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

We acknowledge with thanks the indefinite loan of a Cary 14 spectrophotometer from the Wellcome Trust, London. It is a pleasure to acknowledge the help and discussion of Dr. R. L. Blakeley and Dr. J. K. Stoops, and also the competent technical assistance of Mrs. V. Murray.

References

Augusteyn, R. C., de Jersey, J., Webb, E. C., and Zerner, B. (1969), *Biochim. Biophys. Acta 171*, 128.

Bender, M. L., Begué-Cantón, M. L., Blakeley, R. L., Brubacher, L. J., Feder, J., Gunter, C. R., Kézdy, F. J., Killheffer, Jr., K. J., Marshall, T. H., Miller, C. G., Roeske, R. W., and Stoops, J. K. (1966), *J. Amer. Chem. Soc. 88*, 5890.

Benöhr, H. C., and Krisch, K. (1967), *Z. Physiol. Chem. 348*, 1102

Heymann, E., and Krisch, K. (1967), *Z. Physiol. Chem. 348*, 609.

Horgan, D. J., Dunstone, J. R., Stoops, J. K., Webb, E. C., and Zerner, B. (1969b), *Biochemistry 8*, 2006 (this issue; paper 7).

Horgan, D. J., Stoops, J. K., Webb, E. C., and Zerner, B. (1969a) *Biochemistry 8*, 2000 (this issue; paper 6).

Kirkland, R. J. (1963), Ph.D. Thesis, University of Cambridge, Cambridge.

Krisch, K. (1963), *Biochem. Z. 337*, 531.

Kristjansson, F. K., and Hickman, C. G. (1965), *Genetics 52*, 627.

Ouellet, L., and Stewart, J. A. (1959), *Can. J. Chem. 37*, 373.

Poulik, M. D. (1957), *Nature 180*, 1477.

Runnegar, M. T. C., Webb, E. C., and Zerner, B. (1967), *Australian Biochem. Soc. Sydney*, 8.

Runnegar, M. T. C., Webb, E. C., and Zerner, B. (1969), *Biochemistry 8*, 2018 (this issue; paper 9).

Stoops, J. K., Horgan, D. J., Runnegar, M. T. C., de Jersey, J., Webb, E. C., and Zerner, B. (1969), *Biochemistry 8*, 2026 (this issue; paper 10).

White, B. T. (1956), *J. Dairy Sci. 39*, 547.

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

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

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.

In previous papers of this series on carboxylesterases (Horgan *et al.*, 1969a,b), pig liver carboxylesterase was shown to dissociate, on dilution at neutral pH, from a dimeric species (two active sites) of mol wt ~ 160,000 to a monomeric active species (half-molecule) of mol wt ~80,000.[1] It has also been shown that this dissociation is not rapid (Blakeley *et al.*, 1969). The previously reported results of Barker and Jencks (1967) are totally supported by our findings.

Boguth *et al.* (1965) reported the dissociation of the pig liver enzyme to a species of mol wt ~40,000 in 0.2% sodium dodecyl sulfate and a similar, although less extensive, effect was interpreted to occur from ultracentrifugal data obtained in 6.5 M urea. However, we have obtained no evidence for such a species, even in 8 M urea (Horgan *et al.*, 1969b). Barker and Jencks (1967) have reported that in 6 M guanidinium chloride, 0.1 M in mercaptoethanol, the pig liver enzyme has an apparent molecular weight of 66,000, no correction being made for specific solvent interactions.

Benöhr and Krisch (1967a,b) have reported the purification of ox liver carboxylesterase (dimer, mol wt 167,000) and also the separation of two enzymatically active components on DEAE-Sephadex. These components were identified as dimeric and monomeric forms of the enzyme.

In this paper, we report a series of experiments designed to define the extent and character of the dissociation of ox liver carboxylesterase under a variety of conditions.

Experimental Section

Materials and Methods. The major procedures have been described previously (Horgan *et al.*, 1969a,b; Runnegar *et al.*, 1969). Urea was recrystallized from 70% and then from 90% ethanol (Steinhardt, 1938) at temperatures not exceeding 40°. Buffers in urea were prepared immediately before use by adding the calculated quantity of urea to prepared buffers; pH and molarity of the buffer solutions refer to the buffer before addition of urea. Free sulfhydryl groups were determined by the method of Ellman (1959) at pH 7–8 using a Cary 14 spectrophotometer equipped with a 0–0.1-absorbance slidewire.

DEAE-Sephadex Chromatography. Ox liver carboxylesterase from CM-cellulose chromatography (Runnegar *et al.*, 1969) was concentrated with ammonium sulfate, dialyzed *vs.* 5 mM Tris base, 1 mM in EDTA (pH ~8.6), and applied to a column (2.0 × 40 cm) of DEAE-Sephadex A-50 (Pharmacia, Sweden) equilibrated with the same buffer. The gradient system (Krisch, 1963) consisted of three identical cylindrical vessels joined at the base and on the same level. The two closest to the column contained a volume of 5 mM Tris base (1 mM in EDTA and 40 mM in NaCl); the third vessel contained the same volume of the same buffer, 0.5 M in NaCl.

Effect of Lyophilization. Purified "mixed" carboxylesterase (42 mg) was dialyzed *vs.* 5 mM Tris-HCl buffer (pH 8.6), 1 mM in EDTA. After lyophilization, the enzyme was dissolved in 1 l. of the same buffer and allowed to stand at 3° for 24 hr (measured pH, 8.3). The sample was applied over a period of 70 hr to a column (0.9 × 30 cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. The column was washed with the same buffer for 48 hr, and enzyme was eluted stepwise with 50 ml of the same buffer (0.06 M in NaCl, pH 8.6) and then with 0.15 M NaCl in the same buffer system until all activity had been eluted.

