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Carboxylesterases (EC 3.1.1). The Molecular Weight and Equivalent Weight of Pig Liver Carboxylesterase*

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ABSTRACT: The molecular weight of pig liver carboxylesterase (~88% pure) has been determined as 163,000 (±15,000). The enzyme has two active sites per molecular weight of 163,000 as shown by titration with *p*-nitrophenyl dimethylcarbamate and *p*-nitrophenyl diethyl phosphate. The enzyme undergoes slow irreversible inactivation at pH 5, but on dilution at pH 7.5, the enzyme dissociates apparently into half-molecules which are active.

No evidence has been obtained for the dissociation of the enzyme into species of mol wt ~40,000, either at pH 8 or in 8 M urea.

The physicochemical properties of mammalian liver carboxylesterases have not been extensively investigated. Moreover, only a few enzymes from different animal sources have been studied. This is a direct consequence of the lack of large quantities of these enzymes in highly purified form.

Adler and Kistiakowsky (1961) estimated the molecular weight of pig liver carboxylesterase purified from an acetone powder as 150,000–200,000, using only sedimentation coefficients as the basis for their estimate. The molecular weight of pig liver "microsomal" carboxylesterase as measured by sedimentation and diffusion, by approach to sedimentation equilibrium (Archibald, 1947), and by gel filtration has been given as 174,000 ± 9,000 (Boguth *et al.*, 1965) with reasonable agreement between the three methods used. These authors assumed a partial specific volume of 0.75. Deviations from this value would considerably affect the calculated values of the molecular weight. Further, their experiments using the Archibald method indicated some degree of heterogeneity, as the molecular weights calculated at the air–solution meniscus were invariably less than those calculated at the bottom of the centrifuge cell. No attempt to evaluate the extent of this heterogeneity or to demonstrate the homogeneity of the major component was made. More recently, Barker and Jencks (1967) have reported a molecular weight of 168,000 at pH 7.4, measured by sedimentation equilibrium, for pig liver carboxylesterase purified apparently by the procedure of Adler and Kistiakowsky (1961). Their figure is based on a partial specific volume calculated from amino acid analysis of 0.740.

In contrast to the above results, molecular weights of 180,000 at pH 7.2 and 45,000 at pH 8.0 have been reported for pig liver carboxylesterase (Kibardin, 1962).

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Boursnell and Webb (1949) produced the first estimate of the equivalent weight (96,000) of partially purified horse liver carboxylesterase, using [^{32}P]DFP. In a recent study of the active-site peptides of liver carboxylesterases, from this laboratory, Augusteyn *et al.* (1969) obtained equivalent weights of 79,000, 70,000, and 82,000 for purified preparations of pig, ox, and chicken liver carboxylesterases, respectively, again by using [^{32}P]DFP.

Boguth *et al.* (1965) concluded that pig liver carboxylesterase dissociates into four subunits (mol wt ~40,000) in 0.2% sodium dodecyl sulfate and a similar, although less extensive, effect was interpreted to occur from data obtained in 6.5 M urea. Barker and Jencks (1967) have reported that pig liver carboxylesterase has a molecular weight of 86,000 at pH 2.3, results consistent with our observation of irreversible denaturation on standing the enzyme for 16 days at pH 5.0 (Horgan *et al.*, 1969). Krisch (1966) independently confirmed our earlier observation that pig liver carboxylesterase has two active sites per molecular weight of ~160,000 (Horgan *et al.*, 1966b). Finally, the enzyme has been reported to undergo reversible dissociation into active half-molecules under suitable conditions of salt and of protein concentration (Barker and Jencks, 1967).

In this paper, we report on some of the physicochemical properties of pig liver carboxylesterase purified as described in the preceding paper (Horgan *et al.*, 1969).

Experimental Section

Enzyme. The enzyme used in these studies was purified as described in the preceding paper and had a specific activity of 503.

Materials. *p*-Nitrophenyl dimethylcarbamate was synthesized by the reaction of dimethylcarbamoyl chloride (Aldrich Chemical Co.) with *p*-nitrophenol in dry pyridine. The product was multiply crystallized from chloroform–hexane, mp 106–107°. *Anal.* Calcd for $C_9H_{10}N_2O_4$: C, 51.43; H, 4.80; N, 13.33. Found: C, 51.90; H, 4.86; N, 12.86. *p*-Nitrophenyl diethyl phosphate (paraoxon, E600) was a gift from Albright and Wilson, London. *p*-Nitrophenyl butyrate was prepared by reacting 1 mole of *p*-nitrophenol with ~1.2 moles of *n*-butyryl chloride (bp 102°) in dry pyridine for 1 hr. The crude product was separated by pouring onto ice and dried over anhydrous sodium sulfate. The ester was then taken up in chloroform, rapidly extracted once with cold 10^{-3} N HCl, twice with cold borate buffer (pH 9), twice with cold distilled water, and then dried over anhydrous sodium sulfate. Chloroform was removed on a rotary evaporator and the ester was purified by repeated molecular distillation. The final product was a very pale yellow liquid (*cf.* Huggins and Lapides, 1947). Stock solutions of the esters were prepared in Spectro grade acetonitrile (Eastman Organic Chemicals).

