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The Sulfatase of Ox Liver. X. Some Observations on the Intermolecular Bonding in Sulfatase A*

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ABSTRACT: Several independent items of evidence strongly indicate that hydrophobic bonding significantly contributes to the formation of the tetramer (mol wt 411,000) of sulfatase A. Notable in this respect is the increase in weight-average sedimentation coefficient with increasing temperature at pH 5.0. Electrophoretic mobility data are used to show that the isoelectric point of the protein is 3.4 ± 0.05 in sodium chloride–sodium formate–formic acid buffer at ionic strength 0.10. Weight-average sedimentation coefficient results obtained in the pH range 3.47–5.00 are interpreted in terms of an expansion of the tetramer structure with increasing pH, caused by electrostatic repulsive forces acting in opposition to the postulated hydrophobic bonding. As the pH is further increased the increased net negative charge results ultimately in dissociation to the monomer (mol wt 107,000) at pH 6.6. A dissociation is also brought about by the addition of dioxane to a solution of the tetramer. The effect of sodium dodecyl sulfate on the system has been investigated at pH values of 7.5, 6.7, and 5.0. The approach of the anionic detergent has been shown to be governed by the net charge on the molecule. Results obtained at pH 5.0, where the action was most pronounced, have been chosen for detailed study; in particular, the slow sedimenting material (s 3.6) evident in the presence of detergent has been shown to be essentially homogeneous with respect to sedimentation coefficient and has been characterized by estimating its partial specific volume, its sedimentation coefficient to diffusion coefficient ratio, and the amount of bound detergent. The results show that detergent dissociates the tetramer into subunits of approximately equal size (mol wt 24,000) and the several assumptions required for the latter estimation are discussed. The effect of removing the detergent by dialysis under various conditions has been examined.

Aryl sulfatase A (an arylsulfate sulfohydrolase, EC 3.1.6.1.) from ox liver exists in 0.10 ionic strength solutions as a monomer (mol wt 107,000) at pH 7.5 and as a tetramer (mol wt 411,000) at pH 5.0 (Nichol and Roy, 1964, 1965). The latter work showed that an increase in ionic strength resulted in more extensive polymerization at both pH 7.5 and pH 5.0, suggesting that the net charge at the former pH is sufficiently large to prevent polymerization in 0.10 ionic strength buffers, but that it can be masked either by lowering the pH or by increasing the ionic strength. In this study electrophoretic mobility data are presented to support the hypothesis. The data also show that the isoelectric point of the enzyme is surprisingly low and this has necessitated the determination of weight-average sedimentation coefficients, $\bar{s}$, and enzymatic activities at pH values lower than previously studied (Nichol and Roy, 1964, 1965). It is found that $\bar{s}$ values increase as the pH is lowered toward the isoelectric point.

An increase of $\bar{s}$ with temperature at pH 5.0 is also reported, suggesting that the tetramer structure becomes more compact as the temperature is increased. This finding [together with the failure to detect electrostatic bonds or the more common covalent linkages (Nichol and Roy, 1964, and unpublished observations)] suggests that hydrophobic interaction (Kauzmann, 1959; Scheraga, 1963) may contribute toward polymer

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formation. The action of sodium dodecyl sulfate (SDS)[1] on sulfatase A at various pH values was therefore investigated by sedimentation velocity experiments and the pronounced effect of the detergent under certain conditions is correlated with the electrophoretic mobility data. In addition, the effect of the removal of the SDS by dialysis on the enzymatic and hydrodynamic properties of the enzyme has been investigated.

In the presence of detergent a boundary is evident with s 3.6, lower than that of the monomer. The material contributing to this slowly sedimenting peak has been shown to be essentially homogeneous with respect to sedimentation coefficient. In an attempt to determine the size of the new species the ratio of the sedimentation coefficient to the diffusion coefficient has been estimated. The partial specific volume has been determined, with limited accuracy, by the differential sedimentation method of Martin *et al.* (1956, 1959). These results, together with an estimation of the number of moles of bound SDS, permit a discussion of the subunit structure of the enzyme.

Experimental Section

Enzyme and Buffers. Samples of sulfatase A were prepared as described by Nichol and Roy (1964): sedimentation velocity analysis of each sample at pH 5.0, ionic strength 0.10, showed a single symmetrical peak with s 14. Enzyme assays were carried out in the pH-stat using potassium 2-hydroxy-5-nitrophenylsulfate as substrate (Nichol and Roy, 1964). Buffers were made using analytical grade materials and their composition has in most cases been given previously (Creeth and Nichol, 1960; Nichol and Roy, 1964, 1965), but those of the following compositions were also used

	pH 3.67 (M)	pH 3.57 (M)	pH 3.47 (M)	pH 3.36 (M)	pH 2.90 (M)
Sodium chloride	0.05	0.05	0.05	0.05	0.05
Sodium formate	0.05	0.05	0.05	0.05	0.05
Formic acid	0.04	0.06	0.08	0.10	0.30

The pH of the buffer solutions was determined at 20° using a Vibron electrometer, Model 33B.

Dioxane. Dioxane was freed from peroxides by treatment with stannous chloride and dried with sodium. The calculated amount of dioxane was added directly to the protein solution in acetate buffer, pH 5.0, ionic strength 0.1, and allowed to stand for 1 hr prior to sedimentation velocity analysis. The density and viscosity of the mixtures were calculated from the data of Geddes (1933).

Sodium Dodecyl Sulfate. Sodium dodecyl sulfate was obtained commercially in a purified form and used without further treatment. Weighed amounts of detergent were dissolved in water and measured volumes of the solutions were added directly to buffered protein solutions so that the final ionic strength in each case was 0.05. Prior to the addition of the SDS, the concentration of the protein solution was determined spectrophotometrically at 280 mμ, employing the value of 6.6 for $E^{1\%}_{1cm}$ at pH 5.0 (Nichol and Roy, 1965). These determinations permitted the initial mixing ratio of SDS–protein to be given on a base-molar scale (SDS, mol wt 288 and protein monomer, mol wt 107,000). The protein–SDS solutions were allowed to stand for 2 hr at room temperature before use. Preliminary experiments at pH 5.0 had shown that the analysis was unaffected by extending the time of standing to 36 hr.