Enzymatic Activity on Treatment with Urea and Sulfhydryl Reagents. A dilute enzyme solution was thermostated with urea buffer and either assayed directly (involving a large dilution of an aliquot) or dialyzed exhaustively. The assay was carried out using *o*-nitrophenyl butyrate as substrate in 0.1 M phosphate buffer (pH 7.5), 10% (v/v) acetonitrile.

Gel Filtration in 6.5 M Urea. Swollen Sephadex G-200 was equilibrated with several changes of 6.5 M urea (reagent grade, not recrystallized) in 0.05 M phosphate (pH 6.6), 0.2 M in NaCl and 1 mM in EDTA, prior to preparing a column (2.1 × 36 cm). Ox liver carboxylesterase was dialyzed into the same buffer at 3°, and 0.5 ml of the resulting solution (A_{280} = 21) was applied to the column.

Ultracentrifugation in the Presence of Urea. Uncorrected s_{20} values were determined from sedimentation velocity runs in a Beckman-Spinco Model E analytical ultracentrifuge equipped with a Spinco RTIC temperature control unit. A double-sector schlieren cell, containing the urea buffer as well as enzyme solution, was used. The total area under the schlieren peak(s) arising from the esterase in 6.5 M urea containing mercaptoethanol increased with time as the solution aged; *e.g.*, the areas in Figure 9A,B are ~2.3 and ~10 times, respectively, the area under the peak for a similar sample (after 8 hr) which contained no mercaptoethanol (*cf.* Figure 9C).

Results

DEAE-Sephadex Chromatography. Ox liver carboxylesterase which had been purified up to and including the CM-cellulose step was chromatographed on DEAE-Sephadex, and was eluted with a three-vessel gradient system (Table I), each vessel containing 125 ml. Figure 1 and Table I show the results of this experiment. Carbon dioxide absorption caused the pH of the system

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[1] In this paper, carboxylesterases of mol wt ~160,000 are referred to variously as molecules or dimers; carboxylesterases of mol wt ~80,000 are referred to as half-molecules or monomers. This usage does not comment on the ultimate subunit structure of these enzymes. It merely implies that one active site is associated with the species of mol wt ~80,000.

TABLE I: DEAE-Sephadex Chromatography (First Preparation).[a]

Fraction	Act. (units/ml)	Sp Act. (units/ml)/A_{280}	A_{280}/A_{260}
[Load		32	1.73]
25[b]	4	13	0.86
29	21	28	1.22
33	93	58	1.36
37	108	61	1.31
41	62	54	1.20
45	40	49	1.11
49	53	57	1.18
54	45	56	1.16
58	32	54	1.13
62[c]	18	41	0.96

[a] Applied load, 10,800 units. The gradient vessels contained 125 ml of buffer. Each fraction contains 4.9 ml. [b] 200 ml of gradient. [c] End of gradient.

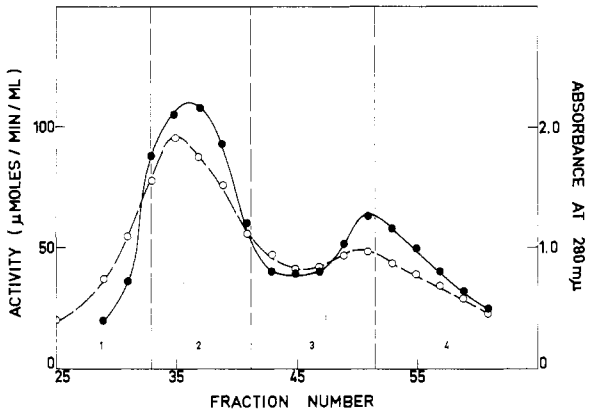

FIGURE 1: DEAE-Sephadex chromatography (first preparation, Table I). (●—●—●) Activity and (○---○---○) A_{280}.

(initially 5 mM Tris base, 1 mM in EDTA, pH ~8.6) to drop to ~8.0. After application of the salt gradient, the pH of the freshly eluted tubes rose to above 9. This probably accounts for the low A_{280}/A_{260} ratio. The eluted enzyme was combined in four fractions (1–4, Figure 1).

The procedure was repeated, except that each gradient vessel contained 150 ml of solution (Table II and Figure 2), and a similar elution profile was obtained. Fractions A–E (Figure 2) were concentrated and dialyzed against the electrophoresis buffer. The zymograms (stained for activity) of suitably diluted samples are shown in Figure 3.

Purified carboxylesterase ("mixed" enzyme) was applied to DEAE-Sephadex after lyophilization and dilution. The elution profile is shown in Figure 4. Figure 5 shows the results of starch and polyacrylamide gel electrophoresis on some fractions from Figure 4.

TABLE II: DEAE-Sephadex Chromatography (Second Preparation).[a]

Fraction	Act. (units/ml)	Sp Act. (units/ml)/A_{280}	A_{280}/A_{260}
[Load		34	1.743]
34[b]	0.8	8	0.300
36	4.0	25	0.503
38	8.8	31	0.691
40	15.0	39	0.819
42	20.4	55	0.804
44	14.0	49	0.736
46	10.0	39	0.688
48	7.6	32	0.636
50	11.0	39	0.703
52	23.6	53	0.898
54	17.0	49	0.828
56	5.8	34	0.607
58	1.8	18	0.510
60[c]	1.0	~10	

[a] Applied load, 8740 units. The gradient vessels contained 150 ml of buffer. Each fraction contains 6.5 ml. [b] 280 ml of gradient. [c] End of gradient.

