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Human Brain and Placental Choline Acetyltransferase: Purification and Properties[†]

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ABSTRACT: Choline acetyltransferase (EC 2.3.1.6) catalyzes the biosynthesis of acetylcholine according to the following chemical equation: acetyl-CoA + choline \rightleftharpoons acetylcholine + CoA. In addition to nervous tissue, primate placenta is the only other animal source which contains appreciable acetylcholine and its biosynthetic enzyme. Human brain caudate nucleus and human placental choline acetyltransferase were purified to electrophoretic homogeneity using ion-exchange and blue dextran-Sepharose affinity chromatography. The molecular weights determined by Se-

phadex G-150 gel filtration and sodium dodecyl sulfate gel electrophoresis are 67000 ± 3000 . *N*-Ethylmaleimide, *p*-chloromercuribenzoate, and dithiobis(2-nitrobenzoic acid) inhibit the enzyme. Dithiothreitol reverses the inhibition produced by the latter two reagents. The pK_a of the group associated with *N*-ethylmaleimide inhibition is 8.6 ± 0.3 . A chemically competent acetyl-thioenzyme is isolable by Sephadex gel filtration. The enzymes from the brain and placenta are thus far physically and biochemically indistinguishable.

Acetylcholine is the neurotransmitter at the vertebrate neuromuscular junction and at specific synapses in the autonomic nervous system. It is also an alleged neurotransmitter in the vertebrate central nervous system. The primate placenta is the only firmly established nonneuronal source of acetylcholine in animals; its function in placenta is unknown (cf. Potter, 1970). Choline acetyltransferase (EC 2.3.1.6) catalyzes the bioformation of acetylcholine with the stoichiometry given in the following chemical equation: acetyl-CoA + choline \rightleftharpoons acetylcholine + CoA. Thiol reagents inhibit the transferase prepared from rat (Potter et al., 1968) and bovine brain (Choa and Wolfgram, 1973; Roskoski, 1974a), human placenta (Schuberth, 1966), torpedo (Morris, 1967), and squid head ganglia (Reisberg, 1954). Previous studies with the bovine brain enzyme support the notion that an active site -SH group mediates the transfer of the acetyl group from donor to acceptor substrate (Roskoski, 1974b). Currier and Mautner (1974) were unable to isolate the postulated acetyl-enzyme intermediate from a purified preparation from squid head ganglia. These results may be related to species differences.

Choa and Wolfgram (1973) and Singh and McGeer (1974) reported methods for purifying the bovine and human brain transferase, respectively, to electrophoretic homogeneity. Using modifications of these procedures, and blue dextran-Sepharose affinity chromatography (Ryan and Vestling, 1974; Thompson and coworkers, 1975), we have purified the human brain caudate nucleus and human

placenta choline acetyltransferase to electrophoretic homogeneity. The variable yield, however, is only 1-10%; this seems to be related, inter alia, to enzyme instability in the latter stages of the purification.

Experimental Section

Materials. Normal caudate nuclei were obtained post-mortem. The placentas were obtained after normal term deliveries. The tissues were stored at -20° for periods up to 3 months without loss of enzyme activity.

Buffers, protein standards, Sepharose 4B, and blue dextran were purchased from Sigma Chemical Co. The blue dextran-Sepharose resin was prepared by the procedure of Ryan and Vestling (1974). C.I. (Color Index) Reactive blue 2 (Cibachron Blue 3-GA), the chromophore of blue dextran, was a generous gift of Ciba-Geigy (Basel, Switzerland). The sources of other materials and the methods for radioactivity determination and Sephadex G-50 gel filtration have been documented previously (Roskoski, 1973, 1974c).

Choline acetyltransferase activity was measured as previously described (Roskoski, 1973) except that $100 \mu\text{M}$ [^{14}C]acetyl-CoA (40 Ci/mol) and 7.5 mM choline (final concentrations) were used and incubations were 10 min at 30° .

