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Pig Liver Esterase. Physical Properties*

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ABSTRACT: The molecular weight of pig liver esterase, purified by the method of Adler and Kistiakowsky, was found to be 168,000 by equilibrium sedimentation, approach to equilibrium, and sedimentation–diffusion, based on a partial specific volume of 0.74 that was calculated from amino acid analysis; this molecular weight is in agreement with that of other esterase preparations. Near pH 4.5 the enzyme dissociates reversibly in a few minutes to active half-molecules of mol wt 85,000 according to equilibrium sedimentation, and *ca.* mol wt 75,000 according to sucrose gradient sedimentation and measurement of the diffusion coefficient by immunodiffusion. Dissociation to half-molecules, of mol wt 85,000–90,000 by gel chromatography on Sephadex G-100, occurs at pH 7–8 over several hours in extremely dilute solutions or in the presence of salts. The same equilibrium constant of approximately 4×10^{-7} M was found in the presence of 0.5 M sodium chloride or 0.5 M lithium bromide, suggesting that interactions with peptide groups are not responsible for dissociation. Concentrated solutions of enzyme give a single band upon electrophoresis in polyacrylamide gel at pH 8.3, but dilute solutions undergo slow dissociation upon standing to subunits with an increased mobility. Below pH 4 the enzyme undergoes irreversible denaturation to inactive half-molecules of altered shape. In 6 M guanidine hydrochloride–0.1 M mercaptoethanol, the uncorrected molecular weight is 53,500. This is interpreted as evidence for dissociation of the enzyme to unfolded quarter-molecules of mol wt 42,000, which exhibit a preferential interaction with guanidine hydrochloride. Different preparations of pig liver esterase exhibit closely related, but significantly different, physical, immunological, and kinetic properties.

We report here some of the physical properties of pig liver carboxylesterase (EC 3.1.1.1) and its subunits, purified by the method of Adler and Kistiakowsky (1961), and a preliminary study of the conditions, rates, and equilibria for the reversible dissociation of the whole molecule into half-molecules. In experiments carried out in parallel to those reported here, Boguth *et al.* (1965) determined a molecular weight of 174,000 and Horgan *et al.* (1966a) a molecular weight of 163,000 for very similar enzymes isolated from pig liver by different methods, and similar properties have been found for enzymes from beef liver (Benöhr and Krisch, 1967a–c) and pig kidney (Franz and Krisch, 1966, 1968). Active-site titrations with *p*-nitrophenyl diethyl phosphate, bis(*p*-nitrophenyl) phosphate, and *p*-nitrophenyl dimethylcarbamate have indicated a minimum molecular weight of 78,000–86,000, showing that there are two active sites on each whole molecule of enzyme (Krisch, 1966; Heymann and Krisch, 1967; Horgan *et al.*, 1966b). A preliminary report of some of this work has appeared (Barker and Jencks, 1967).

Experimental Procedure

Inorganic salts, buffers, solvents, and sucrose were of the highest purity available and were not purified further. β-Mercaptoethanol was redistilled before use and guanidine hydrochloride was purified by recrystallization from ethanol–benzene and from methanol according to the procedure of Y. Nozaki (personal communication). Glass-distilled water was used for all experiments with the purified enzyme; ordinary distilled water was used for enzyme purification. *m*-(*n*-Heptanoyloxy)benzoic acid (mC_7)[1] was prepared from heptanoyl chloride and *m*-hydroxybenzoic acid in pyridine at 0°. After

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[1] Abbreviations used are: mC_5, *m*-(*n*-pentanoyloxy)benzoic acid; mC_7, *m*-(*n*-heptanoyloxy)benzoic acid.

TABLE I: Summary of Esterase Purification.

Purifcn Step	Protein[a] (mg/ml)	Sp Act.[b]	Total[b] Act.	Recov (%)	Purifcn Factor
1. 0.025 M NH_3 extract	31	0.0185	74,100	(100)	(1.0)
2. pH 5.6 supernatant	11.3	0.054	61,400	83	2.9
3. $(NH_4)_2SO_4$ precipitation[c] and dialysis	47	0.183	55,900	76	9.9
4. 25–45% acetone precipitation and dialysis	35	0.383	54,000	73	21
5. DEAE, concentration,[d] and dialysis	10.0	2.34	17,700	24	126

[a] Protein concentration was estimated spectrophotometrically by the method of Layne (1957), except for the pure enzyme (step 5) for which $\epsilon_{1\,cm}^{1\%}$ 13.8 was used. [b] Activity was measured as ΔA_{300}/min per 0.1 ml of enzyme in 3.0 ml of 1.67 mM mC_7–0.05 M Tris, pH 8.0, 25°. Specific activity is defined as ΔA_{300}/min per 0.1 ml of enzyme divided by A_{280} for the enzyme solution. [c] Fraction obtained between 30 g/100 ml and an additional 7.5 g/100 ml of ammonium sulfate. [d] By 75% saturation with ammonium sulfate.

addition of ether, the solution was washed with cold 20% hydrochloric acid and with saturated sodium chloride, dried over magnesium sulfate, and evaporated to dryness. The product was crystallized from petroleum ether (bp 60–110°) and from 40% ethanol–water: mp 78.5–79°, lit. (Hofstee, 1954) mp 78°. *m*-(*n*-Pentanoyloxy)benzoic acid (mC_5) was synthesized by an analogous procedure: mp 83–84°, lit. (Hofstee, 1954) mp 84°. Electrofocusing ampholyte (pH 4–6 (LKB)) was a gift of Dr. W. Murakami. Dialysis tubing was boiled in neutral 10^{-3} M EDTA and washed with distilled water before use.

The enzyme was purified from pig liver acetone powder by the method of Adler and Kistiakowsky (1961). All operations were carried out at 0–4°, except that pH measurements were made at 25°. In the final step the enzyme was eluted with a linear gradient of two column volumes each of 0.02 M potassium phosphate (pH 7.4) and 0.05 M potassium phosphate (pH 5.2) containing 0.25 M sodium chloride, after an initial washing with two column volumes of 0.02 M potassium phosphate buffer (pH 7.4). The purified enzyme was dialyzed against this buffer and stored at −20°. The purification procedure is summarized in Table I.

Enzyme activities were measured spectrophotometrically at 25°, at 300 nm for *m*-hydroxybenzoate esters and at 400 or 347 nm for *p*-nitrophenyl acetate. Corrections were made to account for incomplete ionization of *p*-nitrophenol, if required.

Amino acid analysis was performed by the method of Spackman *et al.* (1958) in a Beckman amino acid analyzer. Cysteine was determined as cysteic acid. Tryptophan was determined spectrophotometrically by the method of Bencze and Schmid (1957).

Gel chromatography of the enzyme was carried out on 2 × 45 (or 50) cm columns of Sephadex G-100 gel (Pharmacia) according to the procedure of Andrews (1964). The gel was swollen at 100° in 0.1 M potassium chloride for 10 min and then washed with buffer before use. Samples were applied in a 1-ml volume containing 10 mg of sucrose just below a layer of buffer at the top of the gel with a Pasteur pipet bent 90° at the tip. Fractions of 3 ml were collected and assayed for enzyme activity with 4.0 mM mC_5 in 0.5 M Tris buffer (pH 8.0).

