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Carboxylesterases (EC 3.1.1). Purification and Titration of Ox Liver Carboxylesterase*

Maria T. C. Runnegar,† Keith Scott, Edwin C. Webb, and Burt Zerner

ABSTRACT: Ox liver carboxylesterase has been reproducibly purified on a large scale from an acetone powder. The enzyme, while significantly purified, is not electrophoretically homogeneous and consists of three major proteins, here called electrophoretic variants 1, 2, and 3. All three variants are very similar protein molecules, apparently distinguished by small charge differences.

The equivalent weight of the enzyme has been estimated at ~68,000 based on titration with *o*- and *p*-nitrophenyl dimethylcarbamates and *p*-nitrophenyl diethyl phosphate.

While there are early reports on the partial purification of ox liver carboxylesterase (White, 1956; Kirkland, 1963), the first substantial purification was that of Benöhr and Krisch (1967) whose method is similar to that reported earlier for the pig liver enzyme (Krisch, 1963) and suffers from the same disadvantages (Horgan *et al.*, 1969a). As part of an extensive investigation of plant and animal carboxylesterases, the purification of ox liver carboxylesterase was undertaken, and the results are reported in this paper.

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Experimental Section

General Methods. Spectrophotometric protein determination, pH-Stat rate assays, starch gel electrophoresis using a discontinuous buffer system (Poulik, 1957; Kristjansson and Hickman, 1965), polyacrylamide gel electrophoresis at a constant current of

TABLE I: Purification of Ox Liver Carboxylesterase from an Acetone Powder.

Step	Total Protein[f] (g)	A_{280}/A_{260}	A_{280}/A_{410}	Sp Act.	% Yield
Extraction[a]	255	1	4	1.5	100
45–70% saturated ammonium sulfate cut[b]	39	1.34	6	6.8	67
CM-cellulose[c]	7	1.10	62	32.6	58
CM-Sephadex[d]					
Fast component	0.412	1.74	190	73	7.6
Mixed component	0.954	1.69	120	67	16.2
Slow component	0.245	1.60	150	68	4.2
					28
Sephadex G-100[e]					
Fast component	0.277	1.73	>200	77	5.4
Mixed component	0.692	1.78	>200	78	14
Slow component	0.153	1.67	>200	73	2.8
					22.2

[a] 395,000 units were extracted from 1200 g of powder in three extractions. [b] The ammonium sulfate cut was dialyzed *vs.* 0.025 M citrate (pH 5.6). [c] Column dimensions, 5 × 90 cm. The column buffer was 0.025 M citrate (pH 5.6) and the esterase did not bind. The step was repeated three times. [d] Column dimensions, 5 × 90 cm. Enzyme was concentrated with 80% saturated ammonium sulfate and dialyzed against the starting buffer (0.05 M acetate, pH 5.30). After thorough washing with starting buffer, the bound esterase was eluted over the region 4.4–5.0 l. of a linear gradient consisting of 3 l. of starting buffer and 3 l. of 0.2 M sodium acetate. [e] Column dimensions, 3.3 × 70 cm. The "fast," "mixed," and "slow" regions of Figure 1 were individually concentrated with 80% saturated ammonium sulfate, dialyzed *vs.* 0.05 M Tris (pH 7.6), and gel filtered in the same buffer, each component being handled in two stages. [f] For the present purpose, a solution having an $A_{280} = 1$ is defined as containing 1 mg/ml of protein.

2 mA/gel (~400 V) for 2 hr in 7% gels, the preparation of acetone powders, ammonium sulfate precipitation, and the source, preparation, and regeneration of chromatographic media are described by Horgan *et al.* (1969a).

One unit of carboxylesterase activity is defined as the amount of enzyme required to catalyze the hydrolysis of 1 μmole of ethyl butyrate/min under the defined assay conditions (Horgan *et al.*, 1969a). Specific activity is equal to activity (in units/ml)/A_{280}.

Extraction of Carboxylesterase from Acetone Powders. Acetone powder (400 g) was extracted with 3.6 l. of 0.1 M citrate buffer (pH 4.0) at 3° for 45 min with constant stirring. The suspension was centrifuged at 2° for 45 min at 3000*g*, giving a reddish-brown clear supernatant with a pH of 4.5–4.7. The pH of the solution was raised to 7.5 by the dropwise addition of 2 N NaOH, giving a turbidity which was not readily removed by centrifugation.