Hydroxylamine solutions were prepared from hydroxylamine hydrochloride (J. T. Baker, Reagent Grade) which had been crystallized from 90% ethanol. Solutions were prepared just before use by the addition of equimolar amounts of NaOH to the hydrochloride. Cysteine solutions were similarly prepared from L-cysteine hydrochloride immediately before use.

All other materials were analytical reagent grade.

Titration of the Enzyme. The kinetics of the hydrolysis of *p*-nitrophenyl dimethylcarbamate catalyzed by pig liver carboxylesterase were followed at 400 mμ in the thermostated cell compartment of a Cary 14 spectrophotometer fitted with a 0–0.1-absorbance slidewire, at 25 ± 0.1°. A typical run is described: 3 ml of 0.15 M Tris buffer (pH 8.16) was equilibrated at 25° in a cuvet in the sample compartment of the spectrophotometer. An aliquot of stock substrate solution was added, followed by an aliquot of acetonitrile, so as to maintain the acetonitrile concentration constant throughout a series of runs. The hydroxide ion catalyzed hydrolysis of the ester is completely negligible at this pH (k_{OH} = 4.4×10^{-4} M^{-1} sec^{-1} at 25°). Recording was commenced to provide a base line for the experiment. An aliquot of enzyme was added and the liberation of *p*-nitrophenolate ion was followed for periods of up to 1 hr.

Assays of the *p*-nitrophenyl dimethylcarbamate inhibited enzyme were similarly run using *p*-nitrophenyl butyrate as substrate. Because of the insolubility of this substrate, these runs were done in buffers containing 10% (v/v) acetonitrile.

The kinetics of reactivation of the *p*-nitrophenyl dimethylcarbamate inhibited enzyme were followed by assaying aliquots of the reaction mixture at suitable times with *p*-nitrophenyl butyrate, a "specific" substrate for the enzyme.

Amino Acid Analysis. An amino acid analysis was obtained using standard methods with the aid of a Beckman-Spinco amino acid analyser. No corrections were made for the destruction of threonine and serine during acid hydrolysis. Tryptophan was estimated spectrophotometrically (Bencze and Schmid, 1957).

Sedimentation Experiments. These experiments were carried out in a Spinco Model E analytical ultracentrifuge. The temperature was controlled at 20° with the RTIC unit. All determinations were carried out in a buffer solution of composition 0.01 M KH_2PO_4, 0.01 M K_2HPO_4, and 0.06 M KCl, pH 6.78, μ = 0.1. The protein solutions were exhaustively dialyzed against this buffer at 4° before each experiment.

Sedimentation coefficients were corrected to water at 20°. Base lines for the schlieren patterns were obtained by the method of Baldwin (1957). This allowed the calculation of the areas under the peaks, so that checks could be made of the concentrations of the sedimenting solutes which had previously been determined by measurement of absorbance at 280 mμ. Good agreement was generally obtained. In the determination of the concentration dependence of the sedimentation coefficient, reference was made to average concentrations which existed in the centrifuge cells during sedimentation (Kegeles and Gutter, 1951).

To test the homogeneity of the preparations, a detailed analysis of the schlieren patterns from sedimentation velocity runs was carried out by a method described by Baldwin (1954). A sedimentation coefficient

distribution (a plot of $g^*(S)$ *vs.* S) was obtained at different times during an experiment. The terms $g^*(S)$, an apparent differential distribution function, and S, a reduced coordinate with the same dimensions as the sedimentation coefficient, have been clearly defined by Nichol and Creeth (1963). In all experiments, the curves of $g^*(S)$ *vs.* S were not quite symmetrical and indicated the presence of a small amount of faster sedimenting material. It was necessary to make allowance for the presence of this component, which was carried out by correcting the schlieren diagrams in the manner described by Baldwin (1957, 1959). The range of S decreased progressively with time, indicating a significant contribution of diffusion to the spreading of the boundary. Thus, the procedure of extrapolating to infinite time (Baldwin, 1959) was used to eliminate these effects. The dependence of sedimentation coefficient on concentration should not markedly affect this analysis because of the relative closeness of the measured sedimentation coefficient and that at infinite dilution (Baldwin, 1959).