Preparation of Human Placental Choline Acetyltransferase. Frozen placenta (500 g) was cut into 3-cm cubes. All subsequent steps were carried out at $0-4^\circ$. The placenta was disrupted batchwise in 5 volumes of buffer A (5 mM potassium phosphate-0.1 mM EDTA (pH 7.4)) in a blender (2 min, high speed). The pH was adjusted to 7.4, if necessary, with 1 M K_2HPO_4 prior to centrifugation at 11000g

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Table I: Purification of Choline Acetyltransferase from Human Placenta.^a

Fractionation Step	Total Protein (mg)	Total Activity (units)	Recovery (%)	Specific Activity (units/mg)	Purification
1. Homogenate	49500	16300	100	0.36	1.0
2. Supernatant (11,000g)	36200	12700	78	0.35	1.0
3. CM-Sephadex column	4530	8100	50	1.76	4.9
4. (NH ₄) ₂ SO ₄ fraction	2940	6740	41	2.29	7.7
5. DEAE-Sephadex column	320	5350	33	16.7	46
6. Blue dextran-Sepharose column	37.5	1470	9	39.1	109

^aProcedures described in Experimental Section. One unit is 1 nmol min⁻¹ at 30°.

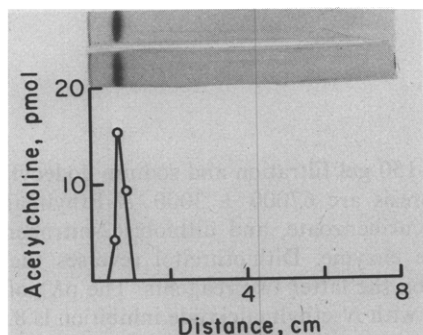


FIGURE 1: Comigration of protein from human brain (upper) and placenta (lower) and enzyme activity on polyacrylamide gels. Gel electrophoresis was performed by the procedure outlined by Davis (1964) at pH 9.3 with 16 μ g of protein. Protein was stained with Coomassie Blue (Weber and Osborn, 1969). Gel slices (2 mm) were first macerated in test tubes with glass stirring rods. Then choline acetyltransferase activity was assayed with 100 μ l of incubation mixture for 30 min at 30° as previously outlined (Roskoski, 1973). Activity is expressed per 20- μ l aliquot of reaction mixture.

for 20 min. After adjusting the pH of the supernatant to 5.9 with 0.1 M citric acid, CM-Sephadex (A-50) (15 g/500 g of tissue) equilibrated with the same buffer was added. After the mixture was stirred occasionally for 30 min, most of the supernatant was decanted and the red resin was transferred to a column (7 cm \times 24 cm) which was washed with 500 ml of buffer B (5 mM potassium phosphate-0.1 mM EDTA (pH 5.9)). The enzyme was eluted with a 4-l. (total) linear KCl gradient (0.0–0.5 M) in buffer B. The enzyme eluted around 0.3 M KCl. After combining the active fractions and adding dithiothreitol (0.5 mM final), solid (NH₄)₂SO₄ was added batchwise (0.4 g/ml). After 2 hr, the precipitate was collected by centrifugation (11000g, 20 min), and taken up in a small volume of buffer A and dialyzed against 200 volumes for 2 hr. The inactive precipitate was removed by centrifugation (27000g, 10 min). The enzyme is stable at this stage and may be stored at –20° for months without significant loss of activity. Two hours before DEAE-Sephadex chromatography, the extract was dialyzed against 100 vol of buffer (0.045 M Tris-HCl, 10% glycerol (v/v), 0.5 mM dithiothreitol, and 0.5 mM EDTA (pH 7.9)). The extract was passed through a DEAE-Sephadex column (A-50) (4 cm \times 20 cm) equilibrated with the same buffer from which enzyme activity freely elutes. After adjusting the pH to 7.4 with 0.1 M citric acid, if necessary, the combined fractions were charged to blue dextran-Sepharose (50 ml bed volume). After the solution was washed with 200 ml of buffer A, containing 10% glycerol and 0.5 mM dithiothreitol, the enzyme was eluted with a 500-ml

(total) linear KCl gradient (0.0–0.5 M). After combining active fractions, the enzyme was concentrated by pressure dialysis and stored at –20°.