Ultracentrifugation was carried out with a Spinco Model E ultracentrifuge using the An-D rotor and either schlieren or interference optics. Sedimentation coefficients and frictional ratios were calculated according to Tanford (1961). Diffusion coefficients were determined by the methods described by Ehrenberg (1957). The Archibald approach to equilibrium experiments (Ehrenberg, 1957) were carried out at 10,000 or 10,589 rpm; after synthetic boundary spreading the rotor speed was increased to 59,780 or 60,000 rpm to obtain the sedimentation coefficient. Meniscus depletion equilibrium sedimentation experiments were carried out at speeds calculated according to the equations of Yphantis (1964). Molecular weights were obtained by the least-squares slopes method, using groups of five equally spaced points. Measurements of ultracentrifuge photographic plates were made on a Gaertner microcomparator or a Nikon Model 6C profile projector.

Sucrose density gradient centrifugation was carried out according to Martin and Ames (1961) in the SW 25.1 rotor of a Spinco Model L ultracentrifuge at 23,000 rpm for 35 hr. Enzyme solutions in 0.5 ml were layered on a linear 5–20% sucrose gradient.

Diffusion coefficients were also determined immunologically by the method of Allison and Humphrey (1959) from the position of the precipitation line formed by diffusion of antigen and antibody in agar gel. Two linear wells, one containing antibody and the other purified enzyme, are placed in agar at right angles. The angle θ between the precipitin line and the antigen well permits an estimation of the diffusion coefficient of the enzyme Dp from the equation $\tan\theta = (Dp/Da)^{1/2}$, in which the diffusion coefficient for rabbit antibodies $Da = 3.8 \times 10^{-7}$ cm²/sec. Antibodies to esterase preparations were obtained by injecting rabbits with a total of 2 mg of the enzyme preparation in complete Freund's adjuvent in the toepad and intramuscularly. The rabbits were bled at 3 weeks. Additional bleedings were carried out 1 week after booster injections of 1 mg of antigen at approximately monthly intervals. Complement fixation measurements were carried out by the method of Levine (1968).

The extinction coefficient of the esterase was determined by comparison with that of bovine serum albumin ($\epsilon_{1\,cm}^{1\%}$ 6.67, Foster and Sterman, 1956) based upon the refractive index of the two proteins. After determination of the absor-

TABLE II: Amino Acid Composition of Pig Liver Esterase.

Amino Acid	Residues/Molecule[a]
Lysine	98
Histidine	31
Arginine	49
Aspartic acid	129
Threonine	76[b]
Serine	80[b]
Glutamic acid	150
Proline	93
Glycine	129
Alanine	117
Half-cystine	14[c]
Valine	110[d]
Methionine	36
Isoleucine	52[d]
Leucine	147[d]
Tyrosine	40
Phenylalanine	78
Tryptophan	60[e]

[a] Based on a molecular weight of 168,000. [b] Values obtained after 24-hr hydrolysis. [c] Determined after performic acid oxidation. [d] Values obtained after 74-hr hydrolysis. [e] Determined spectrophotometrically by the method of Bencze and Schmid (1957).

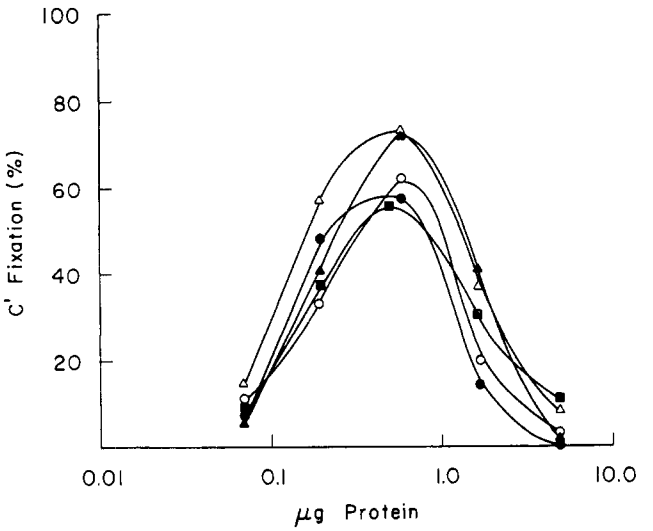


FIGURE 1: Complement fixation curves obtained with antibody to purified esterase, diluted $^1/_{1500}$ (first bleeding). The antigens are purified enzyme (closed circles), enzyme from the leading and tailing edges of the DEAE peak (open circles and closed triangles, respectively), and two enzyme fractions obtained by the procedure of Horgan *et al.* (1966a) (open triangles and squares).

bance at 280 nm the concentration of the two protein solutions was compared by counting interference fringes across a protein–solvent boundary (Richards and Schachman, 1959; Schachman, 1963).

Results

The enzyme purified by the Adler and Kistiakowsky (1961) procedure appears to be essentially homogeneous, confirming the results of these workers, but exposure to acid and other conditions may give rise to altered forms of the enzyme with different physical and catalytic properties. The enzyme was eluted from DEAE-cellulose in a peak of constant specific activity. The preparation used for most of the kinetic experiments reported here showed a suggestion of shoulders on both sides of the main peak, but fractions obtained from these regions showed the same specific activity as the central fractions. A single, symmetrical peak was obtained in all ultracentrifugation experiments at neutral pH. Attempts at crystallization gave precipitates containing approximately equal amounts of thin, platelike crystals and amorphous material; pure crystals were not obtained. The purified enzyme has an absorbance ratio A_{280}/A_{260} of 1.68 and an extinction coefficient $\epsilon^{1\%}_{1\,cm}$ 13.8 ± 0.3 at 280 nm, pH 8.0. Horgan *et al.* (1969a) have reported $\epsilon^{1\%}_{1\,cm}$ 13.05.

The purified enzyme, the enzyme isolated from the leading and trailing edges of the peak eluted from DEAE-cellulose, and two purified enzyme fractions from pig liver which were obtained by the purification procedure of Horgan *et al.* (1966a)[2] were examined immunologically by L. Levine and E. Wasserman. All of the purified enzymes were found to be strongly immunogenic in rabbits. The esterase activity is quantitatively precipitated in the presence of antiserum. Analyses of the five rabbit antisera from the initial bleeding by Ouchterlony double diffusion in agar at pH 7.5 with all five enzyme preparations demonstrated immunologic purity of the immune systems (a single band of precipitation was observed). When all five enzyme preparations diffused toward each of the five antiserums, patterns of identity (no spurs) were observed. Thus, a similar set of antigenic determinants is present on the five enzyme preparations, including the leading and trailing edges of the peak eluted from DEAE-cellulose. A weak secondary band of questionable significance was observed in some fractions with antisera from a second bleeding, but a third bleeding gave only a single band. Complement fixation curves were found to exhibit maximal fixation at the same concentration of antigen for all fractions, but show small differences in shape and in maximum degree of fixation (Figure 1). These results indicate that the different fractions are almost, but not completely, identical. Hain and Krisch (1968) have shown that their esterase preparation is homogeneous by immunological criteria.

The amino acid composition of the enzyme is shown in Table II. The values are averages of determinations after hydrolysis for 24, 48, and 72 hr in constant boiling hydrochloric acid at 110°; the replicate determinations generally agreed within 5%. A partial specific volume of 0.740 cm^3/g was calculated from the amino acid composition (Schachman, 1957; McMeekin and Marshall, 1952; Cohn and Edsall, 1943).