Control experiments in which 0.013 M and 0.004 M solutions of SDS at pH 5.0, ionic strength 0.05, were sedimented at 20° showed the presence of a slowly sedimenting peak, corresponding to micelles of SDS with s 1.5. The presence (or absence) of this peak in experiments involving both detergent and protein was used as a marker of micelle formation.

Electrophoresis. All electrophoresis experiments were conducted at 3 ± 0.1° in a Perkin-Elmer electrophoresis apparatus, Model 38A, equipped with a cold-water circulating system. An open Tiselius cell (2 ml) was used. After the electrophoretic migration had proceeded for *ca.* 2 hr in one direction, the current was reversed for an equal period of time; the return of the boundaries was to the starting position, thus providing a sensitive means of detecting minor cell leaks. At least five photographs, using schlieren optics, were taken to record the movement of the boundary in the descending limb. The regression of the observed boundary position (taken as the maximum ordinate) upon time was used to determine the mobility. The conductivities of the buffer solutions were measured at the temperature of the experiment using a Philips conductivity bridge. As the concentration of protein in each experiment was low (~0.1%), the error in assuming that the conductivities of the buffer and the protein solutions are the same is small.

Ultracentrifugation. Sedimentation velocity experiments were performed using a double-sector filled epoxy centerpiece in a Spinco Model E ultracentrifuge at a variety of speeds and temperatures which are reported in the text. The schlieren patterns obtained were measured with a two-coordinate comparator (Gaertner Toolmakers microscope, Type M 2001 AS-P). The resulting data were used either to calculate the rate of movement of the square root of the second moment of an entire schlieren peak [and hence the weight-average sedimentation coefficient, $\bar{s}$ (Goldberg, 1953)] or the rate of movement of the maximum ordinate to give s. Both types of sedimentation coefficient were corrected to 20° in water (Svedberg and Pedersen, 1940) using the measured partial specific volume of 0.71 (Nichol and Roy, 1965). Areas under peaks were found by trapezoidal integration.

In some instances the data from the plates were also used to evaluate the apparent (differential) distribution function $g^*(S)$, for the range of S (a reduced coordinate with units of sedimentation coefficient) encountered. These terms have previously been defined and discussed

[1] Abbreviation used: SDS, sodium dodecyl sulfate.

fully (Signer and Gross, 1934; Baldwin, 1954; Williams *et al.*, 1958). This procedure corrects the experimental pattern for the effects of radial dilution and the inhomogeneity of the centrifugal field. In the investigation of the nature of the *s* 3.6 peak observed in the presence of SDS at initial SDS–protein ratios of 300, 370, and 480, the calculations were extended to obtain plots of $(S - \bar{s})^2$ *vs.* $1/te^{\bar{s}\omega^2 t}$ by the method of Baldwin (1959), where ω is the angular velocity of the rotor, t is the time from the start of the experiment, and $\bar{s}$ is the weighted mean of the distribution of S. It should be stressed that in all boundaries analyzed in this way, the latter quantity and the weight-average sedimentation coefficient were identical within experimental error and accordingly the single symbol $\bar{s}$ is used throughout (*cf.* Nichol and Creeth, 1963). The method permits a test for homogeneity with respect to sedimentation coefficient, allowing for the effects of diffusional spreading but not for any marked concentration dependence of the sedimenting material.

The boundary analysis procedure of Fujita (1956), described in detail by Baldwin (1957), does account for small linear concentration-dependence effects and permits not only a test for homogeneity but also the estimation of an apparent diffusion coefficient. The Fujita procedure has been applied to determine the apparent ratio of sedimentation coefficient to diffusion coefficient, s/D, for the material sedimenting with *s* 3.6 when the initial SDS–protein ratios were 300, 370, and 480 (the same as used for the Baldwin analyses).

A sedimentation technique (Martin *et al.*, 1956, 1959) was also used to estimate the apparent partial specific volume, $\bar{V}$, of the *s* 3.6 material. Values of the sedimentation coefficients at infinite dilution were found by extrapolating data obtained at three different concentrations of protein in two different environments, acetate buffer, pH 5.0, ionic strength 0.05 in water and the same buffer in 50% (v/v) D_2O. The SDS–protein ratio was held constant at 350. Application of eq 5 of Martin *et al.* (1956) gave a $\bar{V}$ of 0.78 for the *s* 3.6 material. The effects of hydrogen–deuterium exchange were neglected.

Peptide Mapping. This was carried out by Dr. D. C. Shaw. A dry salt-free sample of sulfatase A, prepared by lyophilization of a solution dialyzed *vs.* 0.001 N acetic acid, was oxidized by preformed performic acid. The oxidized protein was suspended in 0.5% ammonium bicarbonate solution and *ca.* 1% (w/w) of trypsin (Worthington, TRL 6246) was added. Indole was included in the reaction mixture to act as a competitive inhibitor of any chymotrypsin contaminating the trypsin. The protein had completely dissolved after incubation for 10 min at 37° and, after a further 20 min of incubation, the mixture was again lyophilized.

A 2-mg sample of the hydrolyzate was applied to Whatman 3MM paper and subjected to two-dimensional electrophoresis, first in pyridine–acetic acid buffer, pH 4.7, and secondly in acetic acid–formic acid buffer, pH 1.9. On staining the dried electropherogram with ninhydrin, *ca.* 36 peptides were detected.