This methodology is efficacious in preparing the human brain caudate nucleus enzyme (after disruption by homogenization) and the bovine brain enzyme by proportioning the quantity of materials used to the weight of the starting tissue. The human caudate nucleus enzyme was also prepared using the methodology of Singh and McGeer (1974); the final products were undistinguishable.

Results

General Characteristics of Human Brain and Placental Choline Acetyltransferase. The velocities of the enzyme-catalyzed reactions are linear for more than 15 min at 30°. Because spurious time courses were occasionally obtained at 37°, this temperature was not routinely used. The partially purified enzymes are stable at –20° for months. After DEAE-Sephadex chromatography, the yields for the human brain enzyme (Singh and McGeer, 1974) and the placental enzyme (Table I) are low. This seems in part related to instability of more highly purified enzyme even though 10% glycerol, 0.5 mM dithiothreitol, and 0.5 mM EDTA are included. Specific activity varies in different preparations of the brain and placental enzymes and the explanation is unknown. Our preparations have fivefold higher specific activity than reported by Singh and McGeer (1974) even without correcting for the lower incubation temperature. These workers, however, have revised their values upward in agreement with the present studies (personal communication).

Molecular Weight Estimation by Sephadex G-150 Gel Filtration. Using the general methodology of Andrews (1965) and *Escherichia coli* alkaline phosphatase (80000), bovine serum albumin (67000), and ovalbumin (43000) as markers, molecular weights were estimated by Sephadex G-150 gel filtration. Human brain and placental choline acetyltransferase coelute at the same position as bovine serum albumin. The molecular weight of each enzyme is about 67000. The bovine brain choline acetyltransferase also elutes at this position. No enzyme activity was detected at positions corresponding to higher or lower molecular weights. Choa and Wolfgram (1974) reported that higher molecular weight aggregates result from (NH₄)₂SO₄ fractionation of the bovine brain enzyme as determined by Sephadex gel filtration. We are unable to confirm this observation using the human brain or placental enzymes.

Gel Electrophoresis of Brain and Placental Transferases. Human brain and placental choline acetyltransferase activity comigrated with a single protein band at pH 9.3 (Figure 1) and 6.3 (not shown). Enzyme recovery is lower

at pH 9.3 than at 6.3. Because ammonium persulfate markedly inactivates the enzyme, riboflavine photopolymerization was used to prepare the gels (Orr and coworkers, 1972). Using bovine serum albumin (67000) and catalase (62000 monomer) as molecular weight standards, dodecyl sulfate gel electrophoresis using the methodology of Weber and Osborn (1969) also gave one protein band with a molecular weight of 66000 ± 3000 . The agreement of these values with those obtained by Sephadex gel filtration show that the enzyme is monomeric and is not composed of subunits.

pH Dependence of Brain and Placental Choline Acetyltransferase. As reported by Glover and Potter for the bovine brain enzyme (1971), the human brain choline acetyltransferase exhibits a broad optimum in the alkaline region (not shown). The pH-activity curves for the placental and bovine brain enzymes are similar to that found for the human brain enzyme. Activity decreases sharply and irreversibly above pH 10.2. A shoulder at pH 6.7 is similar to that found for the alkylation of the essential sulfhydryl group of papain (Polgár, 1974), where there is substantial evidence for thiol-imidazole interaction (cf. Glazer and Smith, 1971). Although pH-activity curves for enzymic reactions are the results of many complex interactions, we suggest that the shoulder in the acid pH range may be related to thiol-imidazole interaction. Several lines of investigation support the notion that the sulfhydryl (cf. Roskoski, 1974b) and imidazole groups (White and Cavallito, 1970; Currier and Mautner, 1974; Roskoski, 1974d) play a role in the choline acetyltransferase reaction.