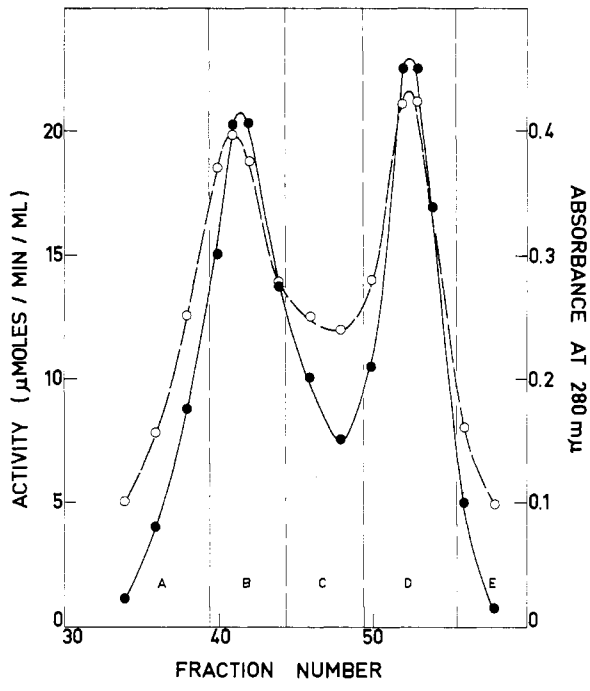

FIGURE 2: DEAE-Sephadex chromatography (second preparation, Table II). (●—●—●) Activity and (○---○---○) A_{280}.

Table III shows the elution volumes of fractions 1 to 4 (Figure 1, Table I) on Sephadex G-200. It is evident that the elution volume varies for fractions 1 and 4

TABLE III: Sephadex G-200 Chromatography of Pooled Fractions from DEAE-Sephadex (First Preparation).[a]

Fraction	Sp Act.	A_{280} Applied	Elution Vol	A_{280} Peak Tube
1	39	14	65.5	0.28
2	66	20	60.0	0.65
3	54	18	59.5	0.49
4	52	9	69.0	0.18
Blue Dextran			40.0	
Cytochrome *c*			102.0	
Bovine serum albumin			68.0	

[a] 0.05 M phosphate (pH 7.4), 0.5 M in NaCl. Column dimensions, 2.0 × 25 cm. Fractions 1–4 (Figure 1) were individually concentrated using ammonium sulfate, dialyzed against the starting buffer, and applied in volumes of ~0.5–1.1 ml.

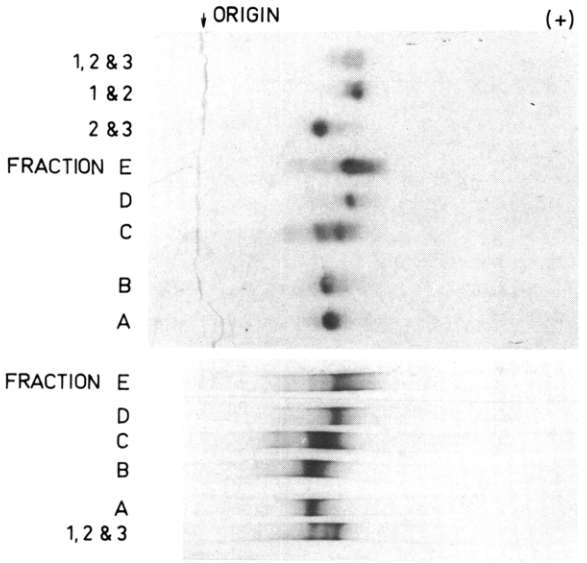

FIGURE 3: Starch and polyacrylamide gel zymograms (stained for activity) of enzyme fractions A–E from DEAE-Sephadex (second preparation, Figure 2). Electrophoretic variants 1 + 2 + 3, 1 (+2), and 3 (+2) are from CM-Sephadex chromatography (Runnegar *et al.*, 1969).

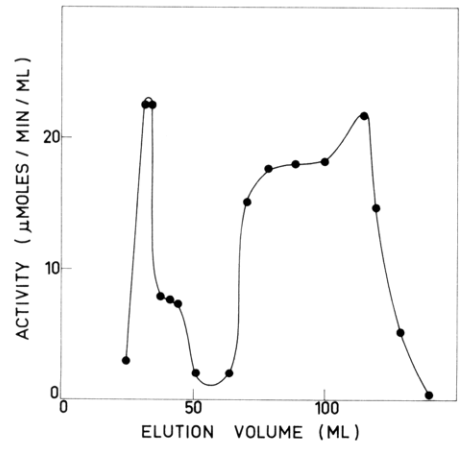

FIGURE 4: DEAE-Sephadex chromatography of purified "mixed" enzyme, after lyophilization and dilution.

which have lower protein concentration at the peak tube. Fractions 1 and 2 have been shown by electrophoresis to contain scarcely any electrophoretic variant 1, while fractions 3 and 4 contain little electrophoretic variant 3. It is therefore clear that the elution volume and, therefore, the state of aggregation of the enzyme depend exclusively upon the concentration of the sample and not on the electrophoretic variants present. This was checked by diluting fractions 2 and 4 and comparing the elution volumes so obtained. Figure 6 shows the elution profiles on Sephadex G-200 of fraction 2 at high and low concentration.

