Role of Tryptophan in Dihydrofolate Reductase†

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ABSTRACT: Dihydrofolate reductase from an amethopterin-resistant strain of *Streptococcus faecium* contains nine tryptophan residues and one cysteine residue per mole of protein (mol wt 20,000). At molar ratios of *N*-bromosuccinimide to enzyme of up to 4:1 a 10–20% *increase* in catalytic activity is observed. This activation is thought to be due to an incomplete oxidation of the single sulfhydryl group on the protein since it can be reversed by dithiothreitol. Higher concentrations of *N*-bromosuccinimide lead to decreases in enzyme activity. Complete loss of activity corresponds to the oxidation of two tryptophan residues. Other residues amenable to attack by *N*-bromosuccinimide are not altered. The oxidation of these two critical tryptophans drastically reduces the aromatic side-chain Cotton effect of the native enzyme in the 260- to 295-nm region of its circular dichroic absorption spectrum. In addition, the evidence indicates that each of these two critical tryptophan residues contributes equally to the aromatic side-chain Cotton effect in this spectral region.

Recent studies from this laboratory have shown that the binding of pyridine nucleotides (TPNH and TPN^+) or folate analogs (dihydrofolate, folate, or amethopterin) to streptococcal dihydrofolate reductase results in large changes in the magnitude of the aromatic side-chain Cotton effect of the enzyme in the 260–310 nm of its circular dichroic (CD) spectrum (Freisheim and D'Souza, 1971; D'Souza and Freisheim, 1972). Decreases in the fluorescence of the protein occur upon formation of such enzyme–ligand complexes.[1] Based on these findings, tryptophan residues have been postulated to be involved in the binding of these folate analogs and pyridine nucleotide coenzymes (D'Souza and Freisheim, 1972).

The present study was undertaken in order to investigate more directly the role of tryptophan residues in substrate and inhibitor binding and in the catalytic mechanism of streptococcal dihydrofolate reductase. NBS[2] has been used successfully to study tryptophan residues in proteins (Spande *et al.*, 1966; Patchornik *et al.*, 1958; Witkop, 1968; Viswanatha and Lawson, 1961). This reagent has also been employed to identify certain critical tryptophan residues essential to catalytic function in ribonuclease T_1 (Kawashima and Toshio, 1969), lysozyme (Hayashi *et al.*, 1965), DNase (Poulos and Price, 1971), and in chicken liver dihydrofolate reductase (Freisheim and Huennekens, 1969). Oxidation of tryptophan residues, changes in catalytic activity, and changes in the aromatic side-chain Cotton effect of streptococcal dihydrofolate reductase were followed as a function of the molar ratio of NBS to enzyme.

Experimental Section

Materials. Dihydrofolate reductase was isolated and purified from an amethopterin-resistant strain of *Streptococcus faecium* according to the procedure of D'Souza *et al.* (1972). TPNH and dithiothreitol were obtained from P-L Biochemicals; NBS was obtained from Matheson Coleman & Bell. Dihydrofolic acid was prepared by the reduction of folic acid (Sigma) with sodium dithionite according to the method of Futterman (1957), as modified by Blakley (1960). Dihydrofolic acid was washed repeatedly with 5×10^{-3} N HCl, lyophilized, and stored in evacuated, sealed tubes. All other chemicals were of reagent or analytical grade.

Methods. In general, the experimental approach consisted of adding increments of NBS to solutions of dihydrofolate reductase and measuring one or both of the following parameters: (a) destruction of tryptophan residues and (b) changes in enzymatic activity.

Oxidation of tryptophan residues was measured by the following procedure. Dihydrofolate reductase (*ca.* $(1–2) \times 10^{-5}$ M in 3 ml of 0.10 M KPO_4 of the desired pH) was placed in a quartz cuvet having a light path of 1 cm. Successive 5–10-μl aliquots of aqueous solutions of NBS were added to each cuvet and mixed, and the absorbance at 280 nm was measured in a Cary Model 15 recording spectrophotometer at 25°. The blank cuvets contained only buffer. Following each addition of NBS, the number of tryptophan residues oxidized per mole of enzyme was calculated from eq 1 (Witkop, 1961; Green and Witkop, 1964),

$$\Delta n = \frac{1.31 \Delta A_{280}}{5500 \times \text{molarity of enzyme}} \quad (1)$$

where Δn is the number of tryptophans oxidized, ΔA_{280} is the decrease in absorbance at 280 nm, 1.31 is an empirical factor based upon oxidation of model tryptophan peptides by NBS (Patchornik *et al.*, 1958), and 5500 is the molar extinction coefficient for tryptophan at 280 nm.

