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# The Sulfatase of Ox Liver. IX. The Polymerization of Sulfatase A*

L. W. Nichol and A. B. Roy

ABSTRACT: Archibald and equilibrium ultracentrifugation experiments have been used to show that sulfatase A exists completely as monomer (mw 107,000) at pH 7.5 and as a tetramer (mw 411,000) at pH 5.0 in 0.10 ionic strength buffers in the protein concentration range 0.005–0.5 g/100 ml. The identical partial specific volume and optical rotatory dispersion of the protein at these two pH values suggest that no marked shape change accompanies the polymerization. Sephadex chromatography experiments revealed that the tetramer dissociated in the concentration range $0.3 \times 10^{-4}$–$0.3 \times 10^{-2}$ g/100 ml at pH 5.0, whereas the monomer at pH 7.5 remained unaltered at these concentrations. Weight-average sedimentation coefficients varied smoothly with pH between pH 5.0 and 7.5, and were concentration dependent at pH 6.3, the intermediate value selected for detailed study.

The results suggest that at pH 6.3 in the concentration range 0.1–1.0 g/100 ml a relatively stable dimer coexists in rapid equilibrium with monomer, trimer, and tetramer. A stable dimer is also formed at pH 7.5 if the ionic strength is increased to 2.0, suggesting that the net charge on the protein at pH 7.5 prevents polymerization in low ionic strength buffers. The results suggest experimental conditions for future kinetic studies which would avoid complications inherent in the study of mixtures of polymeric species. Finally, the amino acid composition of the enzyme is presented and its high proline content is correlated with its unusual optical rotatory dispersion.

In a previous communication (Nichol and Roy, 1964) a method was given for the preparation of sulfatase A (an arylsulfate sulfohydrolase, EC 3.1.6.1) from ox liver. Sedimentation velocity patterns obtained with the enzyme in 0.10 ionic strength buffers of pH 7.5 and 5.0 were subjected to two boundary analyses, those of Baldwin (1959) and of Fujita (1956), and by each method the material was demonstrated to be homogeneous with respect to sedimentation coefficient. However, the s values varied from 6 at pH 7.5 to 14 at pH 5.0, a result tentatively attributed to the polymerization of the enzyme at pH 5.0. The hypothesis has been tested

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in this study by determining the weight-average molecular weights, employing the Archibald procedure (Archibald, 1947) and independent determinations of the partial specific volumes. In addition, optical rotatory dispersion measurements have been made at the different pH values in an attempt to detect any marked change in conformation. The unusual results are in part correlated with the amino acid composition of the protein, which is also presented. The findings, when taken together, confirm that the enzyme exists as a tetramer at pH 5.0 and a monomer at pH 7.5, the conversion being essentially complete in the concentration range 0.1–1 g/100 ml.

It has been suggested by Roy (1953) that the coexistence of two polymeric forms of sulfatase A with different enzymatic activities may explain, in part, the anomalous kinetics found at pH 5.0. However, kinetic experiments were generally performed with enzyme concentrations in the range 0.01–0.1 mg/100 ml and consequently conclusions pertaining to the polymerization formulated from data obtained at high concentrations cannot be applied directly. This problem is general in that kinetic studies with many enzymes are performed at concentrations very different from those used to characterize the material as a protein. Frontal analysis experiments on Sephadex columns (Winzor and Scheraga, 1963) are described which facilitate the correlation of results found in the different concentration ranges. The technique is applicable in the lower concentration region within the limit of detectability of the enzymatic activity and the only limit in the higher concentration region is the amount of enzyme available. The latter restriction has permitted the gel-filtration technique to be extended to a sulfatase concentration of only 0.005 g/100 ml. Accordingly, the equilibrium ultracentrifugation procedure recently described by Yphantis (1964) has been applied to obtain weight-average molecular weights in the concentration range 0.005–0.10 g/100 ml, thus linking the data found from the Archibald and the Sephadex chromatography experiments.

Finally, the system has been examined at intermediate pH values, in the region of pH 6.0. The dependence of the weight-average sedimentation coefficients on pH, temperature, and total concentration are used, together with the theory presented by Gilbert (1959), in an attempt to characterize the system under these conditions.

## Experimental

*Enzyme Preparations.* Samples of sulfatase A were prepared as described by Nichol and Roy (1964) and the numbering of the samples follows that previously adopted.