Results

Electrophoresis. In each electrophoresis experiment performed with sulfatase in the absence of SDS, a single peak with sulfatase A activity was observed and the patterns were essentially enantiographic. As the experiments were conducted only at ionic strength 0.10 and no detailed analyses of boundary shapes were attempted, it is not possible to draw firm conclusions concerning the homogeneity of the material with respect to electrophoretic mobility, but it can be stressed that there was only one reasonably sharp peak. All relevant information on the mobility experiments is summarized in Table I. If the mobility values are plotted *vs.* pH in the range 2.90–6.64 a smooth curve results interpolation of which gives the value for the isoelectric point in sodium chloride–sodium formate–formic acid buffer of ionic strength 0.10 as pH 3.4 ± 0.05. The curve markedly increases in slope above pH 6.6. It is unlikely that the latter effect can be attributed to specific ion binding as the mobility found in Tris–HCl buffer agreed closely with that found at similar pH values in diethylbarbiturate buffer. Previous findings have shown that in the pH range 5.5–6.6 in 0.10 ionic strength buffers, appreciable amounts of higher polymers coexist in equilibrium with the sulfatase monomer and it is, therefore, probable that the change in slope of the mobility curve at pH 6.6 reflects this property of the system. A further complication in the detailed interpretation of the observed mobilities becomes apparent on examining the sedimentation properties of the enzyme in the pH range 2.9–5.0.

TABLE I: Electrophoretic Mobility Data on Sulfatase A in Buffers of 0.1 Ionic Strength and at 3°.

Buffer Type	pH	Descending Mobility ($\times 10^5$, $cm^2\ sec^{-1}\ v^{-1}$)
Diethylbarbiturate	7.54	−9.9
Diethylbarbiturate	7.41	−7.3
Tris–HCl	7.35	−7.6
Diethylbarbiturate	7.30	−7.8
Cacodylate	6.64	−5.8
Cacodylate	6.32	−6.2
Acetate	5.40	−5.7
Acetate	5.00	−5.3
Acetate	4.52	−3.9
Acetate	3.98	−2.9
Formate	3.67	−1.9
Formate	3.57	−1.2
Formate	3.36	2.3
Formate	2.90	3.3

Sedimentation Velocity. A. THE EFFECT OF pH. The

TABLE II: The Effect of pH in the Acid Region on the Weight-Average Sedimentation Coefficient and Enzymatic Activity of Sulfatase A.

	Sedimentation Data			Relative Enzymatic Activity[a] (hr)		
pH	Concn (g/100 ml)	Temp (°C)	$\bar{s}_{20,w}$ (S)	0	5	26
5.00	0.3	6	13.55	100	107	91
4.24	0.3	3	13.96	89	100	92
3.67	0.4	6	14.27	90	59	28
3.57	0.2	7	14.97			
3.47	0.3	8	15.46	78	28	0
3.47	0.3	2	15.55			

[a] The enzymatic activities are expressed as relative to that obtained at pH 5.0 (100) immediately after dilution of the stock enzyme to a concentration of *ca.* 0.06 g/100 ml. It was also determined in each case after the enzyme had been standing at the cited pH value for 5 and 26 hr. The final enzyme concentration in the reaction mixtures was 3×10^{-5} g/100 ml.

weight-average sedimentation coefficients obtained in the acid region above the isoelectric point are summarized in Table II. At pH values of pH 3.57 and above a single sharp and symmetrical peak was observed, but at pH 3.47 some degradation occurred, giving a sharp clearly resolved peak with s 3.5. After dialysis for 5 days *vs.* formate buffer, pH 3.47, the s 3.5 material accounted for about 20% of the total protein; subsequent dialysis of the mixtures against acetate buffer, pH 5.0, gave virtually complete reversal of the degradation and only a single sharp peak with s 14 was observed. It is clear that the corrected values of $\bar{s}_{20,w}$ for the tetramer increase systematically as the pH is decreased toward the isoelectric point and that the trend cannot be explained by the small variation in the protein concentrations used in the experiments (Nichol and Roy, 1964). The change in $\bar{s}$ with pH was confirmed by a differential sedimentation experiment in which one sector of a double-sector cell was loaded with solution at pH 5.00 and the other sector with an identical amount of solution of the same protein concentration (0.2%) but at pH 3.66; as expected, the interferogram revealed a peak of curved fringes (Richards and Schachman, 1957; Schachman, 1959). The data in Table II also show that the increase in $\bar{s}$ is accompanied by a time-dependent decrease in enzymatic activity. It is expected that any time-dependent change in hydrodynamic properties would have reached completion before the sedimentation velocity experiments were performed because a 3-day dialysis in the cold preceded each run. At pH 2.90 and 3.36, below the isoelectric point, no enzymatic activity was detected and sedimentation velocity runs performed at these pH values in 0.10 ionic strength buffers at 4° showed a spread peak, the distribution centering around s ~3. The patterns were, therefore, in sharp contrast to those obtained in the acid region immediately above the isoelectric point.

B. THE EFFECT OF TEMPERATURE. In a separate series of sedimentation velocity experiments it was found that the $\bar{s}_{20,w}$ for the sulfatase tetramer in pH 5.0, ionic strength 0.10 buffers increased with increasing temperature; details are reported in Table III. It is for this reason that the values of $\bar{s}_{20,w}$ reported in Tables II and III at pH 5.0 and at low temperature are significantly lower than those previously reported at 20° (Nichol and Roy, 1965); the value of 14.02 found in this work at 20°, nevertheless, agrees closely with the previous value of 14.10 at 21°. In applying the standard temperature correction (Svedberg and Pedersen, 1940) it was assumed that $\bar{V}$ (as measured in the buffer at 25° by Nichol and Roy, 1965) was temperature independent; account was taken of variations with temperature of the relative viscosity of the buffer and of the absolute densities of water and buffer.

TABLE III: The Effect of Temperature on the Weight-Average Sedimentation Coefficient of Sulfatase A in an Acetate Buffer, pH 5.0, Ionic Strength 0.1.