The quantity $(S - \bar{s})^2$, where $\bar{s}$ is the weighted mean of the distributions of S, corrected for the presence of the small amount of faster sedimenting material, was plotted against $1/te^{\bar{s}\omega^2 t}$ at fixed values of $g^*(S)/g^*(S)_{max}$, where ω is the angular velocity and t the time.

As a further test of homogeneity, and in order to obtain a value for the diffusion coefficient, the method of Baldwin (1957) based on Fujita's (1956) mathematical analysis was also applied to the schlieren patterns from sedimentation velocity experiments. Corrections for the presence of the faster sedimenting material were again made using the method indicated by Baldwin (1957).

Diffusion coefficients were also obtained from slow-speed ultracentrifuge runs by the reduced height–area method. Calculations were made in the manner described by O'Donnell and Woods (1962) and the calculated diffusion coefficients were corrected to water at 20°.

Determinations of the molecular weight by the Archibald (1947) method were also carried out. The somewhat faster centrifuging speeds, recommended by Ehrenberg (1957), were used. This method permitted measurements at the air–solution meniscus, but prohibited measurements at the bottom of the cell because of the steepness of the refractive index gradient curves. The calculation method was essentially that of Klainer and Kegeles (1955). Refractive index gradients were determined from schlieren patterns, but concentrations were obtained from Rayleigh interference patterns. This procedure was recommended by Kekwick and Creeth.[1] Photographs were taken at 8-min intervals over a period of at least 2 hr and values for the ratio s/D were calculated from each photograph.

Chromatography and Electrophoresis. The major experimental details have been described previously (Horgan *et al.*, 1969). Enzyme which had been treated with 8 M urea was gel filtered on a column of Sephadex G-100 (1.4 × 60 cm) which had been equilibrated with 0.1 M Tris buffer (pH 8.0), containing 8 M urea and 1 mM EDTA. The enzyme sample was prepared by concentrating a 500-μl aliquot of 2.09×10^{-4} N enzyme with 80% ammonium sulfate. The precipitate was taken up in 500 μl of Tris buffer containing 8.3 M urea (pH ~ 8) and allowed to stand at room temperature (~25°) for 1.5 hr.

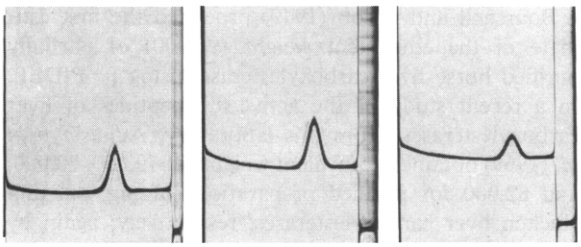

FIGURE 1: Sedimentation diagrams of solutions of pig liver carboxylesterase at pH 6.78. All photographs were taken 32 min after reaching a speed of: (A) 59,780 rpm in the An-D rotor; (B, C) 50,740 rpm in the An-E rotor. The phase-plate angle was 60° in all photographs and the concentrations were: (A) 0.47 g/100 ml, (B) 0.235 g/100 ml, and (C) 0.113 g/100 ml.

The sample was then loaded onto the column. Fractions (~1 ml) were measured by weighing tubes before and after collection, and were assayed for protein by measuring the absorbance at 280 mμ. A solution of α-chymotrypsin, similarly prepared, was also run as a marker.

Dry Weight Determination. The enzyme was exhaustively dialyzed against distilled water and dried using standard high vacuum procedures ($<10^{-5}$ mm), until no trace of water could be detected (5 hr). The dry protein was transferred under vacuum to a drybox and a sample (9.20 mg) was weighed out. This sample was dissolved in 10 ml of 0.15 M Tris buffer (pH 8.16) to give an optically clear solution, and the absorption spectrum was run on a Cary 14 spectrophotometer at 25°.

Results

Molecular Weight Determination. The amino acid composition of the protein was used to calculate a value of 0.733 for the partial specific volume using the method outlined by Cohn and Edsall (1943) and applied by Schachman (1957). Schlieren patterns obtained in sedimentation velocity experiments with the enzyme at pH 6.78 are shown in Figure 1. Single apparently symmetrical peaks were obtained at all concentrations indicating the absence of any gross heterogeneity. At pH 8.0, similar schlieren patterns and sedimentation coefficients were obtained. Careful measurement of the areas of the schlieren peaks indicated the presence of a leading component. By calculating the corrected areas under the schlieren peaks as indicated by Baldwin (1957), the fractional amount of the leading component was estimated to be about 0.1.