*Buffers.* Analytical grade materials were used throughout. Generally 0.10 ionic strength uniunivalent buffers were employed and in most cases the composition for a particular pH value has been reported previously (Nichol and Roy, 1964). Buffers of the following composition were also used:

| | pH 5.7 (M) | pH 6.1 (M) | pH 6.3 (M) |
|---|---|---|---|
| Sodium chloride | 0.088 | 0.078 | 0.076 |
| Sodium cacodylate | 0.012 | 0.022 | 0.024 |
| Cacodylic acid | 0.028 | 0.018 | 0.016 |

In experiments designed to test the effect of ionic strength on the system the buffer compositions were essentially those given previously but with the sodium chloride content suitably adjusted.

*Partial Specific Volumes.* Measurements were made using three concentrations of the enzyme, at both pH 7.5 and 5.0. Protein concentrations were determined at 25° using a differential refractometer (Cecil and Ogston, 1951) in which each protein solution was compared with the buffer against which it had been exhaustively dialyzed. In the absence of an experimentally determined value for the specific refractive increment of the sulfatase a constant value of $1.8 \times 10^{-3}$ dl $g^{-1}$ was assumed (Perlmann and Longsworth, 1948) and the calculated values of the apparent specific volumes are therefore to a certain extent uncertain. It may be estimated that a 10% error in the specific refractive increment could result in an approximately 4% error in the apparent specific volume. The required density data were obtained using a 12-ml pycnometer at 25.00 ± 0.01°; weighings accurate to 0.01 mg were made with a Mettler microbalance, Model M5. In cases where the apparent specific volume, $V_1{}^a$, is independent of concentration, its value is that of the partial specific volume, $\bar{V}$, and values of $V_1{}^a$ were computed from equation (113) presented by Svedberg and Pedersen (1940). In order to check the stability of the preparation, a sample was sedimented in an acetate buffer, pH 5.0, before and after the measurements. In each case a single peak with an $s$ of 14 was apparent.

*Amino Acid Analysis.* Approximately 2-mg samples of sulfatase A which had been exhaustively dialyzed against $1.2 \times 10^{-4}$ M sodium bicarbonate were hydrolyzed *in vacuo* for 22 hours at 110° with 6 N hydrochloric acid. The resulting hydrolysate was analyzed by the standard procedure adopted with the Beckman amino acid analyzer. In calculating the results no allowance was made for possible destruction during the hydrolysis. The tyrosine and tryptophan content was determined separately using the spectrophotometric method of Goodwin and Morton (1946).

*Optical Rotatory Dispersion.* Enzyme solutions (0.2 g/100 ml) were dialyzed against Veronal and acetate buffers, pH 7.5 and 5.0, respectively, for 3 days at 5° and were allowed to warm to room temperature 1 hour before use. The optical rotations were determined at 25° in a 10-cm jacketed cell using a Perkin-Elmer polarimeter, Model 141, after which the enzyme solution was removed from the cell and its protein concentration was determined using the differential refractometer.

*Chromatography on Sephadex.* Sephadex G-200 was prepared as recommended by the manufacturers,

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equilibrated with Veronal buffer, pH 7.5, and packed in a column to give a bed of approximately 1.5 × 50 cm. The column was maintained at 25° throughout and was thoroughly washed with buffer before use.

A stock solution of sulfatase A (0.3 g/100 ml) was dialyzed for 3 days at 5° against Veronal buffer, pH 7.5; 24 hours before chromatography the stock solution was diluted to the required concentration with the appropriate buffer and left at room temperature (20–25°). Approximately 30-ml samples of these solutions were applied to the column and eluted with Veronal buffer. The eluate was collected manually in 2-ml fractions at a flow rate of about 0.3 ml/min. The volume of enzyme solution applied to the column was sufficient to ensure a plateau region in the resulting effluent profile (Winzor and Scheraga, 1963).

The concentrations of protein used (0.3 × $10^{-2}$–0.3 × $10^{-4}$ g/100 ml) were too low to be detected by conventional methods and thus the elution profile of the enzyme was obtained by determining the enzymatic activity of the effluent by suitable modifications of the method of Roy (1956), using dipotassium 2-hydroxy-5-nitrophenyl sulfate (nitrocatechol sulfate) as substrate. Suitable volumes of eluate (20–500 μl) and of water were added to 0.25 ml of nitrocatechol sulfate in acetate buffer, pH 5.0, to give a total volume of 0.8 ml and a final concentration of 0.003 M nitrocatechol sulfate in 0.5 M acetate buffer, pH 5.0. After incubation for an appropriate time (10–60 minutes) the reaction was stopped by adding 2 ml of 0.2 N sodium hydroxide and the absorption at 510 mμ was taken as a measure of the enzymatic activity, and so of the protein content.