The above results were checked on samples of the purified carboxylesterases and these data are collected in Table IV, which demonstrates that the effect of dilution on the elution volumes applies equally to all electrophoretic variants.[2]

The effect of dilution on mobility on starch gel electrophoresis was also investigated. The purpose was to determine whether electrophoretic variant 3 could possibly represent the monomer of electrophoretic variant 1 or 2. Figure 7 shows the zymogram obtained when increasing dilutions of variants 1 and 3 were applied to the gel.

Effects of Urea, Sulfhydryl Reagents, and Mercaptoethanol on the State of Aggregation of the Ox Liver Enzyme. Ox liver carboxylesterase (2.2×10^{-7} N) was allowed to interact with 6.86 M urea in 0.05 M Tris-HCl buffer (pH 8.0) for 1 hr at 10, 25, and 38°. The recovery of activity after dialysis at the respective temperatures was 57, 28, and <0.5%. Under the same experimental conditions at 10°, variation of enzyme

[2] It should be noted that an enzyme preparation which had been frozen and thawed a number of times over a period of 2 years appeared to be modified. It consisted (originally) of electrophoretic variants 1 and 2. The presteady-state reaction with *o*-nitrophenyl dimethylcarbamate was slowed considerably. There was also a 20% decrease in specific activity toward ethyl butyrate. This enzyme preparation applied to Sephadex G-200 in 0.005 M Tris base, 0.001 M in EDTA (pH ~ 8.6), appeared to be completely "monomeric" at concentrations where the native enzyme is dimeric. The result was confirmed by analytical ultracentrifugal analysis. It appears that the enzyme was modified in such a way that it dissociated more readily. At higher concentrations, a single peak was obtained corresponding to a "dimeric" form.

TABLE IV: Sephadex G-200 Chromatography of Purified Ox Liver Carboxylesterase.[a]

Electrophoretic Variant	A_{280} Applied	A_{280} Peak Tube	V_e/V_0	Sedimentation Coefficient of Peak Tube Protein (S)
1 (+2)	27	0.98	1.31	6.6
3 (+2)	16	0.67	1.32	6.6
1 + 2 + 3	32	1.07	1.33	6.9
1 (+2)	0.98	*b*	1.68	
3 (+2)	1.12	*b*	1.77	
1 + 2 + 3	1.07	*b*	1.81	
1 + 2 + 3	0.20	*b*	1.86	
Bovine serum albumin	15	0.72	1.70	

[a] 0.05 M phosphate (pH 7.0), 0.5 M in NaCl. Column dimensions, 4.0 × 87 cm. V_0 = 372 ml (determined with Blue Dextran). [b] Peak of enzyme elution determined by spectrophotometric rate assay using *p*-nitrophenyl butyrate as substrate.

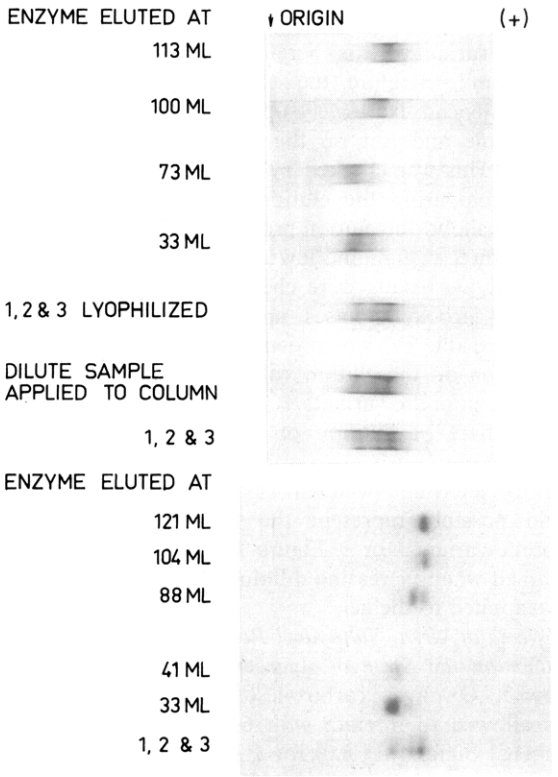

FIGURE 5: Starch and polyacrylamide gel electrophoresis of individual fractions from DEAE-Sephadex chromatography following lyophilization and dilution (Figure 4).

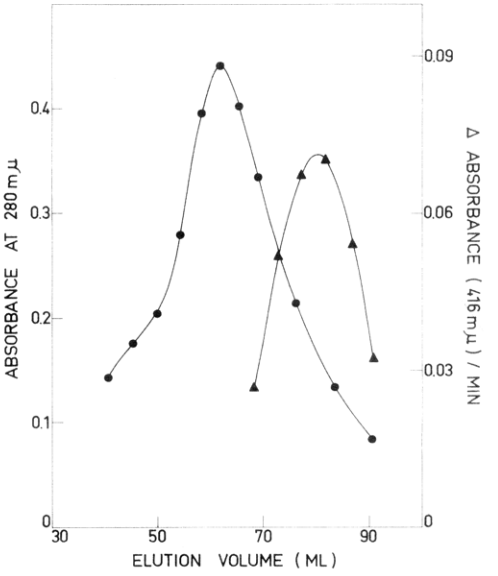

FIGURE 6: Sephadex G-200 chromatography of fraction 2 (Figure 1). See Table III for conditions. (●—●—●) Concentrated sample, A_{280}. (▲—▲—▲) Enzyme applied at A_{280} = 0.40; activity measured by spectrophotometric rate assay using *o*-nitrophenyl butyrate as substrate. Activity and A_{280} of the concentrated sample were coincident.