Disc Electrophoresis. The enzyme was found to be electrophoretically homogeneous upon electrophoresis in 7.5% polyacrylamide gel of a sample at 0.2 mg/ml in 0.05 M Tris–0.38 M glycine buffer (pH 8.5) followed by staining for protein with Amido Black according to the procedure of Holmes and Masters (1967). However, if more dilute solutions of enzyme were allowed to stand before electrophoresis, dissociation into

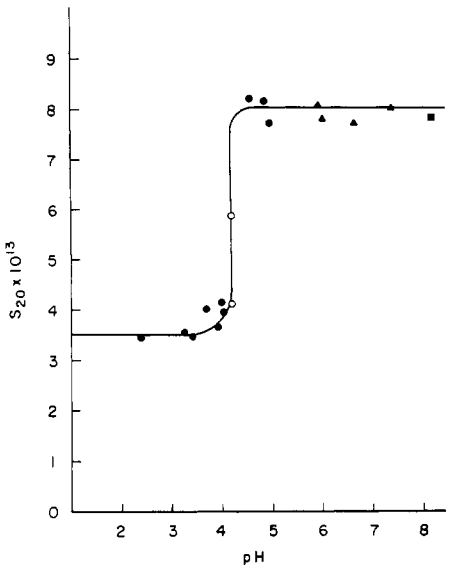


FIGURE 2: The sedimentation coefficient as a function of pH for 2 mg/ml of esterase in 0.1 M potassium chloride–0.02 M Tris (pH 8.2), phosphate (pH 5.9–7.4), or acetate (pH 2.5–5.0) buffer, 20°. The open circles show values determined at the beginning and end of a single run begun 30 min after preparation of a sample at pH 4.2.

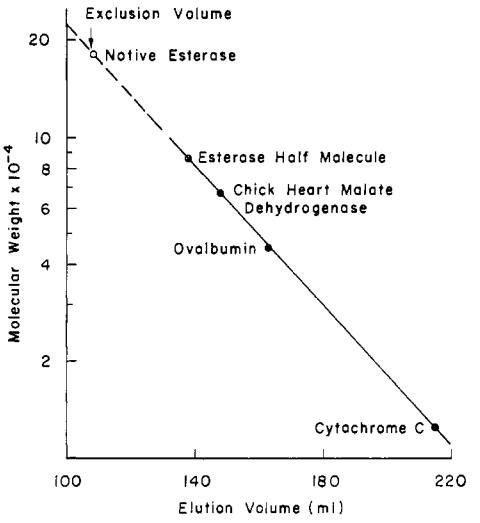


FIGURE 3: Elution positions of esterase whole and half-molecules from Sephadex G-100 (column size 2 × 50 cm) equilibrated with 0.5 M lithium bromide in 0.05 M Tris (pH 8.0). The samples contained 0.2 mg of esterase, catalytic amounts of malate dehydrogenase, 5 mg of ovalbumin, and 3 mg of cytochrome *c* in 1.0 ml of column buffer.

subunits with an increased electrophoretic mobility in this system was observed. In one experiment an enzyme preparation diluted to 2 × 10^{-4} mg/ml gave a single band with the same mobility observed with more concentrated solutions when applied to the gel within 5 min of dilution, but showed progressive dissociation to subunits with an increased mobility upon standing. After 24 hr most of the enzyme activity appeared in these bands of increased mobility. The position of the enzyme after electrophoresis was determined by staining for enzyme activity according to the procedure of Holmes and Masters (1967). A carboxylesterase purified from pig liver by the method of Horgan *et al.* (1966a) showed similar behavior, but dissociated more rapidly (at 2 × 10^{-4} mg/ml) than the enzyme purified by the Adler and Kistiakowsky procedure.[2]

Dependence upon pH of the Sedimentation Coefficient. The sedimentation coefficient in 0.1 M potassium chloride–0.02 M potassium phosphate (pH 7.4) at 20° was found to decrease linearly with increasing protein concentration from 2 to 10 mg per ml, with a slope of −76 S g^{-1} ml and an intercept $s_{20,w}$ = 8.12 S. This is very similar to the values of 8.2 ± 0.1 and 8.3 S reported by Boguth *et al.* (1965) and by Horgan *et al.* (1969b).

As the pH is decreased there is a decrease in the sedimentation coefficient of esterase at 2 mg/ml to a value of 3.5 S, with a sharp transition between pH 4 and 5 (Figure 2). In the transition region of pH the sedimentation coefficient was found to change with time. The open circles in the figure show the sedimentation coefficients of a sample at pH 4.2 which sedimented initially with s = 5.8 S, but slowed gradually over 1 hr and sedimented during the second half of the run with s = 4.1 S. In more concentrated solutions, a second peak with an s value of 5.5–5.9 S was frequently observed at pH 2.0–3.5. In 6 M guanidine hydrochloride containing 0.02 M mercaptoethanol the observed sedimentation coefficient of the enzyme at 5 mg/ml was found to be 0.487 S, which is equivalent to a value of $s_{20,w}$ = 1.3 S after correction for the density and viscosity of the solvent (Kawahara *et al.*, 1965).

The sedimentation coefficient was also estimated by centrifugation of a dilute solution of enzyme in a sucrose gradient at pH 7.4 and 4.5 (Martin and Ames, 1961). At pH 7.4 the activity peak traveled 11.5/25 fractions and at pH 4.5 the peak traveled 7.7/25 fractions. Based on a value of 8.1 S for the whole molecule at neutrality, this gives a value of approximately 5.4 S for the active, dissociated enzyme at pH 4.5 in dilute solution.

Molecular Weight. The meniscus depletion equilibrium sedimentation method of Yphantis (1964) gave a weight-average molecular weight of 168,000 for an enzyme solution initially 0.15 mg/ml in 0.1 M sodium chloride–0.05 M Tris buffer (pH 8.0). A preparation which had previously been dissociated into subunits at pH 4.5 (see below) gave a molecular weight of 173,000 by this method after dialysis for 18 hr against 0.1 M potassium chloride–0.05 M Tris (pH 8.0) at 4°.

The Archibald approach to equilibrium method gave a molecular weight of 165,000 ± 8,000 for an enzyme solution initially 10 mg/ml in 0.2 M sodium chloride–0.02 M potassium phosphate (pH 7.4) in three experiments. Molecular weights were determined at the meniscus only. No trend was seen as a function of time.

The diffusion coefficient for the enzyme at 10 mg/ml was determined to be 4.12 × 10^{-7} cm^2/sec from the synthetic boundary runs in these experiments. Based on the observed sedimentation coefficient of 7.36 S at 10 mg/ml and assuming an approximate cancellation of the concentration dependences of the sedimentation and diffusion coefficients, a molecular weight of 168,000 was calculated from the Svedberg equation.

From the molecular weight of 168,000 and sedimentation coefficient of 8.12 S the frictional coefficient f of the enzyme was calculated to be 8.93 × 10^{-8} dyn sec/cm and the theoretical frictional coefficient for a spherical, unhydrated mol-

[2] P. Greenzaid, unpublished experiments.