The maximum changes in enzyme activity or absorbance at 280 nm occurred in the first 2–3 min following each addition of NBS. The enzyme assays and spectral measurements of the modified protein were performed routinely after 10-min reaction with the oxidant. In substrate protection experiments using either TPNH or dihydrofolate, the decreases in enzyme activity were also maximal after a 10-min reaction time at all concentrations of substrates employed. NBS had no deleterious effect on either substrate at the concentrations used as judged by spectral data and in enzyme rate assays.

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[1] L. D'Souza and J. H. Freisheim, in preparation.

[2] Abbreviation used is: NBS, *N*-bromosuccinimide.

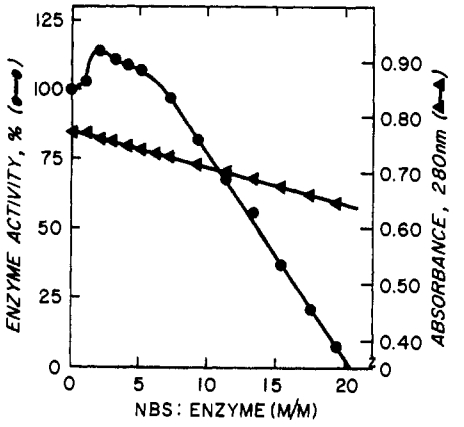

FIGURE 1: Activity and absorbance changes of dihydrofolate reductase as a function of molar excess of NBS. Enzyme concentration was 9.8×10^{-8} M in 0.05 M KPO_4 (pH 6.5). Enzyme activity is expressed as per cent of untreated control.

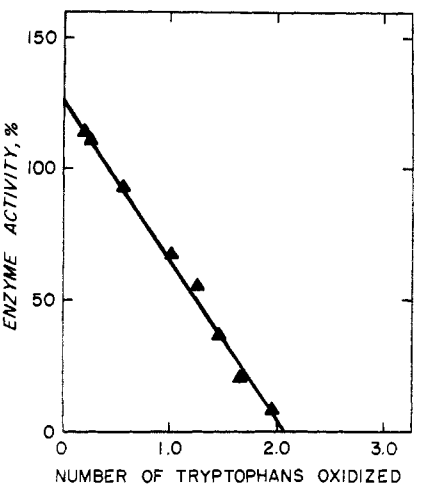

FIGURE 2: Activity of dihydrofolate reductase as a function of the number of tryptophans oxidized. Experimental conditions as in Figure 1.

Enzyme activity was measured spectrophotometrically (Mathews and Huennekens, 1963) by following the decrease in absorbance at 340 nm using a Beckman Model DU spectrophotometer equipped with a Gilford Model 208 auxiliary offset control unit, a Gilford Model 210 automatic cuvet positioner and a Sargent Model SRLG recorder. The assay mixture in a volume of 1.0 ml contained 50 mM KPO_4 buffer (pH 6.5), 33 μmoles of TPNH and 50 μmoles of dihydrofolate. The assays were performed at 25°. The concentrations of NBS in the assay ($<10^{-7}$ M) had no effect on enzymatic activity. The enzyme concentration in the assay was approximately $(2\text{–}8) \times 10^{-9}$ M.

The effect of NBS on the near-ultraviolet CD absorption spectrum of native dihydrofolate reductase was determined using a Cary Model 60 spectropolarimeter with a Model 6001 CD attachment.

Amino acid analyses were performed according to Spackman *et al.* (1958) on the Beckman amino acid analyzer, Model 120C. Hydrolyses were done at 110° for 24 hr in 6 N HCl. Any variations from the above protocols are given in the legends for tables and figures.