Titration of the Carboxylesterase. *o*-Nitrophenyl dimethylcarbamate was a gift from Dr. J. K. Stoops (Bender *et al.*, 1966). The general procedure was described by Horgan *et al.* (1969b), although in the present work, the substrate (titrant) in acetonitrile was generally added last to enzyme in 0.1 M Tris buffer (pH 8.2–8.8). Appropriate controls were done, including the measurement of the apparent molar absorptivities of *p*-nitrophenol (400 mμ) and *o*-nitrophenol (416 mμ) under the conditions of each titration.

Dry Weight Determination. Purified "mixed" enzyme was exhaustively dialyzed against distilled water at 4° and centrifuged. The ultraviolet absorption spectrum of an aliquot was determined in 0.15 M Tris buffer (pH 7.9). An aliquot was dried in a preweighed vessel under high vacuum ($<10^{-5}$ mm) at 100° for 2 hr. The weight of the sample did not change upon further drying at 120° for 1.5 hr.

Results

Enzyme Purification. A reproducible procedure for the purification of ox liver carboxylesterase is given in Table I. The enzyme did not bind to CM-cellulose at pH 5.6, but a significant amount of contaminating protein and red pigment (A_{410}) was retarded. The elution profile from the CM-Sephadex column is shown in Figure 1. The vertical dotted lines in Figure 1 indicate the various fractions which were pooled to give the polyacrylamide electrophoretic patterns in Figure 2. The three distinct bands of activity observed are numbered 1, 2, and 3 in order of decreasing mobility toward the anode. The CM-Sephadex step has resolved bands 1 and 3, each with only slight contamination by 2. In addition, a mixture of bands 1, 2, and 3 is unresolved in the central fractions of Figure 1. The starch gels in Figure 3 show the distribution of electrophoretic variants among the individual fractions of Figure 1.

The three pooled portions ("fast," 1 (+2); "mixed," (1, 2, 3); "slow," 3 (+2)) were individually gel filtered on Sephadex G-100 (Table I), giving a further slight purification.

Each component has been shown to give a single

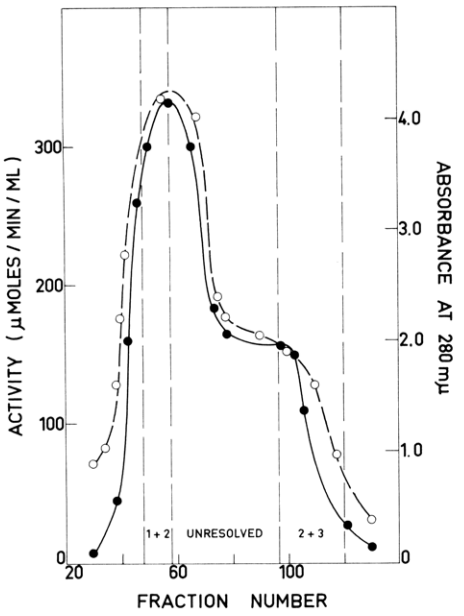

FIGURE 1: CM-Sephadex chromatogram of ox liver carboxylesterase. Each fraction contains 6.2 ml. (○---○---○) A_{280}; (●—●—●) activity. Fractions were pooled as indicated: "fast" enzyme, 1 + 2; "mixed" enzyme, unresolved, 1 + 2 + 3; "slow" enzyme, 2 + 3.

TABLE II: Titration of Ox Liver Carboxylesterase.[a]

o-Nitrophenyl Dimethylcarbamate (mM)	[*o*-Nitrophenol][b] (μM)
1.13	4.59
0.851	4.68
0.567	4.66
0.284	4.59
0.227	4.46
0.114	3.97

[a] 3.12% acetonitrile, 0.1 M Tris, pH 8.53. [b] Calculated from absorbance "burst" extrapolated to zero time, taking account of relevant constants.

symmetrical peak in the analytical ultracentrifuge (Figure 4). The sedimentation coefficient of the "mixed" (1, 2, 3) enzyme in 0.05 M Tris buffer (pH 7.0) obeyed the relationship $s_{20} = 8.3_0(1-0.11_5c)$, over the concentration range 0.3–1.0 g/100 ml, where c is the concentration in g/100 ml and s is in Svedberg units. At protein concentrations below 0.1%, the observed s_{20} value decreased (at 0.025%, $s_{20} = 7.11$). The effect is consistent with dissociation of the dimeric form of the protein. No inert protein bands could be detected on starch or polyacrylamide gel electrophoresis. Refiltration on Sephadex G-100 or G-200 did not increase the specific activity, and other large-scale preparations have reproducibly given specific activities in the same range. There is no appreciable absorbance at 410 mμ and samples of concentrated protein (A_{280} = 10–30) appear colorless.