Choline Acetyltransferase Inhibition by Chemical Modification and Reactive Blue 2. Nbs₂,¹ *p*-chloromercuribenzoate, and *N*-ethylmaleimide inhibit the human brain and placental transferase (Table II). Dithiothreitol (5 mM, 20 min, 30°, pH 7.4) fully reverses inhibition by the former two reagents, but not by the third. These studies provide evidence that sulfhydryl groups are important in the human brain and placental enzymes (Schuberth, 1966). Phenylmethanesulfonyl fluoride, which inactivates papain by reacting with its active site sulfhydryl group (Whitaker and Perez-Villasenor, 1968), and dansyl chloride also inhibit the transferases. Although acetyl-CoA substantially protects against inactivation, dithiothreitol fails to reverse the inactivation (50 mM, 20 min, 30°, pH 7.4). This suggests that inhibition by the sulfonyl halides is unrelated to thiol group modification. Papain inhibition by phenylmethanesulfonyl fluoride, however, is reversed by dithiothreitol.

Reactive blue 2, the chromophore of blue dextran, is an adenosine diphosphoryl nucleotide analog (Thompson et al., 1975). A 100 μ M concentration of chromophore inhibits the enzyme more than 80% (Table II) with 100 μ M acetyl-CoA. With acetyl-CoA as variable substrate (20–150 μ M), the blue chromophore (30–150 μ M) is a linearly competitive inhibitor of the brain and placental enzyme (not shown). With choline as variable substrate (0.25–5.0 mM), the blue chromophore (30–150 μ M) is a linearly noncompetitive inhibitor of the enzymes. The K_m 's for acetyl-CoA are 15–50 μ M and those for choline are 0.75–1.5 mM, depending on the concentration of the alternate substrate, for the brain and placental enzymes. The efficacy of the blue dextran-Sepharose resin apparently is based on this structural analogy.

¹ Abbreviations used are: Nbs₂, dithiobis(2-nitrobenzoic acid); Tricine, *N*-tris(hydroxymethyl)methylglycine.

Table II: Effect of Chemical Modification and Reactive Blue 2 on Choline Acetyltransferase Activity.^a

Substance	Concn	Enzyme Activity (% Control)	
		Brain	Placenta
<i>N</i> -Ethylmaleimide	50 μ M	44	36
<i>p</i> -Chloromercuribenzoate	10 μ M	8	12
5,5'-Dithiobis-(2-nitrobenzoic acid)	10 μ M	13	9
Phenylmethanesulfonyl fluoride ^b	1.0 mM	32	26
Dansyl chloride ^b	50 μ M	18	24
Reactive blue 2	100 μ M	12	19

^a To remove dithiothreitol from the stored enzyme for these experiments, the solution was passed through a Sephadex G-50 (fine) column (1.2 × 20 cm) and used within 24 hr. Then enzyme (0.6 μ g; specific activity 57 units mg⁻¹ for brain and 37 units mg⁻¹ for placenta) was incubated 5 min at 30° in buffer (50 mM potassium phosphate–100 mM KCl–0.1 mM EDTA (pH 7.4)) with the specified concentration of reagent in 10 μ l. Then 10 μ l of the buffer containing 200 μ M [¹⁴C]acetyl-CoA (40 Ci/mol) and 15 mM choline was added and the incubation was continued 10 min. Labeled acetylcholine was removed from unreacted precursor by paper electrophoresis as previously described (Roskoski, 1973). ^b In these experiments, a 1- μ l solution of reagent dissolved in acetonitrile was added to 3 μ g of enzyme in 50 μ l giving the final specified concentration. Acetonitrile was added to the controls. After 5 min at 30°, aliquots (10 μ l) were assayed for enzyme activity.