Results and Discussion

Effect of NBS on Enzyme Activity. The effect of NBS on the catalytic activity of dihydrofolate reductase was examined at pH 6.5, the pH optimum of the enzyme (D'Souza *et al.*, 1972). The results, shown in Figure 1, indicate that the action of NBS occurs in two stages. At relatively low concentrations of NBS (2–4 moles/mole of enzyme), NBS initially *activates* the enzyme (10–20%). At higher reagent concentrations the enzyme undergoes a linear inactivation. Previous studies by Freisheim and Huennekens (1969) have shown that the same behavior (*i.e.*, activation followed by inhibition) occurs upon titration of chicken liver dihydrofolate reductase with NBS. The activation–inhibition response has also been observed previously when dihydrofolate reductases from various sources are treated with various chaotropic agents such as urea or high salt concentrations (Bertino, 1962; Kaufman, 1963; Perkins and Bertino, 1964, 1965; Reyes and Huennekens, 1967). Even at relatively low concentrations of NBS tryptophan residues of streptococcal dihydrofolate reductase are oxidized as evidenced by the linear decrease in absorbance at 280 nm (Figure 1). Complete inactivation of streptococcal dihydrofolate reductase occurs when 20 moles of oxidant is added per mole of enzyme. When loss of enzyme activity is related to decreases in absorbance at 280 nm, the complete inactivation of dihydrofolate reductase corresponds to the oxidation of two tryptophans (Figure 2) of a total of nine such residues (D'Souza *et al.*, 1972).

Mechanism of Activation by NBS. Extrapolation of the linear inactivation of dihydrofolate reductase to the ordinate (Figure 2) suggests that the activated enzyme (128% of the initial activity) is the species which is further modified by NBS leading to a complete loss of catalytic function. Thus, it appeared from these experiments that the activation phase might involve an attack of NBS on some group other than the indolyl moiety of tryptophan. Although NBS is considered to be fairly selective for the oxidation of tryptophan residues, effects on other functional groups have been observed (Ramachandran and Witkop, 1959; Schmir and Cohen, 1961; Ramachandran, 1962). Histidine, methionine, and tyrosine, as well as tryptophan, residues may be modified by NBS depending on the conditions of pH and ionic strength of the reaction (Shaltiel and Patchornik, 1963). Amino acid analysis[3] of NBS-inactivated dihydrofolate reductase showed that the histidine, methionine, and tyrosine residue content of the protein remained essentially unaltered (Table I) when two of nine total tryptophan residues are oxidized (*cf.* Figure 2). However, the single cysteine residue also appeared to be a likely candidate for attack by NBS.

The catalytic activity of the enzyme from several sources is increased when a sulfhydryl group is modified by alkylation with mercurials (Kaufman, 1964; Perkins and Bertino, 1965), oxidation with iodine (Kaufman, 1966), or formation of a mixed disulfide with 5,5′-dithiobis(2-nitrobenzoic acid) (Reyes and Huennekens, 1967). In addition, Freisheim and Huennekens (1969) concluded that the NBS-dependent activation of chicken liver dihydrofolate reductase is due to modification of one of two cysteine residues. Streptococcal dihydrofolate reductase contains one cysteine residue per mole of enzyme (D'Souza *et al.*, 1972). It appeared likely, therefore, that the activation observed with NBS might also be due to some

[3] We are very indebted to Dr. K. A. Walsh (University of Washington) for his aid in the amino acid analyses.

TABLE I: Amino Acid Analysis of NBS-Modified Dihydrofolate Reductase.[a]

	Histidine	Methionine	Tyrosine
Native enzyme	4.7	5.8	5.7
NBS inactivated[b]	4.7	5.6	5.4

[a] Each sample contained 3.8 mg of dihydrofolate reductase in 3.0 ml of 0.05 M KPO_4 (pH 6.5). Following complete inactivation with NBS the samples were extensively dialyzed *vs.* glass-distilled water, lyophilized, and hydrolyzed in 6 N HCl for 24 hr in evacuated tubes. The values indicated are amino acid residues per mole of protein based on 20 aspartic acid residues per molecule from the amino acid composition (D'Souza *et al.*, 1972). Half-cystine was not detected in either the native or NBS-modified enzymes. [b] Average of two determinations.

TABLE II: Effect of Variation of the Molar Excess of Substrates on the Inactivation of Dihydrofolate Reductase.