Protein Concn (g/100 ml)	Temp (°C)	$\bar{s}_{20,w}$ (S)
0.3	1	13.61
0.3	20	14.02
0.3	38	14.23

C. THE EFFECT OF DIOXANE. Sedimentation velocity experiments were performed on three mixtures of dioxane and enzyme (initially as tetramer at pH 5.0). First, at a dioxane concentration of 20% a single sharp boundary was observed with $\bar{s}_{20,w}$ 14.96; in view of the difficulties in evaluating data obtained with multicomponent systems of this type, this value agrees reasonably with that for unaltered tetramer. Secondly, in 33% dioxane a peak pronouncedly asymmetric on the trailing side was evident with $\bar{s}_{20,w}$ 9.52, suggesting that dissociation of the tetramer had occurred. Thirdly, increasing the dioxane concentration to 44% gave a turbid solution which on sedimentation velocity analysis showed a markedly spread boundary with $\bar{s}_{20,w}$ 9.73; the turbidity and the failure to account for the initial amount of protein added suggested that dissociation by dioxane was followed by aggregation in this medium. This hypothesis was supported by the decrease of 66% in the specific activity which occurred in 44% dioxane compared with the drop of only 15% in 33% dioxane. In the last two cases (33% and 44% dioxane) removal of the dioxane by extensive dialysis *vs.* acetate buffer, pH 5.0, ionic strength 0.1, resulted in a single-sharp peak with $\bar{s}_{20,w}$ 14, characteristic of unaltered tetramer.

D. THE EFFECT OF SODIUM DODECYL SULFATE. The

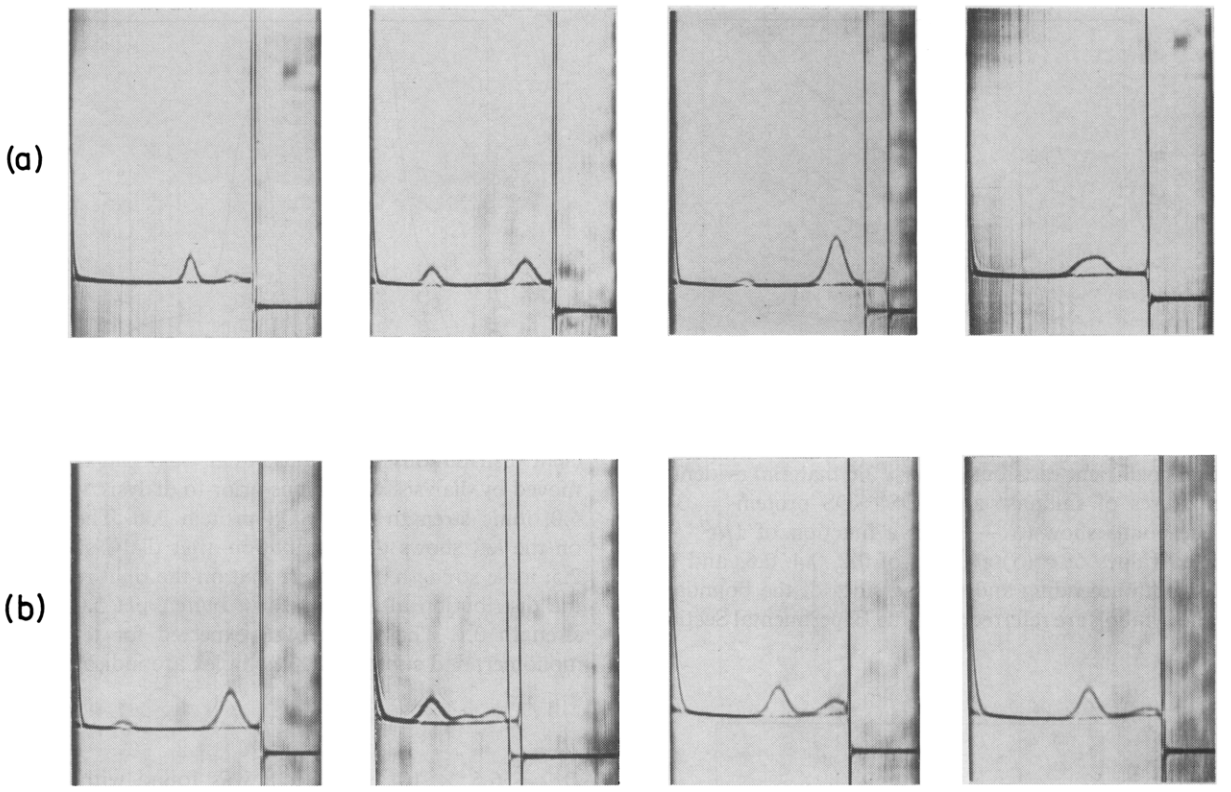

FIGURE 1: Sedimentation velocity patterns of sulfatase A in the presence of sodium dodecyl sulfate at 20° in buffers of ionic strength 0.05. Sedimentation is from right to left and the bar angle was 70° throughout: (a) pH 5.0, SDS–protein = 90, 220, 435, and 560, respectively, from left to right; (b) pH 5.0, 6.7, 7.5, and 7.5, respectively, from left to right. The ratio SDS–protein was 370 for the first three patterns and 220 for the last.

effect of sodium dodecyl sulfate on the enzyme at pH 5.0 and 20° is illustrated by the sedimentation velocity patterns shown in Figure 1a. It is apparent that as the SDS–protein ratio increases the amount of faster sedimenting material (*s* 14) decreases and the amount of slower sedimenting material (*s* 3.6) increases. The *s* 3.6 peak is reasonably symmetrical until the highest ratio (SDS–protein = 560) is reached, when a definite asymmetry is evident. At this stage only traces of *s* 14 material were detected. It is also clear from Figure 1a that the refractive index gradient falls to 0 near the meniscus in each pattern, showing that the concentration of any free detergent in solution must be below its critical micelle concentration. The latter results may be compared with those obtained in the presence of detergent at other pH values and 20°; typical sedimentation velocity patterns are shown in Figure 1b. In the first three patterns the SDS–protein value was held constant at 370. The following points may be noted: (1) the pattern obtained at pH 5.0 is similar to those described above and is included for direct comparison; (2) the pattern obtained at pH 6.7 shows a major fast-sedimenting peak, *s* 6, corresponding to monomer, a peak with *s* 3.6, and an unresolved peak near the meniscus which may be identified with detergent micelles; (3) the pattern obtained at pH 7.5 shows only a monomer peak, *s* 6, and a peak near the meniscus attributable to detergent micelles. The identification of the latter is further substantiated in the fourth pattern, also obtained at pH 7.5, but with the SDS–protein ratio reduced to 220; it is clear that the area under the peak near the meniscus is considerably smaller than in the third pattern.