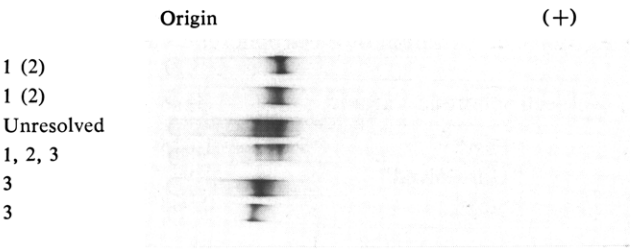

FIGURE 2: Polyacrylamide gel electrophoretogram of pooled fractions from CM-Sephadex (Figure 1). Stained for activity.

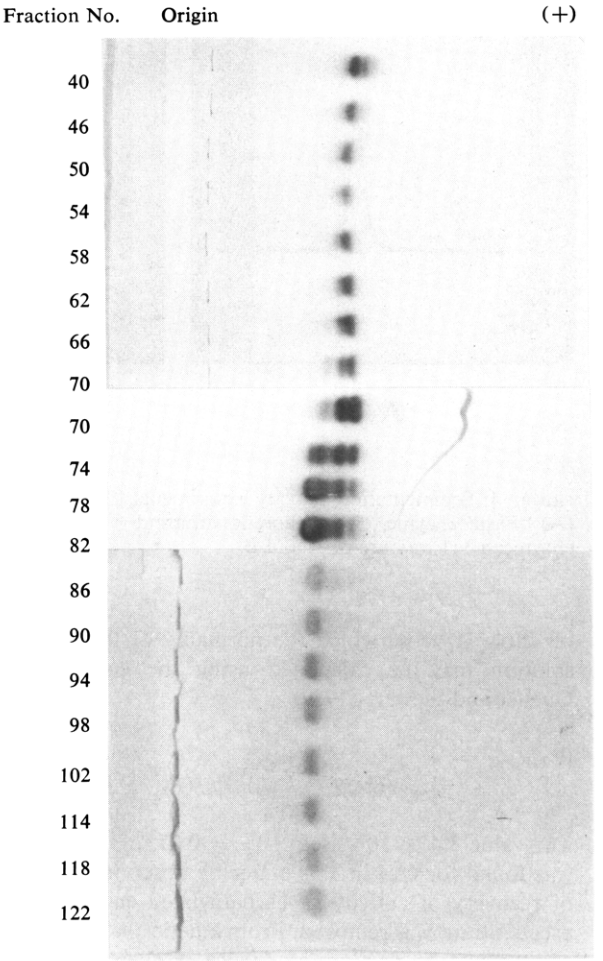

FIGURE 3: Starch gel electrophoretogram of individual fractions from CM-Sephadex (Figure 1). Stained for activity.

Titration of the Enzyme. The hydrolysis of *o*-nitrophenyl dimethylcarbamate by ox liver carboxylesterase at pH 8.2–8.8 gives a "burst" of *o*-nitrophenol followed by a very slow zero-order turnover of substrate (Figure 5). Extrapolation of the zero-order turnover of the substrate to zero time gives the "burst" of *o*-nitrophenol

TABLE III: *Titration of Electrophoretic Variants of Ox Liver Carboxylesterase.*[a]

Electrophoretic Variant[b]	A_{280}	A_{280}/[*o*-Nitrophenol]$_{burst}$	Sp Act.	Equiv Wt[b]
"Fast"	1.625	90,500	68	58,900
"Unresolved"	1.945	86,900	65	54,000
"Slow"	0.933	86,300	68	56,200

[a] [*o*-Nitrophenyl dimethylcarbamate] = 5.6×10^{-4} M, 0.15 M Tris, pH 8.69, and 1.4% acetonitrile. [b] Based on a maximum specific activity of 78 for each electrophoretic variant; see Discussion.