Molar Ratio, Substrate:Enzyme[a]		Molar Ratio, NBS:Enzyme for Complete Inactivation[b]
TPNH:Enzyme	Dihydrofolate:Enzyme	
0	0	14.5
1.8		15.0
	1.9	14.5
5.2		19.0
	5.0	16.0
17.5		31.0
	16	22.2
23		41.0
	23	32.6

[a] The enzyme (1.2×10^{-6} M in 0.05 M KPO_4, pH 6.5) was incubated with either TPNH or dihydrofolate in a total volume of 1.0 ml for 20 min prior to additions of NBS. [b] At enzyme concentrations of *ca.* 10^{-6} M, only 14.5 moles of NBS were required for complete enzyme inactivation. At higher enzyme concentrations the ratio of oxidant to enzyme had to be increased to effect complete inactivation (*cf.* Figure 1).

modification of this sulfhydryl group. NBS is known to oxidize cysteine in proteins (Witkop, 1961) and, moreover, in the case of bovine serum albumin and hemoglobin A (which contain one and eight cysteines per molecule, respectively), 5 and 20–30 moles of NBS are consumed prior to any decrease in absorbance at 280 nm (Hughes and Straessle, 1950; Ingram, 1955; Benesch *et al.*, 1955).

When a fourfold molar excess of reagent was added to the streptococcal reductase (1.2×10^{-6} M) in 0.10 M KPO_4 (pH 8.0) the enzyme activity was 120% that of a control without added NBS. Addition of dithiothreitol (2.2×10^{-5} M in the assay mixture) reversed the activation to the control level suggesting that, indeed, a modification of the cysteine residue had occurred. The chemical nature of the NBS-modified cysteine residue in dihydrofolate reductase has not yet been established. However, the sulfhydryl group might have undergone oxidation to the level of a sulfenic acid, consistent with Kaufman's proposal that activation of the chicken liver reductase by iodine involves the formation of a sulfenyl iodide on the protein (Kaufman, 1966). The maximum activation of dihydrofolate reductase occurs when the molar ratio of NBS to enzyme is between 2 and 4 (*cf.* Figure 1). Since oxidation of the sulfenic acid stage would require at least 2 moles of reagent/mole of enzyme, it is not likely that a higher oxidation state is involved. The ability of dithiothreitol to reverse the activation likewise tends to rule out oxidation states above the level of a sulfenic acid.

One explanation of the data is that NBS oxidation of the enzyme at pH 6.5 (Figures 1 and 2) involves two reactive groups at low concentrations of reagent. Tryptophan is oxidized at low levels of oxidant (Figure 1), but the single cysteine residue appears to be accessible as well. Thus, both tryptophan residues and the single cysteine residue appear to compete for the available reagent at low concentrations. The fact that the activation does not reach the theroetical maximum (compare Figures 1 and 2) supports the assumption that a competition between the two different types of residues for the available oxidant occurs.

Substrate Protection against NBS Inactivation. The results of incubation of the enzyme with various concentrations of either TPNH or dihydrofolate prior to treatment with NBS are indicated in Table II. At molar ratios of either TPNH or dihydrofolate to enzyme of *ca.* 2:1 no protection against enzyme inactivation is afforded. However, as the concentration of either substrate or coenzyme is increased the amount of reagent per mole of enzyme necessary to effect complete inactivation is correspondingly increased. NBS has no effect on the spectral properties of either dihydrofolate or TPNH under the conditions of the experiments. Both TPNH and dihydrofolate afford approximately the same degree of protection against inactivation of the enzyme by NBS, TPNH being slightly more effective in this regard.

Effect of NBS on the CD Spectrum of Dihydrofolate Reductase. Streptococcal dihydrofolate reductase shows a marked aromatic side-chain Cotton effect in the 260- to 310-nm region of its CD spectrum (D'Souza and Freisheim, 1972). Interaction of the enzyme with TPNH results in the generation of an extrinsic Cotton effect at *ca.* 340 nm and a decrease in the magnitude of the aromatic side-chain Cotton effect (Freisheim and D'Souza, 1971). The binding of folate analogs (dihydrofolate, folate, or amethopterin) generates a large enhancement of the molar ellipticity of the protein in the 270- to 315-nm region (D'Souza and Freisheim, 1972). Based on these findings and on enzyme–ligand fluorimetric studies[1] it was suggested that one or more tryptophan residues might play an important role in substrate, inhibitor, and coenzyme binding. The effect of NBS modification on the CD spectrum of the enzyme is indicated in Figure 3. The CD absorption of the native enzyme in the 250- to 300-nm region of the spectrum is markedly decreased following reaction with NBS. Between 265 and *ca.* 295 nm the positive side-chain Cotton effect is obliterated and the ellipticity values reach a minimum when two of the nine tryptophan residues are oxidized. The ellipticity band at 305 nm is virtually unaffected by the oxidation of the two tryptophans which results in complete inactivation of the enzyme. This band may represent contributions from transitions of nonfunctional tryptophan residues, tyrosine residues, or both. For purposes of comparison the changes in