Characterization of Material Evident in the Presence of Sodium Dodecyl Sulfate. A. BOUNDARY ANALYSES. The results which follow refer to the characterization of the *s* 3.6 material in the environmental conditions of pH 5.0 and ionic strength 0.05 (*cf.* Figure 1a). A typical result of a boundary analysis by the method of Baldwin (1959) is shown in Figure 2. Similar plots were found for both ascending and descending sides of the peaks obtained in experiments where the SDS–protein ratios were 300 and 480. The extrapolations to infinite time (0 value of the abscissa) are indicated by dashed lines and it is clear that all values of $(S - \bar{s})^2$ are then close to 0. It may be concluded that, within the limits of accuracy of the test, the *s* 3.6 material does not exhibit any measurable heterogeneity of sedimentation coefficient. This test would obviously fail when the SDS–protein value was 560 because under these conditions the peak is visibly asymmetric (Figure 1a).

In a second analysis of the same patterns the method of Fujita (1956) as described by Baldwin (1957) was used to obtain the apparent diffusion coefficient, D^*,

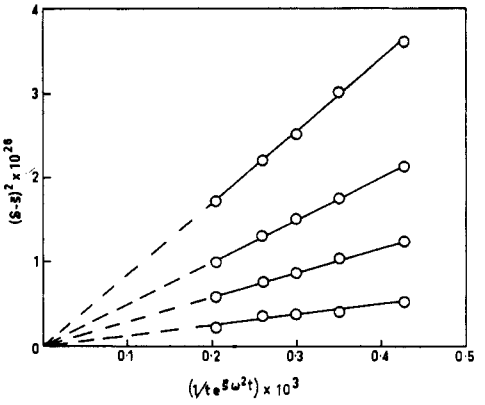

FIGURE 2: Time dependence of the apparent sedimentation coefficient distribution for s 3.6 material evident in mixtures of sulfatase and SDS. SDS–protein = 370. The points show $(S - s)^2$ as a function of $1/te^{s\omega^2 t}$ at fixed values of $g^*(S)/g^*(S)_{max}$ of 0.2, 0.4, 0.6, and 0.8 for both ascending and trailing sides of the boundary. The symbols are referred to in the Experimental Section.

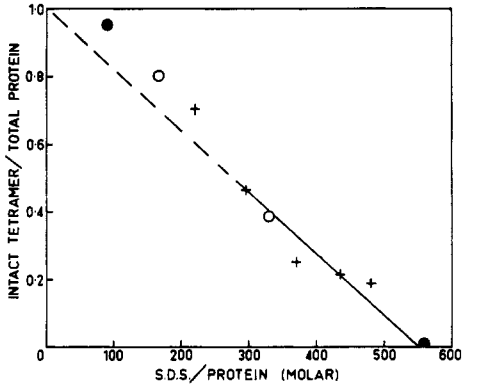

FIGURE 3: The apparent proportion of intact tetramer found from sedimentation velocity patterns of mixtures of sulfatase and SDS in which the initial mixing ratio was varied. The proportion is plotted against this initial ratio, SDS–protein, expressed on a molar scale. The initial protein concentrations were 0.13% (○), 0.19% (●), and 0.29% (+).

account being taken of the linear concentration dependence of the s 3.6 material. This dependence may be expressed as

$$\bar{s}_{20,w} = 4.16(1 - 2.2c) \times 10^{-13}$$

where c is expressed in arbitrary refractometric units to avoid the necessity of assuming a specific refractive increment for the s 3.6 material (see Discussion). Values of D^* computed from the individual patterns in the same experiment were independent of time, confirming the essential homogeneity of the material with respect to sedimentation coefficient. Also, the single value

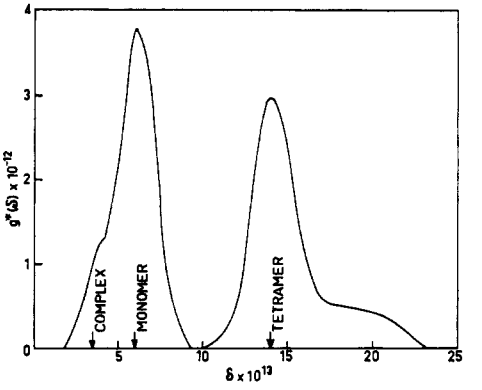

FIGURE 4: Apparent normalized sedimentation coefficient distributions of sulfatase after SDS had been removed by dialysis. Conditions prior to dialysis were pH 5.0, ionic strength 0.05, SDS–protein 250. The curve on the left shows the distribution after dialysis *vs.* pH 7.5, ionic strength 0.1 buffer; that on the right refers to the distribution after dialysis *vs.* buffer, pH 5.0, ionic strength 0.1. The values of S expected for tetramer, monomer, and subunit–SDS complex are indicated.

$D^* = 6.5 \times 10^{-7}$ cm^2 sec^{-1} was found with SDS–protein ratios of 300 and 370; the value increased to 7.0×10^{-7} cm^2 sec^{-1} when the ratio was 480. At the lower ratios the ratio of sedimentation coefficient to diffusion coefficient, s/D, was 0.55×10^{-6} sec^2 cm^{-2}, which must be regarded as an apparent value, especially as both s and D values refer to the same finite protein concentration (Creeth and Nichol, 1960).