At pH 6.78, the concentration dependence of sedimentation coefficient was linear over the range of con-

[1] Personal communication to J. R. D.

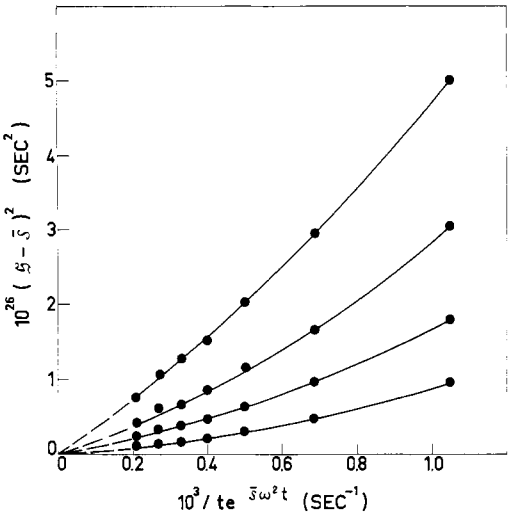

FIGURE 2: Time dependence of the apparent sedimentation coefficient distribution for pig liver carboxylesterase. $(\bar{s} - \bar{s})^2$ is plotted as a function of $1/te^{\bar{s}\omega^2 t}$ at fixed values of $g^*(\bar{s})/g^*(\bar{s})_{max}$ of 0.2, 0.4, 0.6, and 0.8, with the curve for the value 0.2 uppermost. Protein concentration, 0.47 g/100 ml.

centration 0.078–0.47 g/100 ml and is given by $s_{20,w} = 8.3_0(1 - 0.085c)$ which was calculated from the plot of $s_{20,w}$ (Svedberg units, 10^{-13} sec) *vs.* c(g/100 ml) using the method of least squares (Yule and Kendall, 1945).

The results of the boundary analysis (Baldwin, 1959) are shown in Figure 2, where $(\bar{s} - \bar{s})^2$ is plotted against $1/te^{\bar{s}\omega^2 t}$ for various values of $g^*(\bar{s})/g^*(\bar{s})_{max}$. The extrapolations to infinite time are shown by the dashed lines. Although there is uncertainty in the extrapolations owing to the curved plots, it is certain that the values of $(\bar{s} - \bar{s})^2$ at infinite time are close to zero, indicating the absence of measurable heterogeneity in the major component in terms of sedimentation coefficient.

In the second analysis (Fujita, 1956; Baldwin, 1957) the apparent diffusion coefficient, D^*, was calculated, making allowance for the concentration dependence of sedimentation coefficient; the criterion of homogeneity is the constancy of this apparent diffusion coefficient with time. The results of this treatment are given in Figure 3 where D^* is plotted against time. The values obtained, when no allowance was made for the concentration dependence of the sedimentation coefficients, are included for comparison. The diffusion coefficient was assumed independent of concentration and a correction for the small amount of faster sedimenting material was applied (Baldwin, 1957). The constancy of D^* with time confirms the previous result that the major component is free of demonstrable heterogeneity. The diffusion coefficient was estimated as 5.0×10^{-7} cm^2 sec^{-1}.

The diffusion coefficient calculated from several experiments (c, 0.4–0.5% w/v) by centrifuging at slow speed and applying the reduced height–area method was 4.3×10^{-7} cm^2 sec^{-1}. No attempt was made to determine the concentration dependence of the diffusion coefficient.

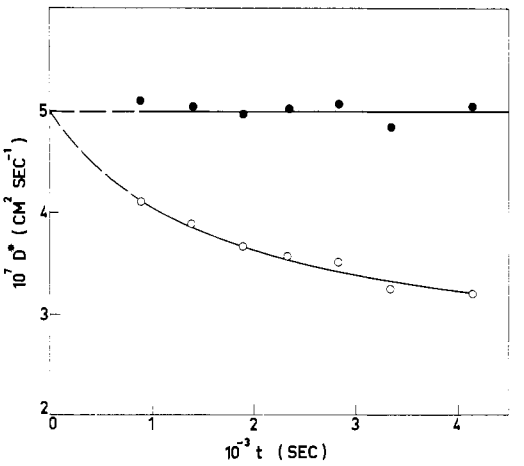

FIGURE 3: Time dependence of the apparent diffusion coefficient for pig liver carboxylesterase. Filled circles show data corrected for concentration dependence, and open circles show the same data without this correction. Protein concentration, 0.47 g/100 ml.