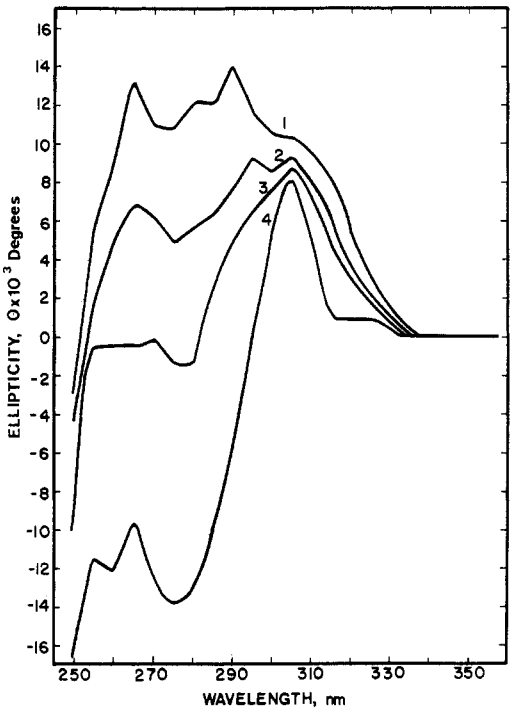

FIGURE 3: Effect of NBS on the CD absorption spectrum of dihydrofolate reductase in the 250- to 350-nm region. Increments of NBS were added to a 3-ml solution of enzyme (3.5×10^{-5} M in 0.05 M KPO_4, pH 6.5). Curve 1 = native enzyme; curve 2 = enzyme with 0.5 tryptophan residue oxidized per mole of protein; curve 3 = enzyme with 1.0 tryptophan residue oxidized per mole of protein; curve 4 = enzyme with 2.0 residues of tryptophan oxidized per mole of protein.

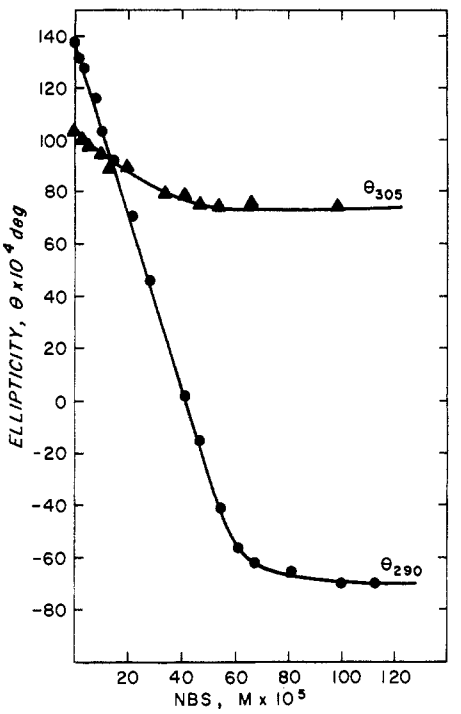

FIGURE 4: Decreases in the ellipticity at 290 and at 305 nm of dihydrofolate reductase as a function of the concentration of NBS added. Experimental conditions were as in Figure 3.

ellipticity at 305 nm and at 290 nm of the native enzyme are followed as a function of NBS concentration (Figure 4). The maximum decrease in ellipticity at 290 nm is about seven times greater than that observed at 305 nm. The complete oxidation of 2 equiv of tryptophan residues of the enzyme (3.5×10^{-5} M) requires approximately 70×10^{-5} M NBS, where the maximum change in ellipticity at 290 nm *vs.* NBS concentration is linear, suggesting that both tryptophans contribute equally to the CD absorption in this spectral region, particularly since tryosines are not modified to an appreciable extent (*cf.* Table I).