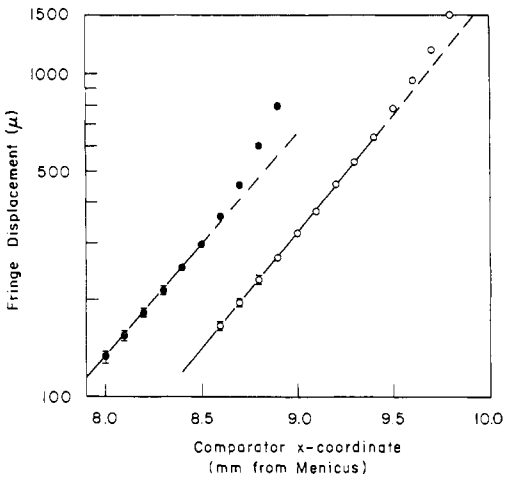


FIGURE 4: Equilibrium sedimentation of esterase at pH 4.5 (solid circles) and pH 2.3 (open circles). The pH 4.5 sample was prepared at a loading concentration of 0.0075% in 0.1 M KCl–0.02 M sodium acetate and was centrifuged at 23,150 rpm for 8 hr at 4°. The pH 2.3 sample was prepared at a loading concentration of 0.015% in 0.1 M KCl–HCl and centrifuged at 23,150 rpm for 9 hr at 20°.

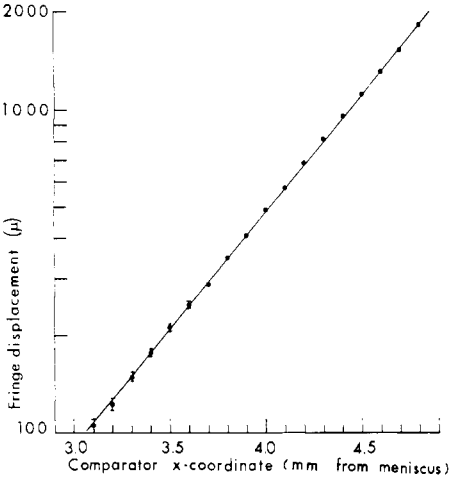


FIGURE 5: Equilibrium sedimentation of 0.05% esterase in 6.0 M guanidine hydrochloride–0.1 M 2-mercaptoethanol (pH 5.8). The run was performed in a three-channel interference cell at 36,000 rpm for 68 hr, 23.0°.

ecule was calculated to be 6.95 dyn sec/cm (Tanford, 1961). If it is assumed that the hydration is 0.3 g of H_2O/g of protein (Mahler and Cordes, 1966), these values give an axial ratio of about 3:1 for the enzyme. Boguth *et al.* (1965) estimated an axial ratio of 5:1 from very similar data for their enzyme preparation, neglecting hydration.

Molecular Weight of Subunits. Upon gel chromatography through columns of Sephadex G-100 of *dilute* solutions of enzyme at pH 8.0, the whole molecule is not retarded but a slower moving peak of dissociated, active enzyme is observed which increases in size in the presence of salt. The molecular weight of the dissociated enzyme was estimated to be 85,000–90,000 by comparison of its elution position with those of malate dehydrogenase, ovalbumin, and cytochrome *c* standards from a column equilibrated with 0.5 M lithium bromide (Figure 3), according to the procedure of Andrews (1964). Identical results were obtained with columns equilibrated with 0.5 M sodium chloride, 0.5 M lithium chloride, and 1.0 M lithium bromide. The elution volume of the dissociated enzyme was found to be independent of the amount of enzyme in the dissociated form. These experiments were carried out with slow flow rates, about 10 ml/hr.

Determination of the molecular weight of the dissociated enzyme in acid solutions was complicated by the fact that at least two different forms of dissociated enzyme exist under these conditions. Evidence was obtained for an initial rapid dissociation into active half-molecules in the vicinity of pH 4.5, followed by a loss of activity and a change in shape of these half-molecules upon prolonged incubation or at lower pH values.

The active half-molecule was examined by equilibrium sedimentation of dilute solutions of enzyme at pH 4.5 with interference optics. Plots of the results gave curved lines indicative of the presence of higher weight material near the base of the cell (Figure 4). Molecular weights of 82,000 and 85,000 were calculated from the linear portions of curves obtained with 0.0075 and 0.003% protein, respectively; in no case was there evidence for any material of lower molecular weight. A similar experiment with 0.015% protein at pH 2.3 gave a molecular weight of 86,000 from the linear portion of the curve (Figure 4). Enzyme solutions incubated under the same conditions as those of the centrifugation gave recoveries of enzyme activities of 60–80% at pH 4.5 and no activity at pH 2.3.

The diffusion coefficient of the active dissociated enzyme at pH 4.5 could not be measured in the ultracentrifuge, because of aggregation at the concentrations required for measurement. The diffusion coefficient was, accordingly, measured by immunodiffusion in 1% agar containing 0.145 M sodium chloride (Allison and Humphrey, 1959). From the single precipitin lines formed between the antibody and enzyme wells in experiments at pH 8.0 (0.01 M Tris) and 4.5 (0.01 M sodium acetate), diffusion coefficients of 4.3×10^{-7} and 6.8×10^{-7} cm² per sec were obtained. The former value agrees satisfactorily with the value of 4.12×10^{-7} cm² per sec for 10 mg of protein/ml at pH 7.4, determined in the ultracentrifuge. The latter value, combined with the sedimentation coefficient of 5.4 S from sucrose gradient sedimentation, gives an approximate molecular weight of 75,000 for the dissociated enzyme.

Ultracentrifugation at lower pH values and higher protein concentrations was complicated by time-dependent changes in the physical constants and by aggregation. From one experiment in which enzyme at 2.0 mg/ml was incubated in 0.4 M sodium acetate buffer (pH 3.7) for 3 hr before centrifugation at 20°, a diffusion coefficient of 4.62×10^{-7} cm² per sec and a sedimentation coefficient of 4.03 S were obtained, giving a molecular weight of 82,000. Higher values of the diffusion coefficient were obtained after shorter times at this pH value. Equilibrium sedimentation of the same sample at pH 3.7 after tenfold dilution and centrifugation at 21,740 rpm for 11 hr gave a curved plot indicative of a mixture of species of molecular weight about 90,000 and larger. No species of lower molecular weight was evident at this time or after an additional sedimentation for 9 hr at 29,500 rpm.

The molecular weight of the fully dissociated enzyme was examined by equilibrium sedimentation in purified 6 M guanidine hydrochloride containing 0.1 M mercaptoethanol. Four

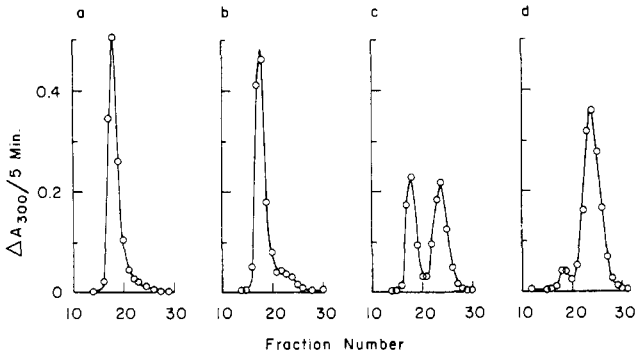


FIGURE 6: Elution patterns of esterase from Sephadex G-100 at 20°. All column buffers contained 0.05 M Tris (pH 8.0). Additional conditions were: (a) native enzyme, 0.1 M KCl. (b) Enzyme in 0.5 M NaCl, applied immediately after preparation. (c) Enzyme incubated in 0.5 M NaCl for 23 hr at 20°. (d) Enzyme incubated in 0.5 ml of 0.01 M sodium acetate for 40 min at 20°. Then 0.15 ml of H_2O, 0.25 ml of 2.0 M NaCl, and 0.1 ml of 1.0 M Tris were added in that order (to give 0.5 M NaCl, pH 8) and the sample was applied immediately to the column equilibrated with 0.5 M NaCl.