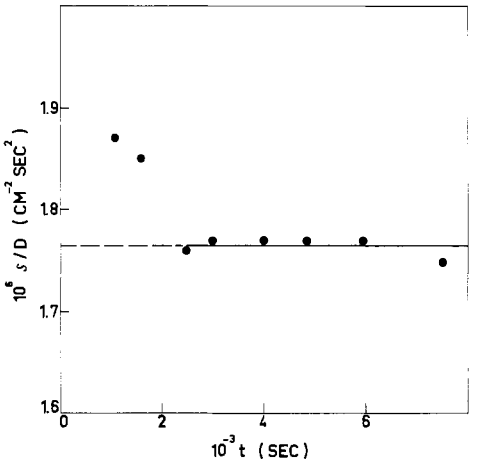

FIGURE 4: Time dependence of the ratio s/D, calculated from successive photographs in an Archibald approach-to-equilibrium experiment. Protein concentration, 0.47 g/100 ml.

Values of s/D obtained from the Archibald approach-to-equilibrium experiments have been plotted against time (Figure 4). An initial decrease followed by constancy with time indicated the presence of a small amount of heavy material which rapidly sedimented from the meniscus and a homogeneous major component with an s/D value of 1.76×10^{-6} cm^{-2} sec^2.

The diffusion coefficient calculated from the slow-speed ultracentrifuge experiments was 4.3×10^{-7} cm^2 sec^{-1}. This value was significantly lower than the value, 5.0×10^{-7} cm^2 sec^{-1}, obtained from the Fujita analysis. The former value represents an average for all species present, whereas the latter value was calculated for the major component only, the contribution of the faster sedimenting material being excluded. For this reason the former value would be expected to be less than the latter. Both these methods are known to give

TABLE I: Titration of Pig Liver Carboxylesterase.[a]

[*p*-Nitrophenyl Dimethylcarbamate] (mM)	A_{burst}[b]	$10^6 \times$ [Enzyme][c] (N)
0.0522	0.0685	3.82
0.1045	0.0715	3.99
0.2089	0.0722	4.03
0.4179	0.0724	4.04
0.5224	0.0724	4.04

[a] 7.46% (v/v) acetonitrile; 0.15 M Tris, pH 8.16. [b] At 400 mμ. [c] Calculated taking account of relevant constants.

values accurate only to about ±5%, and the presence of the faster sedimenting component would introduce further uncertainty into the calculation of D by Fujita's method. Hence, the average of the two values, namely, 4.65×10^{-7} cm^2 sec^{-1}, has been used in the calculation of the molecular weight.

Combination of the data for sedimentation and diffusion (assuming concentration independence for the diffusion coefficient) gives a value for $s^0_{20,w}/D_{20,w}$ of 1.78×10^{-6} cm^{-2} sec^2 which compares favorably with the value 1.76×10^{-6} cm^{-2} sec^2 from the Archibald experiments and the values 1.78×10^{-6} cm^{-2} sec^2 (sedimentation and diffusion) and 1.74×10^{-6} cm^{-2} sec^2 (Archibald) obtained by Boguth *et al.* (1965). This agreement with the results of other workers is to some extent fortuitous in view of the averaging processes used and the fact that the concentration dependence of the diffusion coefficient was ignored, although this factor might be expected to be small (see Tanford, 1961, but compare Boguth *et al.*, 1965).

Combination of the s/D values with that for the partial specific volume ($1 - \bar{v}\rho = 0.264$; $s/D = 1.77 \times 10^{-6}$ cm^{-2} sec^2) gives a molecular weight of 163,000.

Titration of the Enzyme. The hydrolysis of *p*-nitrophenyl dimethylcarbamate catalyzed by pig liver carboxylesterase displays a "burst" phenomenon when the reaction is observed at 400 mμ (pH 8.16). There is an initial rapid release of *p*-nitrophenol, followed by a much slower zero-order turnover of substrate.[2] Extrapolation of the zero-order turnover of *p*-nitrophenyl dimethylcarbamate to zero time gives the quantity A_{burst} of Table I, from which the normality of the enzyme solution may be calculated using the equation of Ouellet and Stewart (1959)

$$[P_1]_{burst} = \left[\frac{k_{+2}}{k_{+2} + k_{+3}} \frac{[S]_0}{[S]_0 + K_m}\right]^2 [E]_0 \qquad (1)$$