Because of the inhibition produced by the nucleotide analog, nucleotides per se were tested for their effect on brain and placental choline acetyltransferase activity. At 1 mM concentrations, where Reactive blue 2 inhibits the enzyme more than 98%, we find that 1 mM AMP, ADP, ATP, and the dinucleotides NAD⁺, NADH, NADP⁺, and NADPH fail to alter enzyme activity. MgCl₂ (1 mM) alone or in combination with these substances also fail to alter enzyme activity. These results suggest that these compounds do not directly regulate enzyme activity in vivo.

Effect of Substrates on *N*-Ethylmaleimide Inactivation of the Brain and Placental Choline Acetyltransferase. Acetyl-CoA near the K_m concentration (15 μ M) completely protects the enzymes against *N*-ethylmaleimide inactivation (Table III). Acetylthiocholine fully protects against inactivation at a concentration two orders of magnitude less than its K_m of 5 mM. Acetylcholine partially protects, and choline fails to protect against inhibition. The greater effectiveness of acetylthiocholine compared with acetylcholine may be related to the higher group transfer potential of the thio ester compared with the oxygen ester so that it more readily forms the postulated acetyl-enzyme form. Under steady-state conditions, when the enzyme is catalyzing the turnover of substrates (100 μ M acetyl-CoA, 7.5 mM choline), the enzyme is resistant to *N*-ethylmaleimide inactivation. When the concentrations of acetyl-CoA and choline are lowered to 25 μ M and 2.5 mM, respectively, the enzyme remains resistant to inactivation. These results are similar to those obtained with the bovine brain choline acetyltransferase (Roskoski, 1974a) and are consistent with the notion that enzyme acylation is rapid and deacylation is slow or rate limiting during turnover. Similar results were obtained by Jencks et al. (1972) for pigeon liver arylamine *N*-acetyltransferase with *p*-nitrophenyl acetate as the acetyl donor. When the acetyl-CoA concentration is 25 μ M the enzyme becomes susceptible to inactivation when the choline concentration is increased to 7.5 mM. The resistant form is

Table III: Effect of Substrates and Reactive Blue 2 on *N*-Ethylmaleimide Inhibition of Choline Acetyltransferase.^a

Addition	Enzyme Activity	
	Brain (%)	Placenta (%)
100 μ M acetyl-CoA	100	100
7.5 mM choline	6	7
5 mM acetylcholine	44	47
50 μ M acetylthiocholine	99	100
100 μ M Reactive blue 2 ^b	9	6
100 μ M acetyl-CoA + 7.5 mM choline	97	98
25 μ M acetyl-CoA + 7.5 mM choline	41	46
25 μ M acetyl-CoA + 2.5 mM choline	96	98

^aThe experiment was carried out as described in Table II, except the enzyme (0.6 μ g) was incubated 5 min at 30° with the specified addition prior to incubation with *N*-ethylmaleimide (50 μ M). Control brain and placental transferase activities were 224 and 209 pmol of product per 10-min incubation, respectively. ^bA fivefold concentrated enzyme solution containing Reactive blue 2 was used for this preincubation. After diluting fivefold to eliminate the chromophore inhibition, transferase activity was determined.

Table IV: pH Dependence of *N*-Ethylmaleimide Inhibition of Human Brain Choline Acetyltransferase.^a

pH	K_{app} (min ⁻¹)	pH	K_{app} (min ⁻¹)
7.8	0.27	8.3	0.54
8.0	0.35	8.5	0.68
8.1	0.40	8.6	0.75

^aThe *N*-ethylmaleimide treatment was performed as described in Table II using 3 μ g of enzyme, 20 mM Tricine, and 100 mM KCl at the specified pH. To adjust the pH for activity measurement, aliquots (40 μ l) of potassium phosphate (0.3 M, pH 7.4) were added to the solution and samples (10 μ l) were assayed for transferase activity. The rate constants were calculated by the procedure of Burstein and coworkers (1974).

converted to an inhibitable form when the rate of deacylation is increased by increasing the choline concentration in the presence of low (25 μ M) acetyl-CoA concentrations.