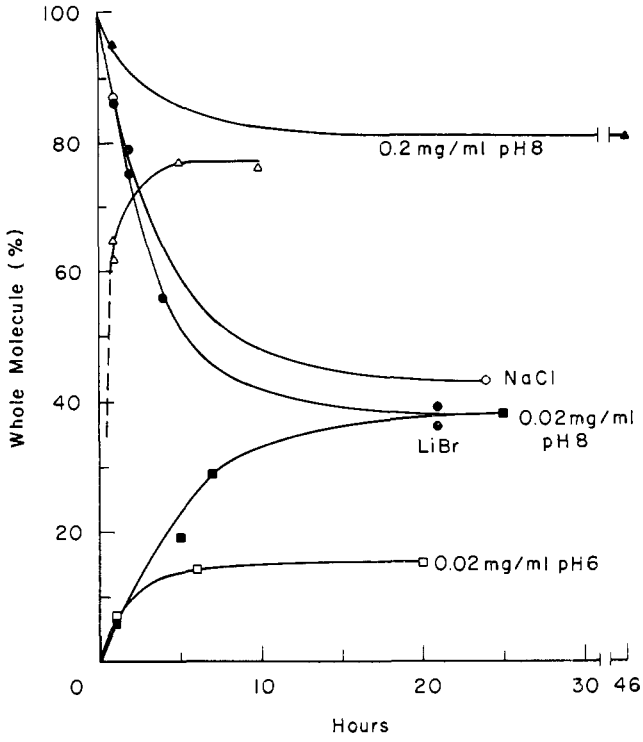


FIGURE 7: Equilibrium for dissociation into half-molecules in the presence of 0.5 M salt at 20°, determined by Sephadex G-100 chromatography; 1 hr was added to the incubation times to approximately compensate for elution times. Dissociated enzyme was prepared by incubation at pH 4.5 for 40 min. The buffers were 0.05 M Tris at pH 8.0 and 0.05 M potassium phosphate at pH 6.0. (△, ▲) 0.2 mg/ml, 0.5 M sodium chloride, pH 8; (○, ■) same, 0.02 mg/ml; (●) 0.02 mg/ml, 0.5 M lithium bromide, pH 8; and (□) 0.02 mg/ml, 0.5 M sodium chloride, pH 6.

experiments with 0.05% protein in the pH range 3.7–6.0 gave linear plots of log fringe displacement against distance (Figure 5) and an apparent molecular weight of 53,500 ± 3,000, based on $\bar{v} = 0.740$. A previously reported value of 66,000 (Barker and Jencks, 1967) was obtained with guanidine hydrochloride which had only been decolorized by passage through activated charcoal and is now believed to have been grossly impure.

Dissociation Studies by Gel Chromatography. The amount of enzyme dissociated into half-molecules was estimated by chromatography on Sephadex G-100 at a rate which is fast relative to the rate of the dissociation–reassociation process. As shown in Figure 6a, there is a small amount of tailing suggestive of dissociation when the native enzyme, initially 0.02 mg/ml, is subjected to gel chromatography in 0.1 M potassium chloride–0.05 M Tris (pH 8.0). If the salt concentration is increased to 0.5 M sodium chloride and the enzyme is immediately subjected to chromatography, a small peak of activity of the half-molecules is apparent, caused by dissociation during the 2 hr required for the chromatographic run (Figure 6b). After 23 hr in the presence of salt, equilibrium had almost been attained and approximately equal amounts of whole and half-moleules were observed (Figure 6c). After dissociation of the enzyme in 0.01 M acetate buffer (pH 4.5) at 0.04 mg/ml for 40 min at 20°, followed by the successive addition of salt and Tris buffer to give 0.5 M sodium chloride (pH 8.0) and immediate chromatography, 94% of the activity was found in the half-molecule peak (Figure 6d). The half-molecule peak always appeared at the same elution position as the second peak in the calibrated molecular weight estimations by gel chromatography (Figure 3).

The dissociation equilibrium was approached from both directions by incubating native enzyme and enzyme which had previously been dissociated at pH 4.5 in the desired buffer and salt solution. The results in Figure 7 show that the same equilibrium position is reached from both directions within 24 hr. An increase in enzyme concentration shifts the equilibrium toward whole molecules and a decrease in pH from 8 to 6 gives an increase in the equilibrium concentration of dissociated enzyme. There is no significant difference between the effects of 0.5 M sodium chloride and lithium bromide on the dissociation equilibrium.

An approximate value of the equilibrium constant for dissociation (eq 1 and 2) was calculated from the ratios of activity in the two peaks at equilibrium. In eq 2, [H] and [W]

$$\text{whole molecule} \rightleftharpoons 2 \text{ half-molecules} \qquad (1)$$

$$K_{eq} = \frac{[H]^2}{[W]} = \frac{4[E_0]}{(A_W/A_H) + (A_W/A_H)^2} \qquad (2)$$

refer to the *molar* concentrations of half and whole molecules, A_W and A_H refer to the measured activities in the first and second peaks, respectively, and $[E_0]$ is the initial molar concentration of undissociated enzyme. The validity of this calculation depends upon the assumptions (a) that the equilibrium position is not perturbed by chromatography on Sephadex and (b) that the activities per active site are the same for the whole molecule and the dissociated enzyme. The results shown in Figure 6 indicate that interconversion of the two forms of the enzyme during chromatography on Sephadex is appreciable, but not large. The activities in the region of the second peak are 5 and 12% of the total activity in the experiments of Figures 6a,b, respectively; the difference of 7% represents the conversion of whole into half-molecules caused by

TABLE III: Equilibrium Distributions of Enzyme Forms Determined by Sephadex Filtration.

Conditions[a]	Enzyme Concn, E_0 (mg/ml)[b]	Activity as Whole (%)	Activity as Half-Molecule (%)	K_{eq} (M)
0.5 M LiBr, pH 8.0	0.02	38	62	4.8×10^{-7}
0.5 M NaCl, pH 8.0	0.02	38	62	4.8×10^{-7}
0.5 M NaCl, pH 8.0	0.2	76	24	3.6×10^{-7}
0.5 M NaCl, pH 6.0	0.02	15	85	2.3×10^{-6}

[a] In 0.05 M Tris-HCl buffer (pH 8.0) or 0.05 M potassium phosphate buffer (pH 6.0). [b] 0.02 mg/ml = 1.2×10^{-7} M.

the salt to which the enzyme was exposed during chromatography in the experiment of Figure 6b. The recovery of 6% of the total activity in the whole molecule peak after dissociation to half-molecules in acid represents an upper limit for the amount of reassociation to whole molecules that takes place during chromatography. The native enzyme and half-molecules which were dissociated in acid and reneutralized in the presence of salt were shown to have the same maximal velocities toward mC_7 at pH 8.0 and toward 0.33 mM PNPA at pH values in the range 3.6–8.4. Furthermore, the relative total activities in three experiments in which the ratio of activities in the first and second peaks was 43/57, 88/12, and 16/84 were found to be 0.96, 1.03, and 1.00, respectively.