From the elution pattern so obtained the elution volume, $V_e$, of the protein was determined, $V_e$ being defined as the volume corresponding to an enzymatic activity of 50% of that in the plateau region. This point corresponds to the maximum ordinate of the first derivative curve obtained as described by Winzor and Scheraga (1963). It should be noted that at the lowest protein concentration used the enzyme was apparently unstable on the column so that the activity of the plateau region was only about 75% of that of the applied solution. When these experiments were completed the column was equilibrated with acetate buffer, pH 5.0, and the procedure was repeated at this pH.

*Sedimentation Velocity Experiments.* Runs were performed in a Spinco Model E ultracentrifuge at 50,740 rpm, employing schlieren optics. The temperature was recorded with the RTIC unit. Each sample was dialyzed in the cold for 3 days against the appropriate buffer and approximately 1 hour was allowed for temperature equilibration before sedimentation was commenced. The individual experimental conditions are given in the text. The weight-average sedimentation coefficients, $\bar{s}$, were calculated from the rate of movement of the square root of the second moment of the entire schlieren pattern (Goldberg, 1953). To facilitate comparison, $\bar{s}$ values were corrected to 20° in water, giving the hypothetical values, $\bar{s}_{20,w}$. It should be noted that, in systems consisting of polymeric species in equilibrium, corrected $\bar{s}_{20,w}$ values will not describe the weight-average proper-

ties at 20° unless the equilibrium position is temperature independent (i.e., $\Delta H°$ is zero). Total concentrations were determined refractometrically using the area under the peak, corrected for radial dilution (Svedberg and Pedersen, 1940), and by assuming that the specific refractive increment of all macromolecular species was 1.8 × $10^{-3}$ dl $g^{-1}$ (Perlmann and Longsworth, 1948). The use of the double-sector filled-epoxy centerpiece in all experiments provided a base line for the schlieren patterns and so facilitated these determinations.

*Archibald Experiments.* The method employed has been outlined in detail by Klainer and Kegeles (1955). In this study only values for the observed refractive index gradient at the meniscus, $(dn/dx)_m$, were used to calculate the weight-average molecular weights. The selected values for the angular velocity, ω, were 7447 rpm for samples in Veronal buffer, pH 7.5, and 6569 rpm for those in acetate buffer, pH 5.0. The temperature was 20° for each run and was recorded but not controlled with the RTIC unit. A maximum variation of 0.1° was recorded during the experiments. Four photographs were taken within the 2-hour duration of the experiment and values of $(dn/dx)_m/x_m c_m \omega^2$ (where $x_m$ is the distance of the meniscus from the center of rotation and $c_m$ is the concentration of the solute at the meniscus, expressed in appropriate units) were computed from each exposure. The values of the ratio were time independent in each case and an average value was used to calculate the molecular weights. Evaluation of $c_m$ from equation (9) of Klainer and Kegeles (1955) requires the refractometric determination of the initial concentration, generally in a separate experiment using a synthetic-boundary cell. As the amount of sulfatase was limited an alternative procedure was adopted in which the rotor was accelerated to 50,740 rpm after the initial photographs had been taken. The area under the peak formed in the sedimentation velocity experiment was determined by trapezoidal integration and corrected for radial dilution. This method has the additional advantage that it provides an immediate analysis of the material.

*Equilibrium Ultracentrifugation.* Experiments were performed in the Spinco Model E ultracentrifuge and the temperature was controlled near 20° with the RTIC unit. Rayleigh interference optics, in which the midpoint of the solution was in focus at the camera lens (Yphantis, 1960), were employed. In all experiments at pH 7.5, standard 12-mm-thick, filled-epoxy double-sector centerpieces with quartz windows were used. Kel-F polymer oil was used as the inert base fluid and equal weights of solvent and solution were employed to give 3-mm columns in each channel.