Unlike acetyl-CoA, Reactive blue 2, the nucleotide analog, fails to protect the enzymes against *N*-ethylmaleimide inhibition. This indicates that the nucleotide binding site is somewhat removed from the residue associated with chemical modification and inhibition. The studies on substrate protection and choline deprotection have been carried out with Nbs₂ (50 μ M, pH 7.4, 5-min incubation) and the results parallel those reported here for *N*-ethylmaleimide. Reactive blue 2 fails to protect against Nbs₂ reagent inhibition. Furthermore, Nbs₂-treated enzyme binds to blue dextran-Sepharose and activity can be regenerated by dithiothreitol after elution with salt.

Determination of the pK_a of the Residue Reacting with *N*-Ethylmaleimide. The pH dependence of *N*-ethylmaleimide inhibition of human brain and placental transferases was measured after a 5-min incubation. This short incubation time was chosen to eliminate appreciable reaction of inhibitor with water under alkaline conditions. The apparent first-order rate constants (K_{app}) were calculated using the methodology of Burstein and coworkers (1974). A pK_a of 8.6 ± 0.3 was calculated for the human brain enzyme from the slope of the plot using the data given in Table IV. The value obtained from the placental choline acetyltransferase is indistinguishable. These experiments were performed in

Table V: Formation of [¹⁴C] Acetyl-Enzyme Complexes from [¹⁴C] Acetyl-CoA and Human Brain Choline Acetyltransferase.^a

Expt	Trichloroacetic Acid Precipitate	Sephadex Gel Filtration
Control	41	44
20-sec incubation	40	
7.5 mM choline, 1 min	38	
7.5 mM choline, 15 min	0.7	
<i>N</i> -Ethylmaleimide, 50 μ M	0.3	0.5

^aEnzyme (20 μ g) was incubated with 100 μ M [¹⁴C] acetyl-CoA for 15 min (unless otherwise specified) at 30° in 50 μ l to prepare the acetyl-enzyme intermediate. The radioactivity associated with enzyme protein after Sephadex gel filtration was measured as previously described (Roskoski, 1973, 1974c). The radiolabel associated with trichloroacetic acid insoluble material was measured by the procedure of Mans and Novelli (1961).

triplicate. The pK_a is in the range expected for a thiol group in protein (Tanford and Hauenstein, 1956).

Stoichiometry of Acetyl-Enzyme Formation. A chemically competent acetyl-enzyme intermediate was isolated by Sephadex gel filtration using the bovine brain choline acetyltransferase (Roskoski, 1973, 1974c). When the human brain enzyme is incubated with [¹⁴C]acetyl-CoA (in the presence of *N*-ethylmaleimide to discharge CoA, Roskoski, 1974c), and the reaction mixture is passed through a Sephadex G-50 column, the [¹⁴C]acetyl group is associated with the enzyme, but CoA is not. In agreement with previous studies (Roskoski, 1973, 1974c), the alleged acetyl-enzyme is chemically competent, and shows the chemical characteristics of a thio ester including trichloroacetic acid stability, cleavage by dilute alkali (pH 10), and performic acid oxidizability. Both the purified human brain and placental enzyme form this chemically competent intermediate. The stoichiometry of the reaction, however, shows that less than 10% of the enzyme protein, assuming a molecular weight of 67000, forms such a linkage (Table V). The stoichiometry determined by trichloroacetic acid precipitation agrees with that determined by Sephadex gel filtration. In either case, performic acid oxidation liberates the labeled acetyl group, which was identified by thin-layer chromatography (Roskoski, 1973), providing firm evidence for the thio ester nature of the bond (Harris and coworkers, 1963). Addition of choline for 1 min, in the presence of [¹⁴C]acetyl-CoA, fails to decrease the label bound as thio ester. This is consonant with the notion that acylation of enzyme is rapid, and deacylation is slow. After a 15-min incubation with choline, which results in the depletion of labeled acetyl-CoA, the amount of bound radioactivity decreases 90%. This indicates that the bound acetyl group turns over. Furthermore, *N*-ethylmaleimide abolishes the formation of the thio ester. The acetyl-enzyme intermediate formation is complete during the shortest period of incubation feasible without rapid reaction techniques (20 sec).