The calculated values of K_{eq} are given in Table III. Similar values are obtained in the presence of sodium chloride and lithium bromide and over a tenfold range of enzyme concentration. There is an approximately fivefold increase in the dissociation constant as the pH is decreased from 8.0 to 6.0.

Because of the kinetic results described in the following paper, attempts were made to determine whether the presence of substrate affects the rate of dissociation; if the presence of substrate affects the enzyme structure in the region of interaction between the two half-molecules, such an effect might be expected. The enzyme was incubated for 1 hr in 0.5 M lithium bromide–0.05 M Tris (pH 8.0) in the presence and absence of substrate. Under these conditions the enzyme would be about 60% dissociated at equilibrium (based on activity), but equilibrium is not reached during a 1-hr incubation; thus, any rapid dissociation brought about by the presence of substrate during even part of this incubation should give rise to an increase in the observed amount of dissociated enzyme. Incubation in the presence of 0.04 M mC_5 (hydrolyzed 95% in about 40 min) and in the presence of 0.015 M mC_7 (hydrolyzed 95% in about 15 min) gave no increase in the amount of dissociated enzyme compared with the control value of 24 ± 3% of the total activity. In a further experiment, enzyme which had been incubated in the presence of 0.04 M mC_5 for 1 hr was chromatographed on a column to which had previously been added 51 ml of buffer containing 0.012 M mC_5. This amount of substrate-containing buffer was chosen so that the enzyme would be exposed to substrate during a large part of the chromatography, but would emerge ahead of substrate and hydrolysis products. The elution pattern of activity showed only 25% half-molecules, again the same as control runs without substrate. Chromatography in 0.1 M potassium chloride–0.05 M Tris (pH 8.0) in the presence and absence of 0.1 M hexanamide indicated no detectable effect of this substrate analog on the amount of dissociation.

Dissociation does not occur to an appreciable extent in the presence of low salt concentration near neutral pH unless the enzyme concentration is markedly reduced. Figures 8a and b show the results of a *slow* chromatographic separation of enzyme applied at concentrations of 2.4×10^{-9} and 0.6×10^{-9} M, respectively. The dashed lines in the figure show the nonenzymatic hydrolysis of substrate, which becomes significant at the very low enzyme concentrations in the eluate from the column. The peak with an elution volume characteristic of the half-molecule is significantly larger at the lower enzyme concentration. A larger amount of dissociation is evident when enzyme, initially 2.4×10^{-9} M, is chromatographed at pH 5.5 (Figure 8c). Application of eq 2 to the results of Figure 8a,b gives values of K_{eq} of 7×10^{-10} and 9×10^{-10} M, respectively. These numbers have no real quantitative significance for obvious reasons (in particular, the fact that the enzyme on the column is much more dilute than the applied enzyme), but they do serve to emphasize the large effect of salt concentration on the dissociation.

Immunological Studies. Dissociation of the enzyme was also detected immunologically by the complement fixation technique in experiments carried out in collaboration with L. Levine and E. Wasserman. Antibody against the purified en-

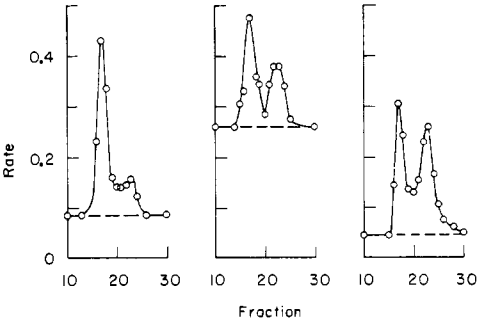


FIGURE 8: Sephadex G-100 elution patterns at very low enzyme concentration, 4°, and a flow rate of 15 ml/hr. Samples were prepared in 1.0 ml of column buffer with 3 mg of cytochrome *c* and 20 mg of sucrose at 20° and were then chilled and applied within a few minutes. Conditions: (a) 0.1 M potassium phosphate (pH 7.7)–2.4×10^{-9} M esterase, (b) 0.1 M potassium phosphate (pH 7.7)–0.6×10^{-9} M esterase, and (c) 0.1 M potassium chloride–0.002 M sodium acetate (pH 5.5)–2.4×10^{-9} M esterase.

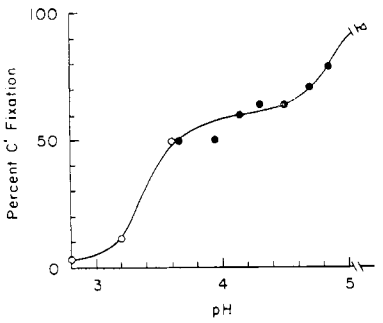


FIGURE 9: Immunological assay of purified esterase by complement (C′) fixation after incubation for 24 hr at a concentration of 0.02 mg/ml in 0.01 M buffer solutions. Buffers: open circles, glycine hydrochloride; closed circles, sodium acetate; triangle, Tris-HCl, 0.14 M sodium chloride, pH 7.5 (not incubated).

zyme reacts less effectively with the half-molecule formed between pH 4 and 5 than with the intact enzyme and less effectively still with the irreversibly inactivated half-molecule formed at low pH (Figure 9). Furthermore, at the lowest pH values the complement fixation curve undergoes a change in shape with maximal fixation at higher antigen concentrations, indicative of a change in conformation of the enzyme under these conditions. Preliminary experiments have indicated that this technique is useful for following the rate and extent of dissociation and have provided qualitative confirmation of the conclusions reached by the Sephadex technique. These experiments are being continued, to provide more complete information about the dissociation–reassociation process.

Electrofocusing on a pH Gradient. The enzyme was examined by the isoelectric fractionation technique of Vesterberg and Svensson (1966), in which the enzyme migrates to its isoelectric point in a pH gradient stabilized by a sucrose density gradient. Isoelectric focusing on a 110-ml column of pH 4–6 ampholyte containing 10 mg of enzyme for 48 hr at 0° gave two closely spaced peaks of enzyme activity and protein at pI 5.02 and 5.10 and a minor peak at pI 5.55, with a total recovery of enzyme activity of 56%. These results are difficult to interpret because of denaturation and the presence of a significant amount of dissociation under the conditions of the experiment, as estimated by the immunological technique. However, they do suggest that the active enzyme has an isoelectric point at pH 5.0–5.1, in agreement with the value of 5.0 found by Adler and Kistiakowsky (1961) and by Horgan *et al.* (1969a).

