies it is often desirable to employ dead end inhibitors which closely resemble the substrate, but do not undergo the catalytic reaction, but for enzymes with Mg nucleotides as substrates this poses a problem. Altering the base or the sugar often produces only a poorer substrate. Changing the oxygen bridge between phosphorus atoms to $-CH_2-$ produces a compound with altered bond lengths and angles, and thus often with rather weak affinity for the enzyme, although the recently synthesized –NH– bridged compounds are much closer analogs and show good promise as inhibitors (Yount *et al.*, 1971). Changing the metal to another inactive one necessitates complex calculations of the resulting metal–nucleotide equilibria and does not allow fixing the levels of free metals or of uncomplexed nucleotides.

However, if one employs a metal that forms inert coordination complexes, these difficulties should be overcome. Cr^{3+} is a paramagnetic transition metal with octahedral coordination geometry that exchanges oxygen ligands very slowly. For example, the rate of exchange of H_2O in the inner coordination sphere is $2–5 \times 10^{-6}$ sec^{-1} at 27° depending on the ionic strength; a half-time of 39–97 hr (Hunt and Plane, 1954). This exchange rate is $10^{10}–10^{13}$ times slower than that seen with Co^{2+}, Ni^{2+}, Mn^{2+} (Swift and Connick, 1962), or Mg^{2+} (Eigen and Wilkens, 1965). Further, dissociation of a

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