By a variety of kinetic procedures (Kézdy and Bender, 1962; Bender *et al.*, 1965), the following approximate values have been found for the kinetic constants under the conditions of the experiments summarized in Table I: $k_{+2} \sim 400k_{+3}$; $K_m \sim 2.5 \times 10^{-6}$ M. In addition, it was found that k_{+2} (carbamylation) was markedly dependent on the acetonitrile concentration, while k_{+3} and K_s were not. When the acetonitrile concentration was lowered to 3.17%, $k_{+2} \sim 1000k_{+3}$. Therefore, for routine estimations of enzyme normalities, the substrate concentration was kept as high as practicable ($\sim 8 \times 10^{-4}$ M) and the acetonitrile concentration as low as possible (~1.6%). Under these conditions, $[P_1]_{burst} = [E]_0$ (very nearly).

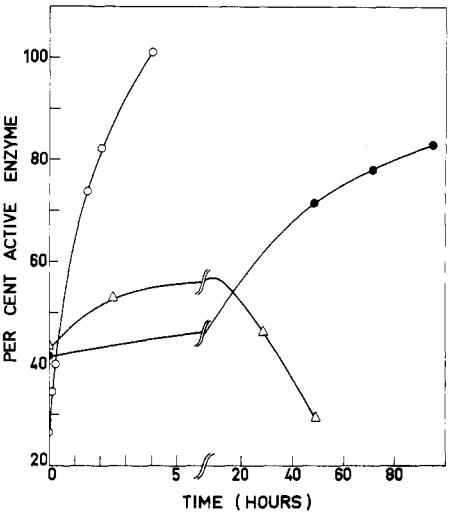

FIGURE 5: Reactivation of *p*-nitrophenyl dimethylcarbamate inhibited pig liver carboxylesterase. (A, ●—●—●) *p*-Nitrophenyl dimethylcarbamate inhibited enzyme, after dialysis against 0.15 M Tris, pH 8.3 (4°). (B, △—△—△) A plus 0.1 M hydroxylamine (4°). (C, ○—○—○) *p*-Nitrophenyl dimethylcarbamate inhibited enzyme, after dialysis against 0.15 M Tris (pH 8.2); 0.25 M hydroxylamine, 5×10^{-3} M cysteine (room temperature ~ 25°).

A similar experiment with paraoxon gives an identical burst (±1%). The titration curve with paraoxon, however, differs in that phosphorylation is faster and dephosphorylation slower than the corresponding reactions with *p*-nitrophenyl dimethylcarbamate. Nonetheless, there is real turnover of paraoxon by this enzyme: $k_{cat} \sim 2 \times 10^{-5}$ sec^{-1} (but *cf.* Keay and Crook, 1965).

Equivalent Weight Estimation. The kinetics of the reaction with *p*-nitrophenyl dimethylcarbamate allowed the first direct determination of the normality of a carboxylesterase solution. Hence, the number of active sites per molecular weight of 163,000 could also be determined. The dry weight determination showed that an enzyme solution containing 1 mg/ml has an absorbance of 1.305 at 280 mμ (0.15 M Tris, pH 8.16). The equivalent weight of the enzyme calculated from the foregoing data is 78,000.

Reactivation of Dimethylcarbamoyl-enzyme. Dialysis of the dimethylcarbamoyl-enzyme against 0.15 M Tris buffer (pH 8.3) leads to the slow regeneration of essentially full activity. Hydroxylamine (0.1 M) causes an initial acceleration of decarbamylation, but long ex-

[2] By contrast, *p*-nitrophenyl diphenylcarbamate is only slowly hydrolyzed by the enzyme.

TABLE II: Effect of Metal Ions on Pig Liver Carboxylesterase.

[Metal ion] (mM)[a]	% Residual Act.[b]		
	Cu^{2+}	Mg^{2+}	Ca^{2+}
0	100	100	100
0.1	100	100	100
0.5	100	100	100
1.0	99	100	96
5.0	96	100	96

[a] All salts used were the chlorides. [b] Activity measured with *p*-nitrophenyl butyrate in the thermostated cell compartment of a Cary 14 spectrophotometer at 400 mμ at 25°; 0.1 M Tris buffer (pH 8.0) containing 10% (v/v) acetonitrile; preincubation time, 30 min.

posure leads to inactivation of the enzyme. This inactivation could be prevented by the addition of 5×10^{-3} M cysteine (Figure 5).

Effect of Metal Ions. The effects of Cu^{2+}, Mg^{2+}, and Ca^{2+} on the hydrolysis of *p*-nitrophenyl butyrate catalyzed by pig liver carboxylesterase are shown in Table II.