B. THE APPARENT MOLECULAR WEIGHT. Incorporation of the apparent value of s/D of 0.55×10^{-6} sec^2 cm^{-2} and the observed $\bar{V}$ value of 0.78 (see Experimental Section) into the Svedberg molecular weight expression results in the value 60,000 for the apparent molecular weight of the s 3.6 material. This result is subject to several assumptions but definitely indicates that SDS dissociates the tetramer of sulfatase to a unit smaller than the monomer (mol wt 107,000). An estimate of the actual size of the corresponding protein subunit clearly requires information on the amount of SDS bound in the s 3.6 material.

In a series of experiments similar to those illustrated in Figure 1a the proportion of intact tetramer (s 14) was determined by dividing the area under the s 14 peak by the area corresponding to the initial protein concentration. The values obtained by this direct means are plotted as the ordinate in Figure 3 *vs.* the initial SDS–protein ratio. The line is an attempt to average the data found with different protein concentrations, and extrapolation to the abscissa shows that *ca.* 550 moles of detergent are bound/mole of protein of mol wt 107,000. The several assumptions in this procedure will be discussed later but it might be noted immediately that the deviation of the points at low SDS-protein ratios indicates that one or more of the assumptions are imperfectly fulfilled. The molecular weight of the protein

subunit, M_s, may be calculated from the observed molecular weight of the complex, M_c = 60,000, by application of the equation, $M_c = M_s(1 + \alpha)$, where α is the number of grams of bound detergent per gram of protein. The resulting value of M_s is *ca.* 24,000.

C. ENZYMATIC ACTIVITY. Determination of the enzymatic activity of sulfatase A showed that it was reduced in the presence of SDS. The remaining activity (expressed relative to that of the untreated enzyme) was similar to, but consistently higher than, the proportion of intact tetramer remaining at the same SDS–protein ratio. For example, at a ratio of 600 where no tetramer remained the relative activity was 0.18. It was nevertheless clear that the enzymatic activity of the protein–SDS complex was greatly reduced and might in fact have been 0 because the thousandfold dilution which necessarily accompanied the determination of the activity could have caused a partial removal of the SDS from the protein with some restoration of the enzymatic activity. The latter hypothesis appears contrary to the results obtained on the dialysis of the complex (see below), although there the removal of the SDS was slow and no substrate was present during the process.

D. THE EFFECT OF REMOVING SODIUM DODECYL SULFATE. The effect of the removal of bound SDS by extensive dialysis *vs.* 0.10 ionic strength buffers at pH 5.0 and 7.5, illustrated in Figure 4, where the apparent normalized distributions of the sedimentation coefficients (not corrected for diffusional spreading) are shown for two separate experiments in which the initial SDS–protein ratio prior to dialysis, was 250. The dialyses were performed in the cold, but the sedimentation velocity analyses were done at 20°. It is seen that maintaining the pH at 5.0 and removing the SDS results in partial conversion of the *s* 3.6 material to *s* 14 material, a value expected for the tetramer in this environment. Similarly, dialysis from pH 5.0 to 7.5, together with the removal of SDS, results in material having the sedimentation properties expected for the monomer. Clearly, however, the reversal is not completely effective as a proportion of faster sedimenting material is evident at pH 5.0, while at pH 7.5, material with an *s* lower than the monomer is apparent. With a higher initial ratio of SDS–protein of 480, removal of the SDS by dialysis at either pH 7.5 or 5.0 gave material with much wider distributions of sedimentation coefficient. In addition, even in favorable cases where the sedimentation characteristics expected for monomer or tetramer were largely restored (Figure 4), no regain of enzymatic activity was found.

E. ELECTROPHORESIS. A solution with an SDS–protein ratio of 300 was subjected to electrophoresis in an acetate buffer pH 5.0, ionic strength 0.05, at 3°. The ascending and descending limbs were essentially enantiographic and showed two moving boundaries, completely separated in the later stages of the run. The mobility of the slower moving boundary was corrected to that expected at 0.10 ionic strength, using the equations of Abramson *et al.* (1942) and assuming a spherical model. As the theory is approximate, the resulting value of -5.8×10^{-5} cm^2 sec^{-1} v^{-1} is in good agreement with that found for tetramer at pH 5.0, ionic strength 0.10 (Table I). In addition, the area under this peak was in accordance with that predicted by interpolation in Figure 3. The faster moving boundary, representing the subunit–detergent complex, was not markedly spread and exhibited a descending mobility of -12.3×10^{-5} cm^2 sec^{-1} v^{-1} at pH 5.0, ionic strength 0.05.

Discussion

The net charge on the sulfatase A molecule at pH 5.0 and 7.5 may be calculated from the observed electrophoretic mobilities (Table I) and diffusion results (Nichol and Roy, 1964) on the basis of models representing extremes in shape (Abramson *et al.*, 1942). If both monomer and tetramer are considered to be spherical, the valence per monomer unit (mol wt 107,000) is −30 at pH 7.5 and −9 at pH 5.0. An alternative model correlating the observed frictional coefficient ratio gives slightly higher values but no significance can be attached to the detailed calculations owing to the approximations made in the theory. Similarly, the coexistence of several polymeric forms in the pH range 5.5–6.6, illustrated by the discontinuity in the mobility at pH 6.6, and the lack of diffusion data at pH values below 5.0 prohibit the interpretation of mobility data obtained in these regions in terms of charge. Nevertheless, the available information on the likely electrostatic forces may be correlated with several structural observations made with sulfatase A and these will be discussed in turn.