concentration from 2×10^{-7} to 2×10^{-6} N resulted in essentially the same recovery of activity (~50%). Activity was lost rapidly at 25° in 6.5 M urea at pH 5.2, *e.g.*, ~85% of the activity was lost in 27 min ($[E]_0 = 3 \times 10^{-5}$ N). It was difficult to estimate recoveries since about two-thirds only of the residual activity can be measured immediately upon dilution from the 6.5 M urea system into the assay buffer system. The remaining activity appears slowly (~30 min).

Ox liver carboxylesterase (0.27%) in 0.01 M phosphate (pH 7.2), 6.5 M in urea, gave no evidence for two peaks in the ultracentrifuge. However, since it was not possible to state in what form the carboxylesterase existed from this run, the effect of increasing concentration of urea on the sedimentation coefficient of carboxylesterase was investigated. For comparison, bovine serum albumin was treated in the same way, since bovine serum albumin has been shown not to associate or dissociate under the experimental conditions (Gutter, 1957). The results of this experiment are shown in Table V.

The elution volume of the enzyme from Sephadex G-200 in the presence of 6.5 M urea was measured as a

TABLE V: Variation in s_{20} of Ox Liver Carboxylesterase and Bovine Serum Albumin with Urea Concentration.[a]

Urea (M)	s_{20} (Esterase)	s_{20} (Bovine Serum Albumin)	s_{20} (Esterase)/ s_{20} (Bovine Serum Albumin)
0	7.48	4.12	1.8_1
1	6.61	3.66	1.8_0
2	6.09	3.32	1.8_2
3	5.37	3.03	1.7_7
4	4.84	2.75	1.7_6
5	3.83	2.08	1.8_4
6	1.71[b]	0.95[b]	1.8_0
7	2.27	1.22	1.8_6

[a] 0.05 M phosphate (pH 6.6), 0.2 M NaCl, 1 mM EDTA, 0.6% protein. [b] The reason for this discontinuity is not apparent. The important point is the constancy of the ratio.

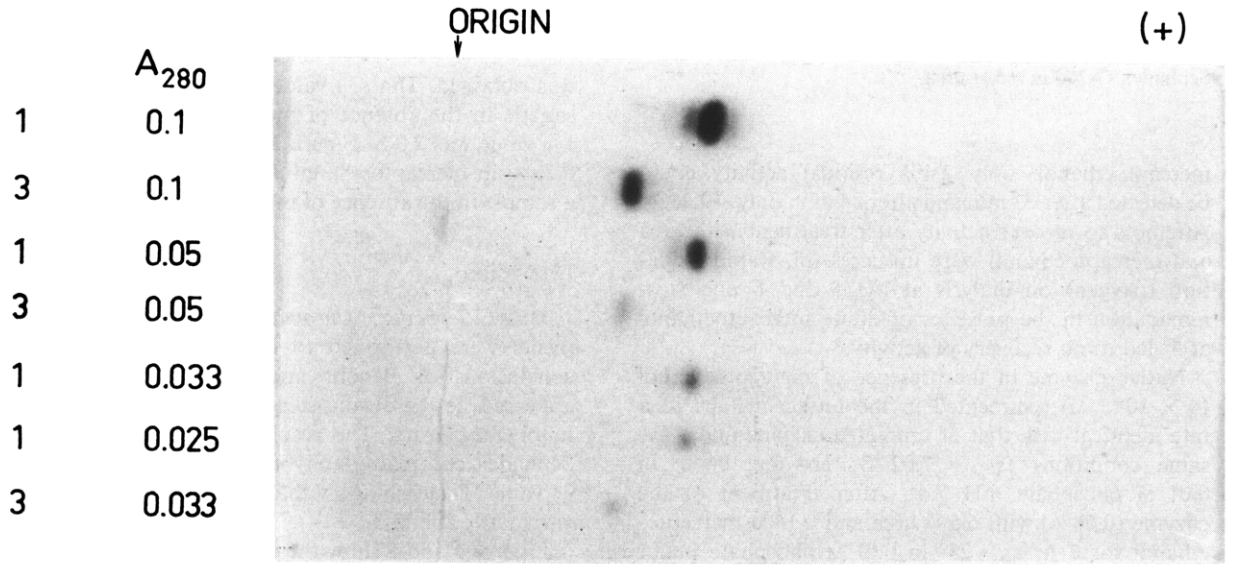

FIGURE 7: Starch gel zymogram of ox liver carboxylesterase electrophoretic variants 1 and 3 at different concentrations.

comment on the preceding experiment. The void volume of the column was 56.8 ml. The elution volume of bovine serum albumin was measured as 76.9 ml. The enzyme (0.5 ml, A_{280} in urea = 21) was eluted in a broad peak (Figure 8). Only 55.6 ml had been eluted to tube 23 and 63.4 ml to tube 27. The elution volume (taken for tube 23 or 27) is considerably less than that for bovine serum albumin (76.9 ml). Neither iodoacetate nor iodoacetamide (9×10^{-4} M) caused the enzyme to lose activity over a period of 1 hr, the assay being made after the removal of the alkylating agents. Further, no significant loss of activity occurred over a period of 6 days at room temperature (~25°). Neither reagent appeared to enhance the denaturing effect of 8 M urea at 4°. No free SH groups could be detected in the native enzyme, or in the enzyme in 6.5 M urea.