The general design and conduct of the experiment followed that recently described by Yphantis (1964). Two separate runs were made at 19,160 rpm in the An-D rotor with sulfatase solutions of concentrations 0.10 and 0.01 g/100 ml which had been dialyzed exhaustively against Veronal buffer, pH 7.5, ionic strength 0.10. In this buffer sedimentation velocity results revealed a single macromolecular component with an apparent molecular weight of 100,000 (Nichol and Roy,

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1964) so that the selected conditions correspond to an effective reduced molecular weight, $\sigma$, of 5 cm$^{-2}$ (Yphantis and Waugh, 1956). For a monodisperse ideal solute at infinite dilution $\sigma$ equals $M(1 - \bar{V}\rho)\omega^2/RT$, where $M$ is the molecular weight, $\rho$ the density of the solvent, $R$ the gas constant, and $T$ the temperature. The term $(1 - \bar{V}\rho)$ is assumed independent of pressure and concentration. In acetate buffers, $p$H 5.0, ionic strength 0.10, a single macromolecular species with an apparent molecular weight of 400,000 was indicated in the previous study and thus in order to keep $\sigma \sim$ 5 cm$^{-2}$ the heavy An-J rotor was run at a speed of 9945 rpm. Three different initial concentrations of 0.10, 0.04, and 0.01 g/100 ml were examined simultaneously by employing three different 12-mm interference cells: a standard cell containing 0.10 g/100 ml solution was counterbalanced against the interference counterpoise, while wedge-centerpiece cells with wedge angles of 1.1° and 0.6°, containing the 0.04 and 0.01 g/100 ml solutions, respectively, were balanced in the remaining two holes.

Estimates of the required equilibrium times in each case were found using the criterion presented by Van Holde and Baldwin (1958), $\epsilon = 10^{-3}$ for $\sigma = 5$ cm$^{-2}$ and 3-mm columns, together with the apparent diffusion coefficients reported earlier (Nichol and Roy, 1964). Figure 1 is typical of all experiments and shows that the criterion was sufficient in that the distributions are time independent within experimental error. The values of the coordinates of Figure 1 were obtained as follows: The plates were measured with a two-coordinate comparator (Gaertner Toolmakers microscope, Type M 2001 AS-P) after alignment of the radial direction along the $x$ coordinate of the comparator. All $y$-coordinate readings were corrected for optical imperfections by using exposures taken at very early times as blanks. The value $y_0$ is the corrected zero-concentration level in the region of the meniscus and its correct choice is fundamental to the method (Yphantis, 1964). In all exposures an average of at least five values showed a scatter of $\pm 3 \mu$, while the fringe width was 287 $\mu$. Measurements were taken at 0.1-mm intervals along three white fringes (minima in the exposure), starting in the region where the fringe displacement was >20 $\mu$ and keeping to the center of the diffraction envelope. Averaging the three values at a particular point gave $y(r)$, the blank-corrected fringe displacement at the point in the cell distance $r$ from the center of rotation. The slope of the line presented in Figure 1, $d \ln [y(r) - y_0]/[d(r^2/2)]$, is the weight-average effective reduced molecular weight, $\sigma_w(r)$, and may be converted to the weight-average molecular weight, $M_w$, by multiplying by $RT/(1 - \bar{V}\rho)\omega^2$. Further treatment of the fundamental data will be discussed under Results.

## Results

*Weight-average Molecular Weights.* The weight-average molecular weights found with the approach to equilibrium and equilibrium ultracentrifugations are,
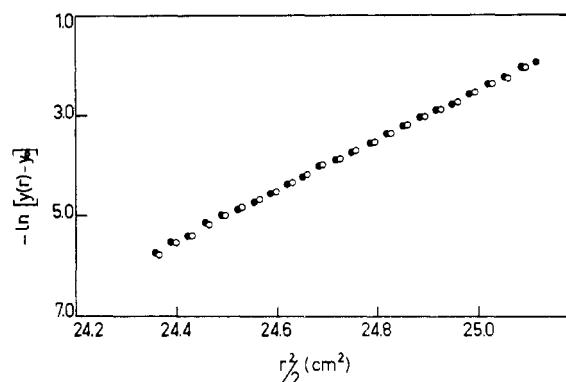