Discussion

Molecular Weight Determination. There is still considerable uncertainty about the absolute value of the molecular weight of the enzyme (163,000) owing to the accuracy with which D, s, and $\bar{v}$ have been determined. A realistic estimate would probably be 163,000 ± 15,000. None the less, the result compares favorably with the only other measurement made which has used a measured partial specific volume (Barker and Jencks, 1967). These authors reported a partial specific volume of 0.740 and a molecular weight of 168,000 at pH 7.4. While the spectral method of tryptophan analysis used in the present work can be regarded only as crude, this should have only a small effect on the measured partial specific volume (0.733), since the partial specific volume of tryptophan is 0.740. The complete amino acid analyses of a series of mammalian liver carboxylesterases will be reported later. It should be noted also that there is no evidence in the present work for the reported dissociation of the enzyme at pH 8.0 (Kibardin, 1962).

The time dependence of the Archibald s/D values (Figure 4), and the results of the Fujita analysis (Figure 3) and of the Baldwin analysis (Figure 2) show that the preparation consists of a homogeneous major component and a small amount of faster sedimenting material. The estimate of the amount of this faster sedimenting material is subject to some error, as no account has been taken of the Johnston–Ogston (1946) effect, which predicts that in such a sedimenting boundary, the amount of trailing component, estimated by area measurement, would be in excess of its true value, while the amount of fast component would be diminished by the amount of the excess. Thus the fractional amount of faster sedimenting material in the particular enzyme preparation under investigation is probably more than the estimated value of 0.1.

These conclusions could apply equally well to the enzyme prepared by Boguth *et al.* (1965) where the results of Archibald experiments indicated a similar degree of heterogeneity, but unfortunately no boundary analysis was available to confirm the homogeneity of the major component.

The presence of a faster sedimenting component in the enzyme (specific activity 503) used in this study is consistent with the results reported in the previous paper (Horgan *et al.*, 1969) where it was shown that a final gel filtration step on Sephadex G-200 is capable of removing a heavier component and raising the specific activity to 550–570. The enzyme used in this study would, therefore, appear to be 88% pure.

Boguth *et al.* (1965) reported that 6.5 M urea caused about one-third of the enzyme to dissociate into subunits of a molecular weight (42,000) similar to that found in the presence of 0.2% sodium dodecyl sulfate. In view of the difficulties inherent in the interpretation of these data, a sample of pig liver carboxylesterase was gel filtered on Sephadex G-100 equilibrated with 8 M urea as described in the Experimental Section. Virtually total recovery of the protein was obtained in a single peak (100 ± 2%) which had an elution volume the same as that of Blue Dextran 2000 (mol wt 2×10^6). That the gel was behaving as a molecular sieve in the presence of 8 M urea was shown by the fact that α-chymotrypsin was clearly retarded on the column. While this result does not exclude dissociation of a species of mol wt 160,000 into half-molecules because of the possibility of a lowered exclusion limit for Sephadex G-100 in 8 M urea (*cf.* Wieland *et al.*, 1963; Nathans, 1965; Přístoupil, 1965), it casts doubt on the existence of species of mol wt 42,000 in 6.5 M urea, because bovine serum albumin (mol wt 65,000) has been shown in an independent experiment to be slightly (but significantly) retarded on Sephadex G-100 in 8 M urea (0.1 M Tris, pH ~ 8) (A. J. Anderson and B. Zerner, unpublished results).

Barker and Jencks (1967) have reported that the enzyme dissociates at low pH. Similar results have been observed in this laboratory for the enzymes from pig and ox livers (P. A. Inkerman and B. Zerner, unpublished data). Barker and Jencks (1967) also reported concentration- and salt-dependent dissociation of the pig liver enzyme at pH to give active half-molecules. On Sephadex G-200 (column dimensions 1.2 × 107 cm; 0.032 M Tris, pH 7.50), we have found that the enzyme is completely dissociated apparently into active half-molecules when the column is loaded at a concentration of 0.8 μg/ml (Figure 6). The recovery of activity in the low molecular weight peak was 71% of the total activity, after correction for loss of activity (17%) of an enzyme sample (0.8 μg/ml) over the time of the experiment (3 days). Since the eluent concentration of the enzyme is 0.1 μg/ml, it is not unreasonable to postulate that the loss of activity is a result of increased denaturation at the lower concentrations ob-