Native enzyme treated with 0.72 M mercaptoethanol for 1 hr at room temperature showed no loss of activity. Further, no free SH groups could be detected after removal of the mercaptoethanol by dialysis.

Enzyme (2×10^{-5} M) which had been treated with mercaptoethanol (0.36 M) for 1 hr at 3° in 6.5 M urea (0.1 M methylamine-HCl and 0.1 M Tris-HCl buffer, pH 8.1) showed measurable free SH content after removal of the reagents by dialysis under nitrogen. Assuming a molecular weight of 150,000, the number of free SH groups found in replicate experiments was 2.6, 4.1, 4.6, and 5.0. The reaction with the Ellman reagent was extremely slow. Also, on occasions, samples after removal of urea tended to be turbid, and even after centrifugation, turbidity would slowly reappear in the solution. These combined effects made the measurements difficult ($\Delta A \sim 0.05$–0.3). Table VI shows the variations in the number of free SH groups as the concentration of mercaptoethanol is varied.

Treatment of the enzyme with urea and mercaptoethanol caused a loss of activity well in excess of that due to urea alone. For example, in 6.5 M urea alone, ~15% residual activity was measured after 27 min at pH 5.2. When the same system was made 0.29 M in

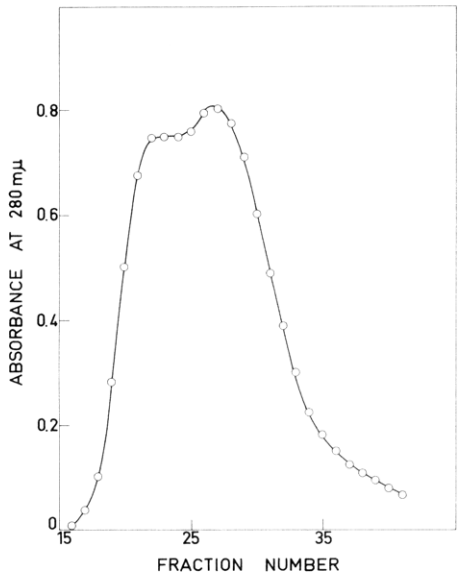

FIGURE 8: Elution profile of ox liver carboxylesterase from Sephadex G-200 in 6.5 M urea.

mercaptoethanol, only 2.4% residual activity could be detected after 23 min and after 84 min, only ~0.33%. Attempts to recover activity after treatment with urea and mercaptoethanol were unsuccessful. Rapid oxidation (oxygen) on dialysis at pH 5 and 8 and slow reoxidation in the presence of dilute mercaptoethanol at 5° led to no recovery of activity.

Native enzyme in the presence of mercaptoethanol (4×10^{-2} M) sedimented in the ultracentrifuge at a rate identical with that of untreated enzyme under the same conditions (s_{20} = 7.02 S; enzyme, 0.8% in 0.01 M phosphate, pH 7.4). After treatment of the enzyme (0.66%) with 6.5 M urea and 0.14 M mercaptoethanol for 3 hr at ~25° in 0.01 M phosphate buffer (pH 7.4), the schlieren pattern shown in Figure 9A was obtained. The s_{20} value was 2.7 S. For a similar sample in the absence of mercaptoethanol (8 hr), the s_{20} value was 2.3 S. Figure 9B shows a similar sample, 0.29 M in mercaptoethanol after 24 hr, and Figure 9C a sample in the absence of mercaptoethanol after 76 hr.

TABLE VI: Variation in the Free SH Content of Ox Liver Carboxylesterase with Varying Mercaptoethanol Concentration.[a]

[Mercaptoethanol] (M)	Moles of SH/150,000 g of Carboxylesterase
0.30	4.6
0.19	5.6
0.10	4.8
0.05	5.8
0.02	4.8
0.002	3.4

[a] 6.5 M urea in 0.1 M Tris (pH 8.1), 0.1 M in methylamine hydrochloride. $[E]_0$ = 5 μM. After 1 hr of reaction at 3° under nitrogen, the solutions were dialyzed under nitrogen *vs.* 0.01 M phosphate (pH 8.0) and the SH content was analyzed.

Discussion

DEAE-Sephadex Chromatography. These experiments were performed to check the validity of the conclusions of Benöhr and Krisch (1967b), which appeared to be inconsistent with our work over a number of years. The results of the second DEAE-Sephadex chromatography experiment (Figures 2 and 3, Table II) clearly show that the column separates the variants 1, 2, and 3.

Figures 4 and 5 show the effect of lyophilization and dilution on the DEAE-Sephadex chromatogram.