It appears that the large net negative charge at pH 7.5 prevents intermolecular bonding but reduction of the repulsive forces by increasing the ionic strength or by decreasing the pH permits polymer formation to occur. It is important that at pH 5.0 (and at protein concentrations >0.0003%) the tetramer alone exists, despite the electrostatic repulsive force, albeit small. Masking of the net negative charge at pH 5.0 by increasing the ionic strength results in more extensive and ill-defined polymerization (Nichol and Roy, 1965), showing that electrostatic forces cannot be directly responsible for the intermolecular binding between four monomer units. However, these forces continue to be important in determining the quaternary structure of the tetramer unit at pH 5.0 because the $\bar{s}_{20,w}$ values shown in Table II increase as the net negative charge is reduced by lowering the pH toward the observed isoelectric point. This variation in $\bar{s}_{20,w}$ which has definitely been shown to be outside experimental error by the differential sedimentation experiment may be directly explained by proposing an intermolecular binding force which is unaffected or strengthened by an increase in ionic strength and which acts in opposition to the prevailing electrostatic force. It seems that the structure becomes more compact as the pH is lowered, and this change is accompanied by a decrease in enzymatic activity (Table II), suggesting that the optimum activity at pH 5.0 (Roy, 1953) is associated with a particular conformation established by the operation of opposing intermolecular

forces. It should be noted that at pH 8.5 and 9.3 in 0.1 ionic strength buffers, there was no significant change in the $s_{20,w}$ value from that found for the monomer at pH 7.5 (unpublished observation).

Although the tetramer structure becomes more compact as the pH is lowered, a stage is reached at a pH value close to the isoelectric point where it partially disaggregates (and/or unfolds) to form material sedimenting with s 3.5. This material is almost certainly of larger molecular weight than the subunit observed in complex with SDS (s 3.6). As the pH is further lowered to values below but close to the isoelectric point the introduction of a net positive charge results in the complete alteration of the tetramer structure to form a range of fragments which are enzymatically inactive. Although the fragments possess similar electrophoretic mobilities when examined in the single environment chosen, the marked heterogeneity, evident in sedimentation velocity experiments, stresses the operational interpretation of the isoelectric point. The low value of the latter may possibly be related to the effect of hydrogen or hydrophobic interactions on the ionization of acidic groups (Scheraga, 1963), but with the present system such discussions are at present hypothetical.

The failure to alter the sedimentation properties of the tetramer by the addition of sulfhydryl and metal-binding reagents and the observation that sulfatase A contains no phosphorus (Nichol and Roy, 1965) exclude more common covalent linkages from the binding of the monomer units. The latter evidence is clearly inadequate in excluding all possible covalent linkages but taken with other observations it lends support to the hypothesis that hydrophobic bonds contribute significantly to the intermolecular binding of monomer units. On the basis of the observation that added electrolytes decrease the solubility of low molecular weight nonpolar materials in water, noncovalent interactions of this type are expected to be strengthened by an increase in ionic strength (Kauzmann, 1959), in accordance with the findings with sulfatase A described above. Providing the assumption is made that small aliphatic hydrocarbons and their polar derivatives may be taken as models, it follows from thermodynamic measurements that the enthalpy change involved in the formation of hydrophobic bonds between nonpolar regions on protein molecules is almost certainly positive (Kauzmann, 1959; Scheraga, 1963). As a consequence, and in contrast to hydrogen bonds, hydrophobic bonds are likely to become stronger as the temperature is increased. The effect of temperature on the sedimentation properties of the sulfatase A tetramer at pH 5.0 is, therefore, of particular interest. The increase in $s_{20,w}$ with temperature (Table III) suggests that the tetramer structure does become more compact as the temperature increases. Similar (but more definite) temperature effects have been observed with other protein systems where hydrophobic interactions are suspected (Murayama, 1956; Fessler, 1957; Lauffer *et al.*, 1958). However, it should be stressed that the small variation reported in Table III cannot be taken by itself as definite proof of hydrophobic bonding, especially as in applying the standard temperature correction $\bar{V}$ has been assumed temperature independent and any variation of the concentration dependence of $\bar{s}$ with temperature has been ignored. The observation, then, merely forms part of the evidence in support of the hydrophobic bond hypothesis.

Other supporting evidence will be presented, but first it is convenient to employ the hypothesis in discussing the formation of the tetramer, which may be visualized as taking place as follows. To avoid the decrease in entropy associated with the change of water structure by nonpolar groups (Frank and Evans, 1945), hydrophobic regions in the monomer come together to form the tetramer, provided that the electrostatic repulsive forces are not too great. On extreme dilution the entropy sacrifice in retaining this ordered polymerized structure may be too large and it will dissociate. The dissociation of the sulfatase tetramer at pH 5.0 and at protein concentrations $<0.0003\%$ (Figure 4 of Nichol and Roy, 1965) finds explanation on this basis. It must be stressed that while hydrophobic bonding between monomer units appears to be a reasonable hypothesis, it has not been demonstrated that this type of bonding acts exclusively.

It has been suggested that a direct test for hydrophobic bonding could be made by demonstrating dissociation in media containing nonaqueous solvents, such as dioxane (Kawahara *et al.*, 1965). Although a detailed interpretation of hydrodynamic data in multicomponent systems of this type is difficult, the results reported above clearly indicate that dioxane does cause an initial dissociation of the tetramer. However a similar effect was caused by increasing the pH to 6.3 in aqueous buffers, thereby increasing the net negative electrostatic repulsive force between monomer units (Nichol and Roy, 1965). As the effect of dioxane on the net charge borne by sulfatase is unknown, the possible operation of a similar mechanism in the case of the dissociation by dioxane cannot be excluded. The result, nevertheless, is not inconsistent with the postulate of hydrophobic bonding between monomer units.