Discussion

Identity and Structure. The pig liver esterases isolated by different procedures in this and other laboratories appear to be almost identical molecules, which have, nevertheless, several significant differences. The enzyme contains two active sites in each whole molecule of mol wt 168,000; the differences between this value and the molecular weights of 174,000 reported by Boguth *et al.* (1965) and 163,000 reported by Horgan *et al.* (1966a) are a consequence of the values for the partial specific volumes of 0.75 and 0.733, respectively, used for the calculation of molecular weight by these workers (Franz and Krisch (1968) have recently reported a value of $\bar{v} = 0.74$, for their preparation, in agreement with our value). The sedimentation coefficients of the different preparations agree, and the diffusion coefficient of 4.1×10^{-7} cm^2 per sec at 10 mg/ml of protein is in reasonable agreement with the value of 4.3×10^{-7} cm^2 per sec obtained by the immunodiffusion method and a value of $4.6 \pm 0.2 \times 10^{-7}$ cm^2 per sec extrapolated to zero protein concentration (Boguth *et al.*, 1965).

Immunological examination has provided the most sensitive measure of the essential similarity and the small differences of the different preparations. The close similarity of the products is shown by the absence of different antigenic determinants (spurs) upon Ouchterlony double diffusion of two fractions prepared by the procedure of the Australian group and three fractions from different regions of the DEAE peak obtained by the Adler and Kistiakowsky procedure against separate antibodies obtained for each of these fractions. Furthermore, it was found that one of the fractions obtained by the Australian procedure adsorbed all of the antibody in a serum specific for the Adler and Kistiakowsky enzyme; *i.e.*, there are no distinct antigenic determinants in the latter preparation which are not also present in the former. The most sensitive test is the microcomplement fixation procedure, which shows that the different preparations are almost identical, with maximal complement fixation at a constant antigen concentration, but nevertheless show small differences in the curves that are definite evidence for differences in the preparations. These differences are smaller than those observed upon substitution of a single amino acid in hemoglobins A_1 and S (Reichlin *et al.*, 1964) and in human compared with *M. mulatta* cytochrome *c* (Margoliash *et al.*, 1967). Finally, Ouchterlony double diffusion of the dissociated enzymes at pH 4.5 gave a single homologous band for the five enzyme preparations, although faint additional bands were observed with some fractions.

The different enzyme preparations move as a single band with the same mobility upon electrophoresis in polyacrylamide gel at concentrations sufficient for staining with a protein stain. However, in much more dilute solutions the enzymes undergo a time-dependent dissociation to subunits with a greater mobility, which can be detected by the sensitive assay for enzyme activity. The rate of this dissociation is different for the different preparations, and the dissociation products, because of differences in structure or conformation, do not move as a single band with a constant mobility. It is presumably these dissociation products that give rise to some of the multiple bands of enzyme activity observed upon electrophoresis of crude tissue homogenates by Holmes and Masters (1968). Multiple bands caused by dissociation and by differences in the proteins have also been observed for beef liver esterase (Runnegar *et al.*, 1969a).

Finally, there are significant differences in the kinetic behavior of the different enzyme preparations, which will be referred to in this discussion as the A and K, and the Aust enzymes, for the enzymes prepared by the Adler and Kistiakowsky (1961) and Horgan *et al.* (1966a) procedures, respectively.

(a) The specific activity of the A and K enzyme toward 12.5 mM ethyl butyrate at 25°, pH 7.4, is less than half that of the Aust enzyme, although the activities toward *p*-nitrophenyl acetate are almost identical in the low substrate concentration range (Barker and Jencks, 1969).[2] The activities of the two enzymes toward ethyl butyrate are 103 and 225, respectively, expressed in the units of Horgan *et al.* (1966a). Furthermore, the specific activity of our preparation of the

Aust enzyme toward 12.5 mM ethyl butyrate at 25 and 38° and toward 1.5 mM phenyl acetate at 25°, pH 7.4, is 20–30% lower than that of the enzyme prepared by the Australian workers according to the same procedure; these activity ratios were confirmed in both laboratories with both enzyme preparations.[2,3] Finally, a second enzyme fraction was obtained from this fractionation procedure in this laboratory with a specific activity of only 36 toward ethyl butyrate, but a higher activity toward benzyl acetate than the "normal" Aust enzyme; these two enzymes have the same sedimentation coefficient and almost identical immunological characteristics.[2]

These differences cannot be explained by the presence of impurities in the enzyme preparations. Our studies have confirmed several of the physical criteria from which Adler and Kistiakowsky (1961) concluded that their method of preparation gave a homogeneous enzyme. In addition we have shown that our preparation of this enzyme (i) exhibits a single peak upon polyacrylamide gel electrophoresis at concentrations above those at which dissociation takes place, (ii) gives evidence for no more than traces of impurities by immunological examination by Ouchterlony double diffusion, and (iii) dissociates completely to ultracentrifugally homogeneous subunits at low pH and in guanidine hydrochloride; *i.e.*, any impurity must undergo dissociation in the same manner as the enzyme. Our preparation of the Aust enzyme is homogeneous according to criteria i and ii and by ultracentrifugation and chromatography.[2] An active site titration of this preparation, kindly carried out by Dr. Stoops, gave a molecular weight of 78,200 per active site, in agreement with the value for the enzyme prepared by these workers (Horgan *et al.*, 1966b).

(b) The A and K enzyme exhibits a lag in its catalysis of the hydrolysis of *p*-nitrophenyl acetate, whereas the Aust enzyme does not (Barker and Jencks, 1969).

(c) The A and K enzyme exhibits substrate activation with *p*-nitrophenyl acetate, whereas little or no such activation is observed with the Aust preparation (Barker and Jencks, 1969). Furthermore, substrate activation is relatively more significant in the hydrolysis of ethyl butyrate catalyzed by the enzyme we have prepared according to the A and K procedure and for the enzyme prepared by Ocken (1967) than for the preparation originally prepared by Adler and Kistiakowsky (1961) and for the Aust enzyme; the ratios of the maximal velocities at high and low substrate concentrations are 2.5, 2.5, 1.3, and 1.5, respectively, for these four preparations.[2]

(d) The hydrolysis of *p*-nitrophenyl acetate catalyzed by the A and K enzyme is activated 75% by 0.1 M acetone and 56% by 0.1 M dioxane, whereas the Aust enzyme shows only a 20% activation by these compounds. In contrast, the rate of *p*-nitrophenol release from *p*-nitrophenyl acetate catalyzed by the Aust enzyme is increased by 80% in the presence of 0.1 M methanol, whereas the same conditions give only a 14% increase in rate with the A and K enzyme (Barker and Jencks, 1969).[2]

The following are possible explanations for these differences. (a) The primary structures of the different preparations differ in one or more amino acids, either intrinsically or because of proteolytic action during isolation. A special case of such a difference would be the existence of differing subunits, which could be combined to give different isozymes. The electrophoresis experiments show a single band in concentrated solutions, but give several bands in very dilute solution, which might be interpreted as components of isozymes. However, the immunological results suggest that large structural differences do not exist among the different enzymes and their subunits. The complement fixation curves indicate that the areas of the surface of the protein with which antibodies may react are very nearly identical for the different preparations; any subunits or other regions of grossly different structure would have to be buried in an inaccessible region of the protein. Ouchterlony double-diffusion experiments with the dissociated enzyme at pH 4.5 reveal no more than traces of impurities and show a single main band without evidence for significantly different antigenic determinants (spurs) upon reaction of each of five different enzyme preparations against the five corresponding specific antibody preparations. It is possible that two different subunits could give a single precipitin line against two corresponding antibodies in one or more cases by coincidence, but, since the position of the precipitin line depends on the concentration as well as the nature of the antibody and antigen, it is unlikely that such a coincidence should occur in all of the 25 cases examined.