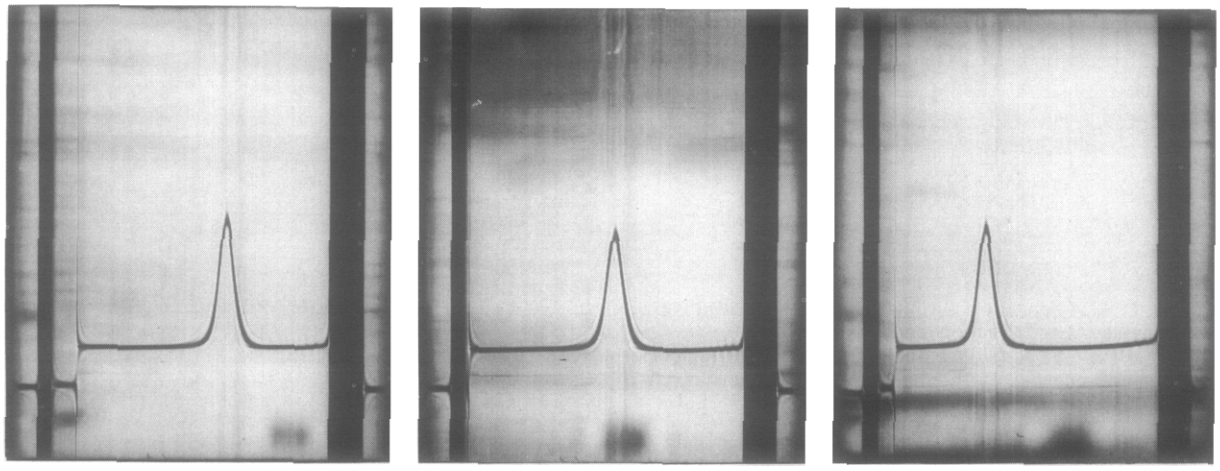

FIGURE 4: *Sedimentation velocity experiments (0.05 M Tris, pH 7.0, 20°) on pooled fractions from CM-Sephadex (Figure 1).* (A) "Fast" enzyme, electrophoretic variant 1 (+2); (B) "slow" enzyme, electrophoretic variant 3 (+2); (A + B) "mixed" enzyme, a 1:1 mixture of A and B.

of Table II, from which the normality of the enzyme solution may be calculated using the equation of Ouellet and Stewart (1959)

$$[P_1]_{burst} = [k_{+2}/(k_{+2} + k_{+3})[S]_0/(K_m + [S]_0)]^2[E]_0$$

The value 3.4×10^{-5} sec^{-1} (μ = 0.07, pH 8.58, 25°) was found for k_{+3} in Tris buffer by observing the rate of recovery of activity of carbamylated enzyme after excess titrant was removed. From kinetic analysis of the presteady-state portion of Figure 4 (Bender *et al.*, 1966) it was found that k_{+2}/k_{+3} was at least 1000 and $K_m \sim 10^{-6}$ M.[1]

All three electrophoretic variants of ox liver carboxylesterase gave very similar "bursts" for equivalent enzyme concentrations, based on absorbance at 280 mμ (Table III). The presteady-state portions of the reactions were also very similar.

Titrations with *o*- and *p*-nitrophenyl dimethylcarbamates and with *p*-nitrophenyl diethyl phosphate gave identical "bursts" (±2%), even though the presteady-state portion of the *p*-nitrophenyl diethyl phosphate reaction was too fast to measure. Turnover of the *p*-nitrophenyl diethyl phosphate inhibited enzyme could not be detected above the slow spontaneous rate of hydrolysis of the titrant (*cf.* Horgan *et al.*, 1969b).

The ultraviolet absorption spectrum of ox liver carboxylesterase is shown in Figure 6. The dry weight determination on this sample showed that an enzyme solution containing 1 mg/ml has an absorbance of 1.34 at 280 mμ (0.15 M Tris, pH 7.92).

Discussion

Purification of the Enzyme. The purification of ox liver carboxylesterase described in this paper is the result of extensive investigations into the best combination of steps. The aim was to produce a large-scale procedure which would reproducibly yield highly purified enzyme, but it is clear that this aim has been achieved only in part, in that the abundant sample of protein contains the "fast," "mixed," and "slow" variants of the enzyme. It is possible to produce electrophoretically pure forms of variants 1 and 3 by vicious cutting of fractions. However, as described in another paper of this series (Stoops *et al.*, 1969), the kinetic behavior of the three electrophoretic variants appears to be very

[1] *p*-Nitrophenyl dimethylcarbamate was a less satisfactory titrant, because the approach to steady state was ~10 times slower than with *o*-nitrophenyl dimethylcarbamate.