In certain polypeptides and proteins molar excesses of NBS to tryptophan of up to 10-fold are required for complete oxidation (Witkop, 1961). Freisheim and Huennekens (1969) have found that over a 10-fold molar excess of NBS is required to oxidize one functionally critical tryptophan in chicken liver dihydrofolate reductase. In the case of the streptococcal reductase, a 20-fold molar excess of reagent is required to oxidize two of nine tryptophans with a concomitant complete loss of enzymatic activity. Thus, it is reasonable to assume that seven tryptophan residues may be buried within the hydrophobic interior of the protein, while two are relatively more exposed to solvent. The indolyl moieties of two essential tryptophan residues may interact in a stacked-card arrangement with the pteridine ring of the folate analogs as well as with the adenine and nicotinamide rings of TPNH through π–π bonding interactions. Such interactions have been proposed to account for the large enhancements in molar ellipticity of certain regions of the aromatic side-chain Cotton effect of streptococcal dihydrofolate reductase upon asymmetric binding of substrates, cofactors, or inhibitors (D'Souza and Freisheim, 1972).

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Acetyl Coenzyme A Carboxylase. Purification and Properties of the Bovine Adipose Tissue Enzyme†

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ABSTRACT: Acetyl-CoA carboxylase has been purified over 1000-fold from bovine perirenal adipose tissue. Sedimentation velocity analysis indicates that the enzyme is homogeneous and can exist in polymeric and protomeric states. The polymeric form in the presence of citrate has a filamentous structure, as revealed by electron microscopy, and a high sedimentation coefficient ($s_{20,w}$ = 68 S). Treatment of the polymeric carboxylase with 0.5 M NaCl at pH 9.0 results in its dissociation to a protomeric species having an $s^0_{20,w}$ of 14.7 S and a molecular weight of approximately 560,000. The bovine adipose tissue enzyme is activated by citrate and this activation is associated with an increase in maximal velocity of the carboxylase-catalyzed reaction with no significant effect on K_m values for ATP (Mg^{2+}), bicarbonate, or acetyl-CoA. Following the addition of the polymeric form of the enzyme to assay reaction mixture minus tricarboxylic acid activator, there is a gradual decline in catalytic activity to a level less than 2% of that obtained in the presence of citrate. This decay appears to be associated with depolymerization to the less active protomeric form. Reversibility of the decay in activity is indicated by the nearly instantaneous reactivation produced by citrate. Reactivation is associated with the activator-initiated transition from a 13–15S protomeric species to a 47–50S polymeric form, respectively, as determined by sucrose density gradient centrifugation under assay conditions. Relatively impure carboxylase preparations are markedly activated by (+)-palmitylcarnitine, bovine serum albumin, and dilution, in addition to the citrate activation normally observed. Together these factors produced an activation effect of over 23-fold. Since these activating conditions have no effect on the homogeneous carboxylase, it is evident that this capacity for activation (or deinhibition) is lost during the course of purification. Dilution, (+)-palmitylcarnitine, bovine serum albumin, and purification probably act by a common mechanism, possibly by the removal of an endogenous inhibitory hydrophobic material which is bound to the impure carboxylase.

Acetyl-CoA carboxylases from animal tissues have been shown to be activated by certain tricarboxylic acids, notably citrate and isocitrate (Matsuhashi *et al.*, 1962; Kallen and Lowenstein, 1962; Waite, 1962; Martin and Vagelos, 1962a,b; Waite and Wakil, 1962; Lane and Moss, 1971b; Moss and Lane, 1971), this activation being associated with an increased sedimentation velocity (Vagelos *et al.*, 1962, 1963; Matsuhashi *et al.*, 1964; Numa *et al.*, 1965a,b). In the case of the avian liver carboxylase, the activation and increased sedimentation velocity were shown to be concurrent with the citrate-promoted polymerization of weight homogeneous protomeric subunits of 410,000 (Gregolin *et al.*, 1966b; Gregolin *et al.*, 1968b). The polymerized form of the liver carboxylase has a molecular weight of 4–10 million and was found by electron microscopy to have a unique filamentous structure (Gregolin *et al.*, 1966a). Acetyl-CoA carboxylase, from bovine adipose tissue, recently obtained in our laboratory in homogeneous form, was also found to have a similar filamentous structure (Kleinschmidt *et al.*, 1969).

It has been reported that relatively impure preparations of acetyl-CoA carboxylase from rat liver were markedly activated by (+)-palmitylcarnitine (Fritz and Hsu, 1967; Green-

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