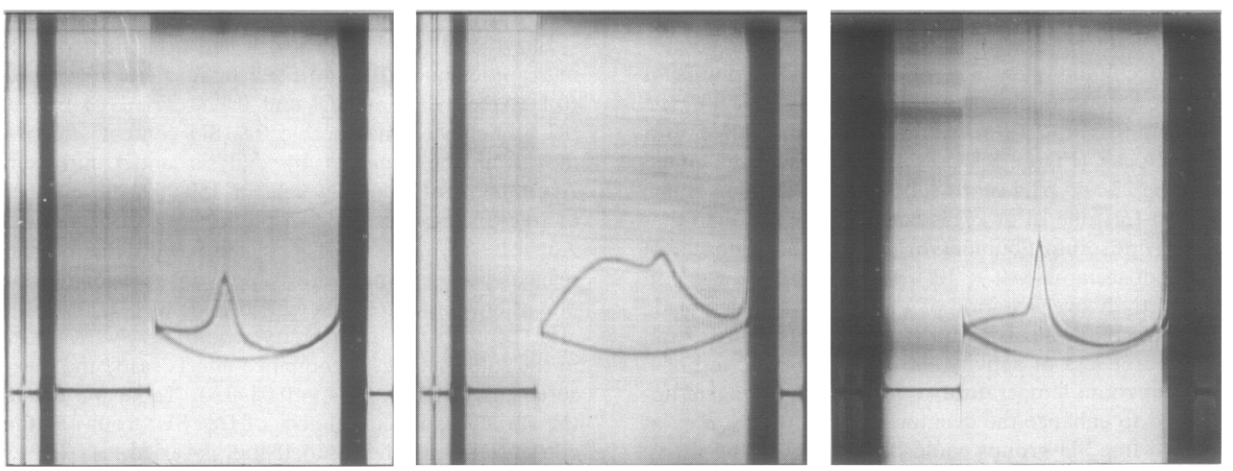

FIGURE 9: Sedimentation–velocity diagram for ox liver carboxylesterase in 6.5 M urea (0.01 M phosphate, pH 7.4) and mercaptoethanol. (A) 3 hr in 0.14 M mercaptoethanol, s_{20} = 2.7 S. (B) 24 hr in 0.29 M mercaptoethanol, s_{20} = 2.7 and 1.2 S. (C) 76 hr, no mercaptoethanol, 2.6 S.

Lyophilization of the enzyme does not cause the disappearance of any electrophoretic variants, the results of Figure 5 being entirely consistent with those of Figure 3.

Benöhr and Krisch (1967b) reported that lyophilization caused the disappearance of the monomer. (Electrophoretic variant 3 (+2) would correspond to the "monomer" while variant 1 (+2) would be the "dimeric" enzyme!)

The results of gel filtration on Sephadex G-200 (Table III) of fractions 1–4 (Table I, Figure 1) clearly establish that the elution volume is dependent upon the concentration of the enzyme and not on the electrophoretic variants present. Any doubt that this is the correct explanation of the phenomena observed is removed by inspection of Figure 6 and Table IV, which establish that the enzyme dissociates on dilution, and that all variants are similarly affected. It is probable that the bands observed on electrophoresis of dilute samples represent the monomeric form of the enzyme, since the *lowest* concentrations applied were of the order of 0.03 mg/ml and further dilution would occur on electrophoresis.

The possibility remains that one of the peaks from DEAE-Sephadex obtained in the present work is monomeric enzyme and the other dimeric. This separation would be superimposed on the separation of the electrophoretic variants. However, it is impossible to comment on the size of the protein in the two peaks from the DEAE-Sephadex column, since concentration will cause association, and dilution, dissociation, of each variant as was shown on Sephadex G-200 (Table IV). It is difficult to understand how Benöhr and Krisch (1967b) determined the molecular weights of the two peaks for DEAE-Sephadex on Sephadex G-200, since they state that the monomer–dimer equilibrium is concentration dependent, and the experimental conditions for the application of the sample to Sephadex G-200 were not described.

The above authors also report that their preparation contained only one major band of activity. One possible explanation for the difference between their observations and the present results could be that differences exist between breeds of cattle in Germany and Australia. The electrophoretic multiplicity of carboxylesterases is well documented. Holmes and Masters (1968) have reported the presence of four major bands of carboxylesterase in the liver homogenate of a *single* ox by polyacrylamide gel electrophoresis. The four bands had similar sensitivity to heat and urea denaturation and had similar specificities. In the present work, a fourth band of esterase activity was detected in the eluate from CM-Sephadex (Runnegar *et al.*, 1969), but was not pooled with the active fractions because of the low specific activity of tubes containing it.

Effects of Urea, Sulfhydryl Reagents, and Mercaptoethanol on the State of Aggregation and Activity of the Enzyme. Although the rate of loss of activity on addition of urea was very fast, it still took a finite time to inactivate the enzyme completely. These results are in contrast to those of Malhotra and Philip (1966) who claimed immediate irreversible inhibition of goat intestinal carboxylesterase.

Ultracentrifugation of ox liver carboxylesterase, using bovine serum albumin as a nonassociating and nondissociating protein marker, establishes that the ratio of sedimentation coefficients of the two proteins remains remarkably constant in the range 0–7 M urea (Table V). This constancy strongly indicates that no change in gross molecular form of the carboxylesterase has occurred in going from 0 to 7 M urea. The gel filtration experiment on Sephadex G-200 (Figure 8) serves to confirm the above results (*cf.* Přistoupil, 1965).

The native enzyme, the enzyme treated with 6.5 M urea, and the enzyme treated with mercaptoethanol have no detectable SH groups. However, the enzyme treated with both urea and mercaptoethanol appears to have a real SH content (Table VI), since only at the lowest concentration of mercaptoethanol used is there a decrease in the Ellman titration. This suggests that the observations are not due to nonspecific binding of mercaptoethanol.