FIGURE 1: The time dependence of the fringe displacements obtained in a sedimentation equilibrium experiment with sulfatase A of initial concentration 0.10 g/100 ml at $p$H 5.0 in 0.10 ionic strength acetate buffer. Times after start of the experiment were 20 hours (○) and 18 hours (●). The natural logarithm of the fringe displacement, $y(r)$, after subtraction of the zero concentration level, $y_0$, is plotted against $r^2/2$, where $r$ is the distance from center of rotation.

together with the experimental conditions, summarized in Table I. It is apparent at both $p$H values that there is no significant concentration dependence of the molecular weights, the values scattering around the arithmetic means of 107,000 at $p$H 7.5 and 411,000 at $p$H 5.0. If the species existing at $p$H 7.5 is termed monomer, there is no doubt that at $p$H 5.0 tetramer is formed. It should be noted that the values were obtained in the equilibrium experiments from the statistically determined slopes, $d \ln [y(r) - y_0]/[d(r^2/2)]$, of the straight lines (similar to that shown in Figure 1), using values of $[y(r) - y_0] > 100 \mu$. The standard deviation of points from the line obtained by the least-squares method never exceeded $8 \times 10^{-3}$ with this limit to the values of the net fringe displacement. However, additional systematic errors are inherent in the method (Yphantis, 1964), and notable in this respect is the random error in the choice of $y_0$ which introduces a large percentage uncertainty in the molecular weight when the measurable net fringe displacements are small (cf. Figure 10 of Yphantis, 1964). Accordingly, the values obtained with the lowest initial concentration, 0.01 g/100 ml, are less reliable than the values obtained with the higher concentrations of the enzyme.

The same restriction applies to the calculation of the weight-average effective reduced molecular weights, $\sigma_w(r)$, at a point $r$ corresponding to a small net fringe displacement (<100 $\mu$), and hence to a low value of the concentration at $r$. This is illustrated in Figure 2, where point values of $\sigma_w(r)$ found by the statistical treatment of five equally spaced points in the ln $[y(r) - y_0]$ versus $r^2/2$ plot (Yphantis, 1964) are plotted against the net fringe displacement and the concentration at the corresponding point $r$ in the cell. It is seen that the values of $\sigma_w(r)$ found from experiments at $p$H 5.0 in-

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TABLE I. Weight-average Molecular Weight of Sulfatase A in 0.10 Ionic Strength Uniunivalent Buffers.

| Sample | Type of Experiment | $\omega$ (rpm) | Initial Concentration (g/100 ml) | $M_w$ pH 7.5 | $M_w$ pH 5.0 |
|---|---|---|---|---|---|
| 3 | Archibald | 7,447 | 0.88 | 99,000 | |
| 3 | Archibald | 7,447 | 0.61 | 105,000 | |
| 3 | Archibald | 7,447 | 0.51 | 100,000 | |
| 5 | Equilibrium | 19,160 | 0.10 | 106,000 | |
| 5 | Equilibrium | 19,160 | 0.01 | 123,000 | |
| 3 | Archibald | 6,569 | 0.45 | | 440,000 |
| 3 | Archibald | 6,569 | 0.42 | | 409,000 |
| 5 | Equilibrium | 9,945 | 0.10 | | 394,000 |
| 5 | Equilibrium | 9,945 | 0.04 | | 394,000 |
| 5 | Equilibrium | 9,945 | 0.01 | | 417,000 |
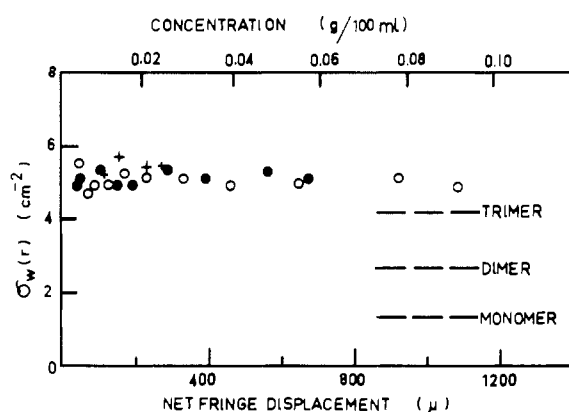


FIGURE 2: The effective reduced weight-average molecular weight, $\sigma_w(r)$, found from an equilibrium experiment in which three concentrations of sulfatase A at pH 5.0 were run in three different cells. Initial concentrations were 0.10% (O); 0.04% (●); 0.01% (+). A direct comparison of the three sets of results is obtained by plotting point values of $\sigma_w(r)$ against the corresponding fringe displacement and concentration.