Discussion

Choline acetyltransferase from bovine brain (Choa and Wolfgram, 1973), human caudate nucleus (Singh and McGeer, 1974), and human placenta (Table I) has been purified to electrophoretic homogeneity. The enzyme absorbs to CM-Sephadex and fails to absorb to DEAE-Sephadex, properties which are used to advantage in the present purification. Thompson and coworkers (1975) propose that blue dextran-Sepharose may be generally useful in the purification

tion of enzymes which contain the dinucleotide fold, a number of examples of which they have documented. More definitive experiments, however, are required to establish the presence of this super secondary structure in choline acetyltransferase. A portion of the chromophore of blue dextran, C.I. Reactive blue 2, is analogous to the adenosine diphosphoryl portion of acetyl-CoA (Thompson et al., 1975). Furthermore, the chromophore is a competitive inhibitor (with acetyl-CoA as variable substrate) of brain and placental choline acetyltransferase. Commercially available affinity resins with CoA linked to matrix by a thio ester linkage (P.L. Biochemicals, Inc.) are also efficacious in purifying these enzymes. However, it is more expensive and offers no advantage over blue dextran-Sepharose.

The finding that only 10% of the protein forms a thio ester with acetyl-CoA as substrate raises the possibility that the acetyl-thioprotein intermediate is adventitious. Further studies are required to demonstrate that the rate constants for the formation and further reaction of the postulated acetyl-enzyme intermediate are adequate to account for the observed rate of the enzymic reaction. The complete protection against *N*-ethylmaleimide inhibition (Table III) indicates that acetyl-CoA converts all active enzyme to a resistant form. Failure to find a 1:1 stoichiometry of acetate and enzyme may be related to breakdown of the intermediate during isolation, to the presence of inactive enzyme or other proteins, or to the possibility that the isolated acetyl-thioenzyme represents a minor pathway. The calculated turnover number for the purified acetyltransferases is very low. Using 67000 as the molecular weight and the reported specific activity, the turnover numbers of purified bovine brain (Choa and Wolfgram, 1973), human brain (R. Roskoski, unpublished), and human placenta (Table I) are 97, 3.06, and 2.61 min⁻¹, respectively. In addition to an inherently low turnover number, these values may reflect the presence of inactive choline acetyltransferase or other proteins in the preparation. The preparation of antibodies to the human brain enzyme, and localization of the enzyme in the known cholinergic anterior horn cells of spinal cord (McGeer and coworkers, 1974), provide evidence that the protein is choline acetyltransferase. Because of the marked loss of activity during the later stages of purification, we favor the view that the preparations contain inactive enzyme. Indeed, two major difficulties in studying this enzyme include low yields and enzyme instability.

The kinetic data from other laboratories with transferase from bovine brain (White and Cavallito, 1970; Glover and Potter, 1971), human brain (White and Wu, 1973), and human placenta (Morris and Grewaal, 1971; Morris and coworkers, 1971; Rama Sastry and Henderson, 1972) show converging double reciprocal plots to the left of the ordinate. These studies, along with the results with product inhibition, are consistent with sequential or Theorell-Chance mechanisms. Sensitivity to thiol reagents, protection by acetyl donor substrates, and protection during turnover provide evidence for an acetyl-enzyme intermediate for bovine (Roskoski, 1974b) and human brain and placental transferase (Table III). The isolation of a chemically competent acetyl-thioenzyme (Roskoski, 1973, 1974c) supports this scheme. These studies suggest that choline reacts with the postulated acetyl-thioenzyme before CoA dissociation. A covalent enzyme-substrate intermediate is not excluded when kinetics are not of the parallel line type. For example, the kinetic studies with *E. coli* succinate thiokinase are consistent with a sequential mechanism in which all three sub-

strates combine with enzyme before release of product (Moffet and Bridger, 1970), even though the reaction involves a kinetically competent phosphoenzyme intermediate (Bridger et al. 1968).