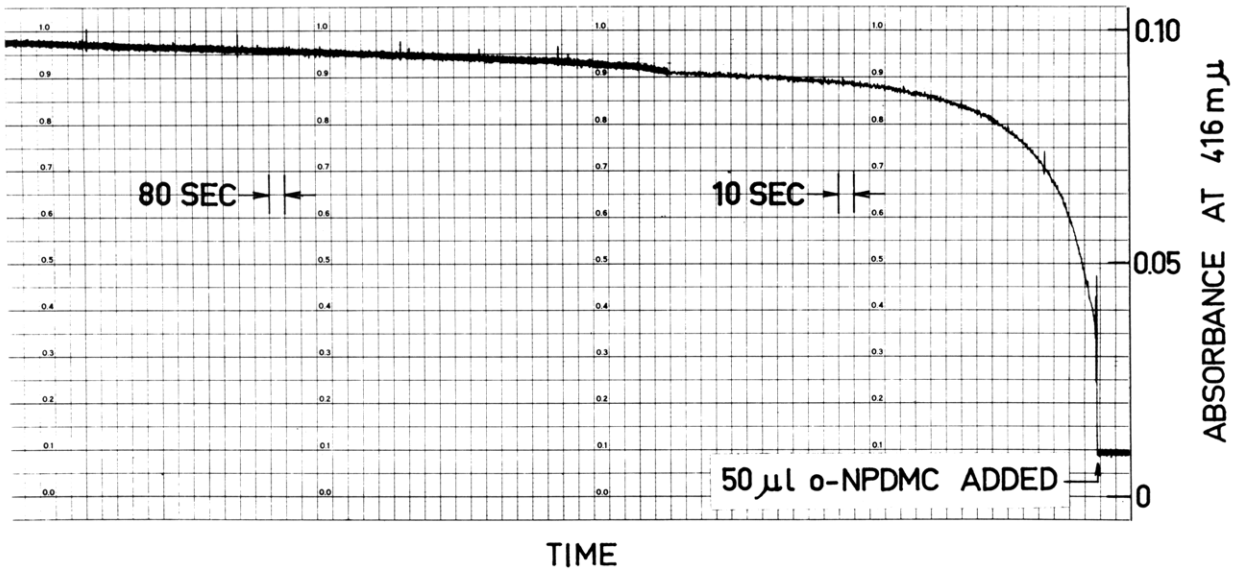

FIGURE 5: Titration of ox liver carboxylesterase (electrophoretic variant 1 (+2)) with *o*-nitrophenyl dimethylcarbamate (pH 8.7)

similar. The problem, therefore, would appear to be of less significance than was originally anticipated.

Difficulties have been experienced with some of the acetone powders over the course of about 4 years. During the period of a heavy drought, acetone and chloroform–acetone powders of pig, horse, ox, and sheep livers decreased markedly in extractable activity. The present method works very well with acetone powder extracts containing about 40 units of activity/ml.

The purification procedure of White (1956) could have yielded little better than a crude extract. Benöhr and Krisch (1967), however, have reported a procedure starting with a microsomal fraction which gives an enzyme purified 70-fold (based on the specific activity of the microsomes) in 20% yield. They report the enzyme to be homogeneous by column chromatography, gel filtration, and paper electrophoresis, but detect a "faster moving esterase-active" band on high-voltage starch gel electrophoresis. As pointed out in a previous paper (Horgan *et al.*, 1969a), starch gel electrophoresis is not a sensitive index of homogeneity. That the three electrophoretic variants of ox liver esterase activity are not artifacts, produced in the acetone–powder step, has been demonstrated by comparison with electrophoresis of crude homogenates in which they (and other) variants are present.

The symmetrical peaks obtained in the ultracentrifuge (Figure 4) for mixtures of the various enzyme forms again point up the insensitivity of this tool as a casual criterion of homogeneity.

The ultraviolet spectrum of the enzyme (Figure 6) demonstrates that the protein is indeed in a respectable state.