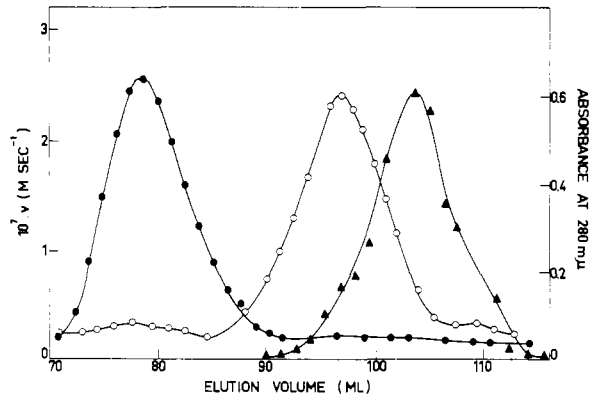

FIGURE 6: The elution profile of pig liver carboxylesterase from a Sephadex G-200 column (1.2 × 107 cm, void volume, 50.4 ml) loaded at 2.8 mg/ml (●) and 0.8 μg/ml (▲). Bovine serum albumin (○), loaded at 5 mg/ml, used as a marker. Column buffer, 0.032 M Tris (pH 7.50). The dilute enzyme solution was eluted at peak activity after ~50 hr.

taining on the column. It is likely therefore that the low molecular weight form of the enzyme is fully active. Absolutely no activity corresponding to the 163,000 molecular weight form of the enzyme was observed in this experiment. However, other experiments (Blakeley *et al.*, 1969) have shown that the dissociation of the enzyme is not rapid.

The V_e/V_0 ratio obtained for bovine serum albumin (1.92) leaves no doubt that it is monomeric (mol wt 65,000) (*cf.* Andrews, 1965). The elution profile shown in Figure 6, therefore, would indicate a molecular weight of ~55,000–60,000 for the low molecular weight form of the carboxylesterase. However, chicken liver carboxylesterase which exists preferentially as a species of mol wt ~ 80,000 (P. A. Inkerman and B. Zerner, unpublished results) is similarly retarded with respect to bovine serum albumin on Sephadex G-200. Therefore, if the low molecular weight form of the enzyme is the half-molecule, as would appear reasonable, the apparent discrepancy may be explained by a differential shape factor.

Titration and Equivalent Weight. While a classical kinetic evaluation of the data has been made in determining approximate constants for the titration system, it must be emphasized that the acylation of this enzyme (and of other carboxylesterases) is not accurately described by a simple carbamylation–decarbamylation scheme. While this system has been extensively investigated, a satisfactory explanation has not yet been found (*cf.* Heymann and Krisch, 1967). Nonetheless, the validity of the titration of the pure enzyme is attested to by the data in Table I, and by the fact that the titration yields reproducible kinetic parameters on a wide range of substrates.

The fact that the diethylphosphoryl-carboxylesterase undergoes slow dephosphorylation raises the question as to whether pig liver carboxylesterase turns over the classical inhibitor, DFP. The alkaline rate constant for the hydrolysis of *p*-nitrophenyl diethyl phosphate is 11 times that for diisopropyl *p*-nitrophenyl phosphate (Ginjaar and Vel, 1958). As these compounds are models for the *p*-nitrophenyl diethyl phosphate- and DFP-inhibited enzymes, respectively, *i.e.*, for the diethylphosphoryl-enzyme and diisopropylphosphoryl-enzyme, it would be expected that the diethylphosphoryl-enzyme would be considerably more reactive and turn over faster than the diisopropylphosphoryl-enzyme. In view of the extremely slow turnover of the diethylphosphoryl-enzyme, it is probable that the DFP-inhibited enzyme would have a negligible turnover ($k_{cat} \sim 2 \times 10^{-6}$ sec^{-1}).

Reactivation of Dimethylcarbamoyl-enzyme. The inactivation of the pig liver enzyme by hydroxylamine has been traced to the combined effect of hydroxylamine, oxygen, and low concentrations of heavy metal ions (in particular, Cu^{2+}). Protection by cysteine is due to the fact that cysteine is acting as a metal chelating agent, rather than as an SH protector, since other chelating agents (*e.g.*, 8-hydroxyquinoline) are also effective. This rules out the possibility that the carboxylesterase is an SH enzyme (Fernley and Zerner, 1968). Further, the effects of Cu^{2+} and other metal ions on the enzyme are, in general, small (Table II). The present results may be compared with those of Keay and Crook (1965) who found 87% inhibition by 10^{-3} M Cu^{2+} of a partially purified "pig liver carboxylesterase." Among other alternatives, it is possible that the enzyme they isolated was an arylesterase.

The carboxylesterases are remarkably efficient catalysts of the hydrolysis of esters, and results based on a reliable enzyme concentration which establish this point are reported in a later paper in this series (Stoops *et al.*, 1969).

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

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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