The human brain and placental enzymes are physically and biochemically indistinguishable. Furthermore, antibodies prepared against the human brain enzyme react monospecifically with the human placental choline acetyltransferase. The bovine brain enzyme also resembles the human enzymes in the parameters which have been examined thus far. Although future experiments may show differences in the primary structures, it seems likely that conclusions obtained from the study of one enzyme will hold for the others.

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Mechanism and Specificity of Rhodopsin Phosphorylation[†]

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ABSTRACT: Partial separation of protein kinase activity from rhodopsin in isolated bovine retinal photoreceptor outer segments was accomplished by mild ultrasonic treatment followed by ultracentrifugation. Residual kinase activity in the rhodopsin-rich sediment was destroyed by chemical denaturation which did not affect the spectral properties of the rhodopsin. The retinal outer segment kinase was found to be specific for rhodopsin, since in these preparations it alone of several bovine protein kinases was capable of phosphorylating rhodopsin in the light. The phosphorylation reaction apparently requires a specific conformation of the rhodopsin molecule since it is abolished by heat denaturation of rhodopsin, and it is greatly reduced or abolished by treatment of the visual pigment protein with potassium alum after the rhodopsin has been "bleached" by light. When kinase and rhodopsin or opsin fractions were prepared from dark-adapted and bleached outer segments

and the resultant fractions were mixed in various combinations of bleached and unbleached preparations, the observed pattern of light-activated phosphorylation was consistent only with the interpretation that a conformational change in the rhodopsin molecule in the light exposes a site on the visual pigment protein to the kinase and ATP. These results rule out the possibility of a direct or indirect (rhodopsin-mediated) light activation of the kinase. Finally, phosphorylation of retinal outer segment protein in monochromatic lights of various wavelengths followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicates that both rhodopsin and the higher molecular weight visual pigment protein reported by several laboratories have the same action spectrum for phosphorylation. This result is consistent with the suggestion that the higher molecular weight species is a rhodopsin dimer.

Rhodopsin, the visual pigment of the vertebrate retinal rod outer segments, undergoes phosphorylation by ATP or GTP in the light in a reaction that requires magnesium but is not stimulated by cyclic nucleotides (Kühn and Dreyer, 1972; Bownds et al., 1972; Frank et al., 1973; Chader et al., 1975). Rhodopsin phosphorylation and a subsequent slow dephosphorylation have been shown to take place in vivo in the retinal rods of the frog (Kühn, 1974), but the role of these reactions in the normal physiology of the vertebrate visual cell is unknown. A previous report from our laboratory (Frank and Bensinger, 1974) and a recent communication from another group of investigators (Weller et al., 1975a) have dealt with the mechanism of the light activation of rhodopsin phosphorylation as well as the specificity of the retinal outer segment protein kinase for rhodopsin. Both reports suggested that the activation of rhodopsin

phosphorylation by light results from the exposure of a phosphate-acceptor site on the visual pigment molecule to the nucleoside triphosphate and the enzyme. In addition, these studies indicated that rhodopsin is the specific substrate for a retinal outer segment protein kinase. However, as we shall discuss, the evidence thus far presented in support of these conclusions is incomplete. We now therefore describe experiments that were designed to examine these questions in further detail. Our results offer more concrete evidence in support of the conclusions of the two earlier reports, and give additional information on visual pigment phosphorylation.

Materials and Methods

Preparation of Retinal Outer Segments. Frozen, dark-adapted bovine retinas obtained from the George Hormel Co., Austin, Minn., were used in all of our experiments. Retinal outer segments were isolated by differential centrifugation on 40% sucrose in 50 mM Tris-HCl buffer (pH 7.4) as described previously (Frank et al., 1973). Outer seg-

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