volving three different initial concentrations fall on an essentially straight line parallel to the $x$ axis and that there is considerable scatter in the dilute regions in accordance with expectation. Multiplication of each value of $\sigma_w(r)$ by the constant factor $RT/[(1 - \bar{V}\rho)\omega^2]$ would give a series of point weight-average molecular weights: the value for the constant factor for experiments performed at pH 5.0 was $7.77 \times 10^4$ cm². It is apparent from Figure 2 that, even with the considerable uncertainty attached to the points at low concentration, dilution at pH 5.0 to a concentration of 0.005 g/100 ml does not result in dissociation of the tetramer. The values of $\sigma_w(r)$ for monomer (mw 107,000), dimer, and trimer are indicated by dashed lines in Figure 2.

*Partial Specific Volumes.* The partial specific volume of the protein, required in the calculation of molecular

weights, was deduced from apparent specific volumes found at pH 7.5 and 5.0, which are given in Table II. The accuracy of these determinations is limited as only small quantities of sulfatase were available. However, it is clear that the values at pH 7.5 and 5.0 are quite close, and that no marked concentration dependence exists. Accordingly, a single value of 0.715 was taken as the value of $\bar{V}$ in all calculations and it was assumed that $\bar{V}$ was independent of temperature. Because of this uncertainty in the value of $\bar{V}$, the absolute, but not the relative, values of the molecular weights shown in Table I are to some extent uncertain.

TABLE II: Apparent Specific Volumes of Sulfatase A at 25°.

| Concentration (g/100 ml) | $V_1{}^a$ pH 5.0 | $V_1{}^a$ pH 7.5 |
|---|---|---|
| 0.182 | | 0.717 |
| 0.160 | 0.711 | |
| 0.080 | | 0.714 |
| 0.077 | 0.716 | |
| 0.045 | | 0.72 |
| 0.044 | 0.73 | |

*Optical Rotatory Dispersion.* A constant value of $\bar{V}$ is not a sensitive test for a possible shape change accompanying the polymerization, and accordingly optical rotatory dispersion studies were made at pH 7.5 and 5.0. The results of these studies are shown in Figure 3 in which the data are treated graphically according to the simple Drude equation by the method of Yang and Doty (1957). It is clear that the curves representing the data found at pH 7.5 and 5.0 coincide. Moreover, the nonlinearity of the plot shows that
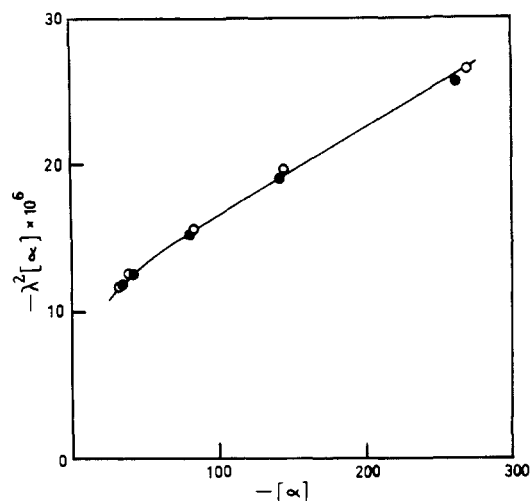
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FIGURE 3: A Yang-Doty plot of optical rotatory dispersion of sulfatase A in 0.10 ionic strength uniunivalent buffers at 25°. The $p$H values were 7.5 (O) and 5.0 (●).



CONCENTRATION ( g/100 ml )

FIGURE 4: The elution volume, $V_e$, of sulfatase A on a 1.5 × 50-cm Sephadex G-200 column at 25° as a function of protein concentration. The samples were in uniunivalent buffers of 0.10 ionic strength; $p$H 7.5 (O); $p$H 5.0 immediately after dilution (●); $p$H 5.0 24 hours after dilution (+).

sulfatase A differs from most proteins in its optical activity. The data likewise did not give straight-line plots with the Moffit equation although several values of $\lambda_0$ in the range 200–220 m$\mu$ were chosen. This again shows that sulfatase A does not have the optical rotatory dispersion of a typical protein.