The action of sodium dodecyl sulfate on sulfatase A is in many respects similar to its degradative effect on a wide variety of other protein systems (*e.g.*, Hersh and Schachman, 1958; Steiner and Edelhoch, 1961). In the present study, comparison of Figure 1a and b shows that the approach and action of the anionic detergent is largely governed by the net negative charge on the various sulfatase A species. It may be estimated from Figure 3 and from the amino acid composition of the enzyme (Nichol and Roy, 1965) that at pH 5.0, *ca.* 1 molecule of detergent is bound/2 amino acid residues; as the protein still bears a net negative charge at this pH, it seems unreasonable to assume that binding is between positive charges on the protein and the negative charge on the detergent (*cf.* Putnam and Neurath, 1944; Rosenberg and Klotz, 1955). Nevertheless, calculations based on the equations of Abramson *et al.* (1942) for a spherical model and on the observed electrophoretic mobility of -12.3×10^{-5} cm^2 sec^{-1} v^{-1} for the s 3.6 material indicate that the valence on this hydrodynamic

unit is −22. As *ca.* 550 moles of detergent are bound/monomer unit (or 140 moles/protein unit of mol wt 24,000), it is clear that two possibilities exist: either all the bound detergent is not ionized (Johnson and Joubert, 1951; Brand and Johnson, 1956), or charges due to the bound detergent do not occur on the surface of the hydrodynamic unit. Therefore, while the binding of SDS may be taken as supporting evidence for the hydrophobic bonding hypothesis, disruption of the tetramer by the electrostatic repulsion of the bound detergent molecules may also contribute.

The estimation of the molecular weight of the *s* 3.6 material was based on data obtained in experiments where the initial SDS–protein ratio was <480. Below this ratio the material was essentially homogeneous (*cf.* Figure 2) while at and above it the reported increase in D^* and the asymmetric nature of the sedimentation velocity pattern (Figure 1a) suggested that further structural modification had occurred. The method used in the estimation of the amount of bound detergent by the extrapolation shown in Figure 3 therefore requires careful examination. It is based on the following three assumptions: (1) that the operation of physical and chemical interactions do not markedly complicate the analysis of the sedimentation velocity patterns in terms of area and velocity measurements (Gilbert and Jenkins, 1959; Nichol and Ogston, 1965); (2) that insignificant amounts of SDS are bound to the intact tetramer which is present at all ratios investigated; (3) that no free SDS remains in solution. If all three assumptions were valid the experimental points would be expected to lie on a straight line passing through the point (1.0, 0), as indicated by the broken line in Figure 3. The fit is reasonably good but the deviation of points at low SDS–protein ratios indicates that one or more of the assumptions are imperfectly fulfilled.

Several independent items of evidence show the first assumption is justified. First, the action of SDS was complete 2 hr after addition and the refractive index gradient fell to 0 between the peaks evident in the electrophoresis and sedimentation velocity analysis (Figure 1a). Secondly, the electropherogram obtained with the SDS–protein mixture examined was essentially enantiographic. Thirdly, the action of SDS was not truly reversible as neither the enzymatic activity nor the expected sedimentation properties were regained on removal of the detergent. Fourthly, the results of the boundary analyses of Baldwin (1959) and of Fujita (1956) suggest that the *s* 3.6 peak is not a reaction boundary. Finally, the sedimentation coefficient of the faster peak is that expected for tetramer (*s* 14), a result incompatible with the postulate that this boundary is a reaction boundary. The small deviation of points is, therefore, attributed to the fact that not all the detergent is bound to the *s* 3.6 material; that is, that assumptions 2 and 3 above are imperfectly fulfilled. As we have seen, the sedimentation coefficient and electrophoretic mobility of the remaining tetramer are essentially unchanged in the presence of the detergent which suggests, in agreement with assumption 2, that little SDS can be bound to this form of sulfatase A. Certainly as the SDS–protein ratio increases, any error in this regard decreases. It appears, therefore, that the most likely error in estimating the amount of bound SDS is that some SDS remains free in solution. Even although the amounts of SDS and protein added to the mixture are known, it is not possible to use the observed area measurements to estimate the latter quantity because the specific refractive increment of SDS and protein are different (Brand and Johnson, 1956) and the value for the SDS–protein complex is obviously unknown. It is for this reason that the concentration of the *s* 3.6 material has not been given in absolute units. However, it is clear from refractometric measurements (Figure 1a) that large amounts of detergent are bound to the *s* 3.6 material, a fact substantiated by the large observed increase in $\bar{V}$. If a significant amount of SDS remained free in solution, not only would the amount of SDS bound be in error, but also it would be necessary to apply more complicated thermodynamic theory (Schachman, 1960; Casassa and Eisenberg, 1964) to obtain the molecular weight of the *s* 3.6 species. Application of these approaches is considered impossible at this time, as the *s* 3.6 material has not been obtained free of tetramer. Fortunately, the amount of SDS free in solution must be below the critical micelle concentration of *ca.* 0.002 M in this environment (Williams *et al.*, 1955; Brand and Johnson, 1956) and that in the experiments used to determine the molecular weight it may be considerably below it as the tetramer continues to exist in the solution.

In summary, although the estimate of the size of the sulfatase A subunit, 24,000, is not free from objection, it appears unlikely that the subunit could be less than a fifth unit (mol wt 21,000) or greater than a third unit (mol wt 36,000). Certainly the basic conclusion that SDS effects the dissociation of sulfatase tetramer to a unit of molecular weight lower than that of the monomer is definitely established. As the resulting material is homogeneous with respect to sedimentation coefficient at reasonably low values of the SDS–protein ratio, it may further be proposed that under these conditions the tetramer is broken into subunits of approximately equal size. It has previously been reported (Nichol and Roy, 1965) that the monomer of sulfatase A contains 14 lysyl and 34 arginyl residues/molecule. If the protein had consisted of a single polypeptide chain, or of a number of subunits all of which were chemically distinct, then 49 peptides should have been produced on tryptic digestion. If, on the other hand, the monomer had been made up of four identical subunits then 13 peptides should have been produced. In fact, approximately 36 peptides were found. If, then, the postulated subunit of mol wt 24,000 exists it is obvious that several chemically distinct species of this must occur, despite its apparent hydrodynamic homogeneity.

The forces involved in binding the subunits together to form the enzymatically active monomer may be partly hydrophobic, but the evidence, resting on the action of SDS, is not conclusive. On the other hand, it appears that the structure of the enzymatically active tetramer at pH 5.0 is largely determined by attractive

intermolecular hydrophobic bonds acting in opposition to electrostatic repulsive forces.

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