Titration of the Enzyme. Because of the absence of an exact agreement between the form of the acylation of pig liver carboxylesterase and the usual kinetic schemes, the ox enzymes were intensively investigated. It can be stated with confidence that the simple kinetic scheme constitutes a very good approximation to but does not

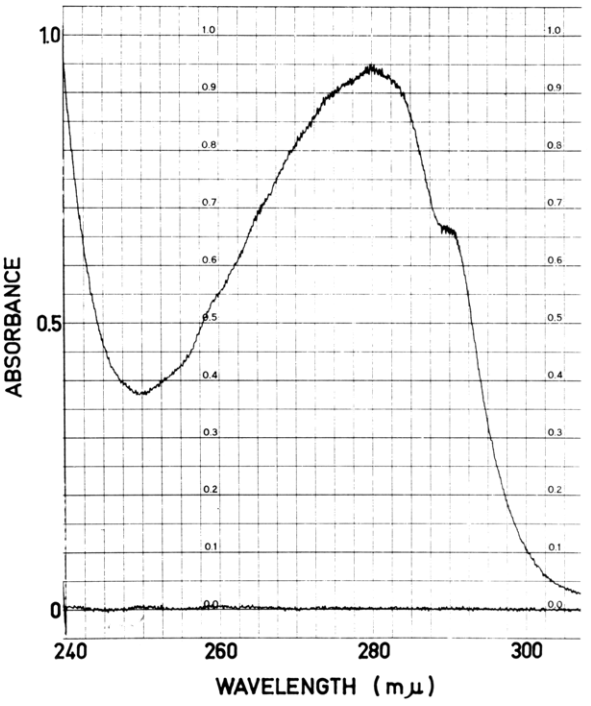

FIGURE 6: Spectrum of ox liver carboxylesterase ("mixed" enzyme) in 0.15 M Tris buffer (pH 7.9).

provide a totally satisfying description of the system. It is possible that this discrepancy could be accounted for by a relatively slow dissociation of the ox liver enzyme dimer. The explanation offered by Heymann and Krisch (1967) for the kinetic behavior of the pig liver enzyme with bis(*p*-nitrophenyl) phosphate does not account, even remotely, for our detailed results. Work on this problem is continuing.

The equivalent weight of the ox enzyme determined from the titration experiments is ~68,000. The molec-

ular weight of the enzyme is ~150,000 (Runnegar *et al.*, 1969), which establishes the enzyme in close relationship to the pig enzyme. However, the two enzymes do differ in active-site sequences (Augusteyn *et al.*, 1969) and in their kinetic behavior (Stoops *et al.*, 1969). The equivalent weight of ~68,000 is derived from three independent titrations (*o*-nitrophenyl dimethylcarbamate, *p*-nitrophenyl dimethylcarbamate, and paraoxon) of "mixed" enzyme of specific activity 74, corrected to a specific activity of 78. Table III shows that the titration of a less pure sample (specific activity ~ 68) gives a low equivalent weight, indicating that the titration is overestimating the active enzyme in the system. This observation has been confirmed on other impure samples, and probably derives from contamination of the active enzyme protein by other protein material whose spectral characteristics are different, since the specific activity, and hence the correction, is dependent upon A_{280}. Because of this possible difficulty, kinetic data obtained with these enzymes (Stoops *et al.*, 1969) were collected with samples of high specific activity. There is no reason to believe, however, that the titration is not producing a valid estimate of the concentration of active sites of the enzyme in solution, since the pig enzyme gave consistent kinetic data when the specific activity of the enzyme varied from 447 to 569.

Related work on the liver enzymes from horse, sheep, and chicken will be published later.

Acknowledgments

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Carboxylesterases (EC 3.1.1). Dissociation of Ox Liver Carboxylesterase*

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ABSTRACT: DEAE-Sephadex chromatography of ox liver carboxylesterase purified up to and including the CM-cellulose chromatography step (Runnegar, M. T. C., Scott, K., Webb, E. C., and Zerner, B. (1969), *Biochemistry 8*, 2013 (this issue; paper 8)) resulted in the separation of electrophoretic variants 1, 2, and 3, variant 3 being eluted first. Gel filtration on Sephadex G-200 established that the dimeric form of the enzyme reversibly dissociates to an active monomeric form.

The native enzyme contains no titratable SH groups, nor does the enzyme treated with mercaptoethanol or 6.5 M urea, separately. Iodoacetate and iodoacetamide ($\sim 10^{-3}$ M) are without effect on the enzyme. After treatment of the enzyme with ~0.3 M mercaptoethanol in 6.5 M urea, approximately 5 SH groups/150,000 molecular weight species may be titrated with the Ellman reagent. Comparative ultracentrifugation of ox liver carboxylesterase and bovine serum albumin in solutions of varying urea concentration (to 7 M) shows that the dimeric enzyme does not dissociate under the conditions of the experiment. This observation is supported by the results of gel filtration on Sephadex G-200 in 6.5 M urea.