*Amino Acid Composition.* The results of the analysis are shown in Table III, and expressed as the number of residues per monomer of molecular weight 107,000. The tyrosine content determined spectrophotometrically (together with the tryptophan) agreed with the value found chromatographically. A striking feature of the composition is the relatively high proline content, which is approached only by some caseins and exceeded by the collagens (Tristram and Smith, 1963). On the other hand, the content of tyrosine and tryptophan is comparatively low, an observation which may be correlated with the low extinction coefficients observed at 280 m$\mu$; the values of $E_{1\,cm}^{1\%}$ were 7.0 and 6.6 at $p$H 7.5 and 5.0, respectively. An amino sugar, almost certainly glucosamine, was consistently associated with the protein, and the presence of other sugars is therefore very likely. In a separate determination, using a 5-mg sample of the enzyme, no phosphorus was detected, even though the method could have detected one phosphorus atom per monomer unit.

An apparent specific volume of sulfatase A was calculated from the data in Table III, together with the specific volumes of the known residues. The value of 0.70 which was obtained is in reasonable agreement with those listed in Table II.

*Chromatography on Sephadex.* The results obtained by the chromatography of sulfatase A on Sephadex G-200 are given in Figure 4. It is clear that the elution volume, $V_e$, of the enzyme at $p$H 7.5 is essentially constant over a range of protein concentration of
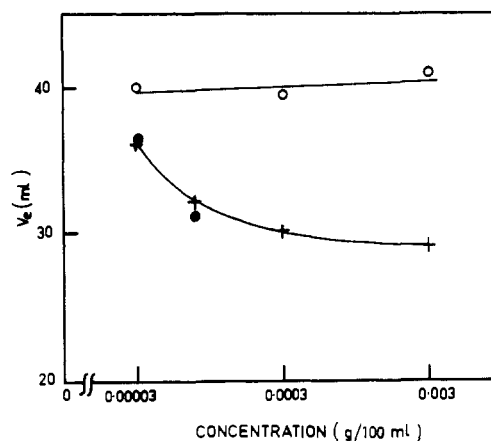
TABLE III: Amino Acid Composition of Sulfatase A.

| | Residues/Monomer Unit |
|---|---|
| Lysine | 14 |
| Histidine | 39 |
| Arginine | 34 |
| Aspartic acid | 72 |
| Threonine | 56 |
| Serine | 59 |
| Glutamic acid | 88 |
| Proline | 90 |
| Glycine | 109 |
| Alanine | 94 |
| Half-cystine | 21 |
| Valine | 49 |
| Methionine | 16 |
| Isoleucine | 16 |
| Leucine | 133 |
| Tyrosine | 28 |
| Phenylalanine | 50 |
| Tryptophan | 11 |
| Glucosamine | 9 |
| Ammonia | 89 |