The synergistic effect of mercaptoethanol on denaturation in urea was examined ultracentrifugally, and casual inspection of Figure 9B might suggest evidence for dissociation of the principal molecular species. However, the area of the sedimentation peak increased significantly throughout the experiment (Figure 9A,B; *cf.* Figure 9C). Mercaptoethanol was necessary to observe the effect. Steinmetz and Deal (1966) have noted a similar effect in their investigation of the dissociation of rabbit muscle pyruvate kinase. It is likely that the two effects are very similar, but neither can be taken at the present time as evidence for a simple dissociation.

All the results therefore point to the absence of fragmentation of ox liver carboxylesterase in urea. Further, the work of Barker and Jencks (1967) in 6 M guanidinium chloride does not provide support for the dissociation of pig liver carboxylesterase into species of mol wt ~ 40,000.

The reduction of the ox enzyme in 6.5 M urea by high levels of mercaptoethanol is not without precedent (Reithel, 1963), but a valid explanation of the ultracentrifugal data (Figure 9) has not yet been found.

Kinetic data for the ox enzyme are reported in the following paper (Stoops *et al.*, 1969).

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References

Barker, D., and Jencks, W. P. (1967), *Fed. Proc. 26*, 452.
Benöhr, H. C., and Krisch, K. (1967a), *Z. Physiol. Chem. 348*, 1102.

Benöhr, H. C., and Krisch, K. (1967b), *Z. Physiol. Chem. 348*, 1115.
Blakeley, R. L., Hinds, J. A., Kunze, H. E., Webb, E. C., and Zerner, B. (1969), *Biochemistry 8*, 1991 (this issue; paper 5).
Boguth, W., Krisch, K., and Niemann, H. (1965), *Biochem. Z. 341*, 149.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
Gutter, F. J. (1957), *Arch. Biochem. Biophys. 72*, 194
Holmes, R. S., and Masters, C. J. (1968), *Biochim. Biophys. Acta 151*, 147.
Horgan, D. J., Dunstone, J. R., Stoops, J. K., Webb, E. C., and Zerner, B. (1969b), *Biochemistry 8*, 2006 (this issue; paper 7).
Horgan, D. J., Stoops, J. K., Webb, E. C., and Zerner, B. (1969a), *Biochemistry 8*, 2000 (this issue; paper 6).
Krisch, K. (1963), *Biochem. Z. 337*, 531.
Malhotra, O. P., and Philip, G. (1966), *Indian J. Biochem. 3*, 7.
Pristoupil, T. I. (1965), *J. Chromatog. 19*, 64.
Reithel, F. J. (1963), *Advan. Protein Chem. 18*, 123.
Runnegar, M. T. C., Scott, K., Webb, E. C., and Zerner, B. (1969), *Biochemistry 8*, 2013 (this issue; paper 8).
Steinhardt, J. (1938), *J. Biol. Chem. 123*, 543.
Steinmetz, M. A., and Deal, Jr., W. C. (1966), *Biochemistry 5*, 1399.
Stoops, J. K., Horgan, D. J., Runnegar, M. T. C., de Jersey, J., Webb, E. C., and Zerner, B. (1969), *Biochemistry 8*, 2026 (this issue; paper 10).

Carboxylesterases (EC 3.1.1). Kinetic Studies on Carboxylesterases*

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ABSTRACT: Kinetic evidence has been obtained which is consistent with the formation of an acyl-enzyme intermediate in the hydrolysis of butyrate esters catalyzed by pig liver carboxylesterase. A study of the pig liver carboxylesterase-catalyzed hydrolysis of phenyl butyrate revealed activation by substrate and by modifiers such as benzene. This activation has been interpreted in terms of a classical kinetic scheme involving sites for substrate and modifier on a single enzyme molecule.

For a series of activated esters, the catalytic rate constants for the carboxylesterase-catalyzed hydrolyses were found to be relatively insensitive to changes in the acyl group, indicating that binding is not responsible for the high reactivity of these systems. Kinetic and other data suggest that there are possibly some different elements of essential chemistry between the esterases and the proteinases.

Mammalian liver carboxylesterases (EC 3.1.1) are highly efficient catalysts of ester hydrolysis. The enzymes differ markedly from the serine proteinases in that high reactivity is exhibited toward simple aliphatic esters. For example, the k_{cat} for the pig liver carboxylesterase-catalyzed hydrolysis of *p*-nitrophenyl acetate is $\sim 10^5$ times the k_{cat} for the same reaction catalyzed by α-chymotrypsin (Horgan *et al.*, 1966). However, α-chymotrypsin exhibits a similarly high reactivity toward "specific" substrates, *e.g.*, *N*-acetyl-L-tyrosine ethyl ester. Since, for these substrates, it has been shown that both the acylation and deacylation steps are sensitive to changes in the acyl moiety, several workers have postulated that a major part of this high reactivity results from the binding of the acyl moiety to the active site (Hein and Niemann, 1962; Koshland, 1962; Bender *et al.*, 1964a).

Investigations of the specificity of carboxylesterases have also shown that changes in the acyl and alcohol moieties affect reactivity. Webb (1964) has extensively studied the specificity of horse liver carboxylesterase. There was only a threefold change in reactivity of acetate and butyrate esters when the chain length of the alcohol moiety was varied from C_1 to C_8. Esters of the C_4 alcohol exhibited the highest reactivity. By contrast, the reactivity of the ethyl ester of *n*-alkyl aliphatic acids increased 14-fold from the acetate to the valerate. Longer chain acyl groups resulted in a small decrease in reactivity. The best substrates were found to contain

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