(b) The preparation may undergo irreversible changes in enzyme conformation during the purification procedure, presumably during exposure to organic solvents or low pH. It is known that the kinetic properties of the enzyme change, with a tendency toward loss of the biphasic substrate concentration curves, upon exposure to heat or acid (Adler and Kistiakowsky, 1961; Barker and Jencks, 1969). The procedure of Horgan *et al.* (1966a) involves an acetone powder extraction at pH 3.9–4.5 and the procedure of Krisch (1963) includes a precipitation and centrifugation step at pH 4.2.

(c) The different preparations may contain different bound nonprotein materials. Porcine pancreatic ribonuclease is isolated as a series of at least eight electrophoretic variants of identical amino acid composition, but with different bound carbohydrate moieties (Reinhold *et al.*, 1968). Several esterases are obtained from tissue homogenates in the form of lipid-containing aggregates that exhibit activity toward longer chain substrates than are hydrolyzed by purified esterase; the change in properties is reversible upon the removal or addition of lipid (Schnatz and Cortner, 1967; Okuda and Fuji, 1968). It is conceivable that traces of tightly bound lipid would survive the purification procedure. This remarkable effect of lipid suggests that the isolated esterases may be drastically different in structure and function compared with the *in vivo* enzyme and that the native enzyme may act principally to catalyze the hydrolysis of long-chain fatty acid esters.

Properties of the Subunits. Dissociation of several esterases into active half-molecules at high dilution has been observed independently in several laboratories and is consistent with the finding of two active sites per whole molecule (Krisch, 1966; Franz and Krisch, 1966; Horgan *et al.*, 1966b; Barker and Jencks, 1967; Horgan *et al.*, 1969b; Runnegar *et al.*, 1969b). This dissociation is greatly increased at pH 4.5. At still lower pH values the enzyme is converted irreversibly into an inactive form which has only a trace of the complement fixing ability in the presence of antibody that is observed with the native enzyme; the active half-molecule fixes complement more effectively, but is still much less active than the native enzyme (Figure 9). The inactive form has a lower sedimentation coefficient than the active half-molecule,

[3] J. Stoops and B. Zerner, personal communication.

but has a correspondingly lower diffusion coefficient and appears to have the same molecular weight. The calculated axial ratios for the whole molecule, active half-molecule, and inactive half-molecule are 3:1, 2:1, and 11:1, respectively. These values are subject to the usual uncertainties of such calculations, but they support the conclusion that low pH brings about an irreversible unfolding to a protein of altered conformation.

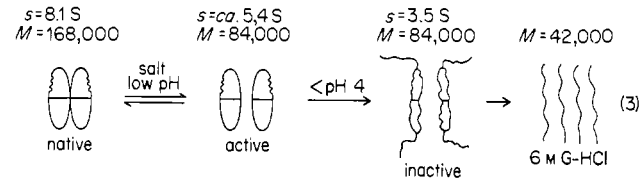
The uncorrected molecular weight of the enzyme in 6 M guanidine hydrochloride–0.1 M mercaptoethanol of 53,500 (assuming the same partial specific volume, 0.740 cm^3/g, as in water) is intermediate between the values expected for half- and for quarter-molecules, although closer to the latter. Based on the treatment of Hade and Tanford (1967), a corrected molecular weight of 84,000 would require the preferential binding of 0.43 g of water/g of protein, whereas the expected molecular weight of 42,000 for the quarter-molecule would require the preferential binding of 0.32 g of guanidine hydrochloride per g of protein (on a molar basis, the required relative binding of guanidine hydrochloride would, of course, be less). Since preferential binding of water has seldom, if ever, been observed and preferential binding of guanidine hydrochloride has been observed in a number of instances (Kielley and Harrington, 1960; Woods *et al.*, 1963; Noelken and Timasheff, 1967; Hade and Tanford, 1967), it appears that in the presence of this denaturing agent the protein dissociates to quarter-molecules. It should, however, be noted that the preferential binding of guanidine hydrochloride required for this interpretation is considerably larger than the amounts, up to 0.2 g/g of protein, that have been reported previously. The presence of only two active sites per whole molecule suggests that the four subunits are not identical; however, no evidence for heterogeneity of the subunits has yet been obtained. Boguth *et al.* (1965) have calculated a molecular weight of 42,000 for the enzyme in the presence of 0.2% sodium dodecyl sulfate from the results of a single approach to equilibrium experiment with 8.7 mg/ml of protein, but it is difficult to draw a firm conclusion from this result in view of the known dependence of apparent molecular weight upon both protein and sodium dodecyl sulfate concentration (Carusi and Sinsheimer, 1963; Gregolin *et al.*, 1968; Franz and Krisch, 1968). Our single attempt to examine the protein in the presence of this detergent resulted in precipitation upon dialysis against 0.2% sodium dodecyl sulfate.

Rates and Equilibria for Dissociation. Dissociation to active half-molecules at neutral pH is a slow process which proceeds to a much greater extent in the presence of salt. The dissociation constant of approximately 4×10^{-7} M in the presence of 0.5 M lithium bromide or sodium chloride may be compared to the crude estimate of 8×10^{-10} M at lower salt concentration, which probably represents only an upper limit. The similar effectiveness of sodium chloride and lithium bromide as dissociating agents is of particular interest. Lithium bromide is an effective dissociating agent for F-actin and decreases the activity coefficient of the model peptide acetyltetraglycine ethyl ester, whereas sodium chloride increases the activity coefficient of this model peptide and does not cause dissociation of F-actin (Nagy and Jencks, 1965; Robinson and Jencks, 1965). This suggests that there is no large change in the degree of exposure of peptide groups upon dissociation. Dissociation of the esterase may represent a case in which dissociation is brought about by interaction of salts with charged groups, a mechanism which has been frequently suggested, but not yet clearly established for salt-induced dissociation of proteins.

It should be noted that the presence of substrate does not affect the dissociation. This fact and the close similarity in the activity of the whole and half-molecules, per active site, suggest that the active site is located on the surface of the enzyme in such a way that it is not directly involved in the interaction region between half-molecules.

At acid pH both the rate and equilibrium constants for dissociation into half-molecules are greatly increased. The half-time for dissociation of several hours at pH 8 is reduced to 5–10 min at pH 4.5. Although the equilibrium constant at low pH has not been determined, the amounts of dissociation observed at various protein concentrations require that it be on the order of 1.4×10^{-5} M, 35 times larger than the value in 0.5 M salt (pH 8.0), and 17,000 times larger than the upper limit in low salt at pH 7.7. It is of interest that dissociation is favored at a pH value close to the isoelectric point. This means that dissociation is not a consequence of electrostatic repulsion of uniformly distributed charges on the protein. If charged groups are involved, as seems very probable, they must be specific groups which are either in the interaction region or which control a conformation change that brings about dissociation. A change in the ionization state of one or more such groups or interaction with ions could lead to dissociation.

Our present understanding of the properties of pig liver esterase may be summarized schematically by eq 3.



Acknowledgments

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