0.3 × $10^{-2}$–0.3 × $10^{-4}$ g/100 ml. In contrast, $V_e$ of the enzyme at $p$H 5.0 was markedly concentration dependent. Thus at a concentration of 0.3 × $10^{-2}$ g/100 ml and $p$H 5.0, $V_e$ was much less than that at $p$H 7.5, showing that the molecular size of the enzyme was greater at the lower $p$H; but as the protein concentration was lowered to 0.3 × $10^{-4}$ g/100 ml $V_e$ increased
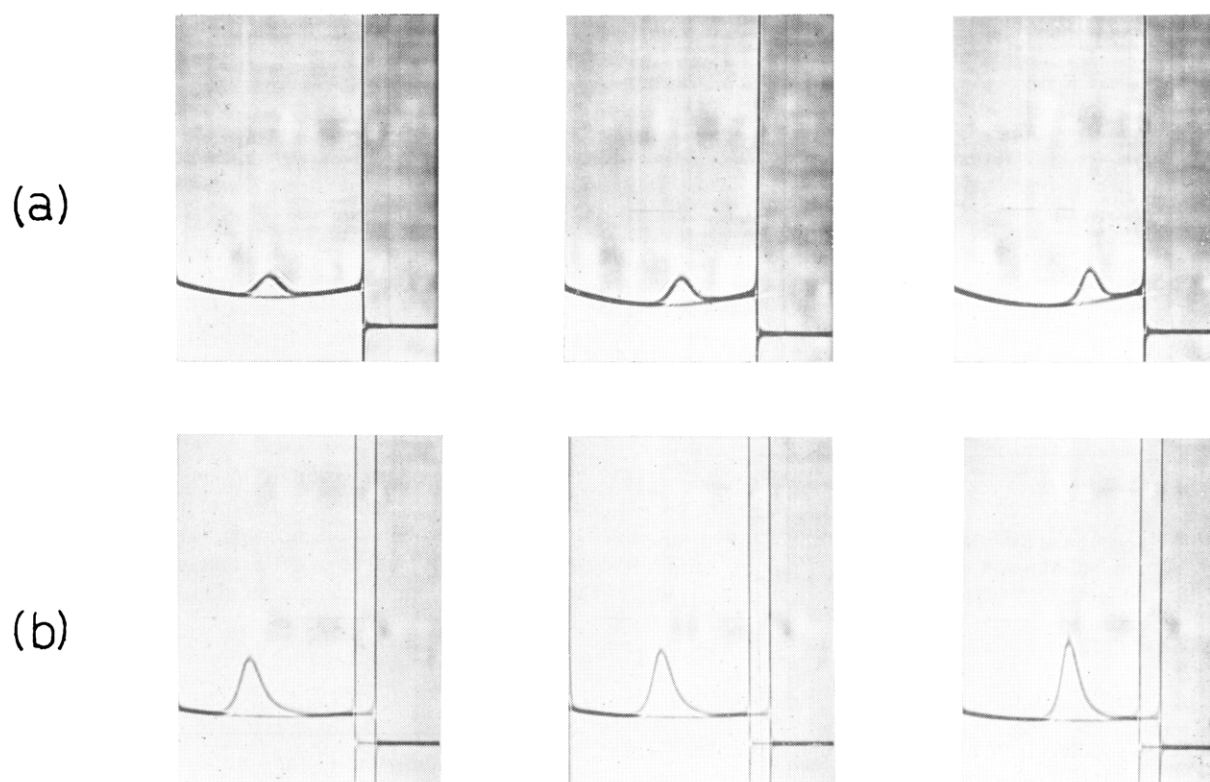
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(a)



(b)



FIGURE 5: Sedimentation velocity patterns of sulfatase A (sedimentation from right to left). (a) Sample 6 in Veronal buffer, ionic strength 2.0, $pH$ 7.5, at a protein concentration of 0.3 g/100 ml. (b) Sample 6 in cacodylate buffer, ionic strength 0.10, $pH$ 6.3, at a protein concentration of 0.81 g/100 ml.

until it was close to that obtained at $pH$ 7.5 at the same concentration.

Figure 4 also shows that the apparent dissociation of sulfatase A on dilution at $pH$ 5.0 is rapid in comparison with the time required for the completion of the experiment (about 2 hours.) The same value of $V_e$ was obtained at $pH$ 5.0 in the low concentration range whether the dilution was carried out 15 minutes or 24 hours prior to the commencement of the chromatography. In this connection it should be noted that at $pH$ 5.0 the elution profile on the leading edge was quite sharp at all concentrations studied.

*Weight-average Sedimentation Coefficients.* The dependence of the corrected weight-average sedimentation coefficient, $\bar{s}_{20,w}$, on $pH$ is shown in Table IV. The values found at $pH$ 5.0, 5.5, 6.6, and 7.5, where single symmetrical peaks were observed, agree closely with $s_{20,w}$ values calculated from the rate of movement of the maximum ordinate (Nichol and Roy, 1964). It is clear from Table IV that $\bar{s}_{20,w}$ decreases systematically with increasing $pH$ and that the transition cannot be explained by the concentration dependence of the sedimentation coefficients. The $pH$-dependent weight-average properties of sulfatase A suggest that electrostatic forces may be involved in the polymerization and accordingly the effect of ionic strength on the system was determined at two $pH$ values. At $pH$ 5.0, with a protein concentration of 0.4 g/100 ml, a peak with an $\bar{s}_{20,w}$ of 14 S was observed both in buffers of ionic strength 0.01 and 2.0; but whereas the pattern at the lower ionic strength was that normally obtained, the pattern at the higher ionic strength showed the presence of aggregated material which sedimented to the bottom of the cell during the early stages of the run. At $pH$ 7.5, ionic strength 2.0, a single symmetrical peak with $\bar{s}_{20,w}$ of 8.72 S (protein concentration of 0.3 g/100 ml) was obtained and is shown in Figure 5a.

In the intermediate $pH$ range single peaks asymmetric on the trailing side were evident: Figure 5b shows patterns obtained at $pH$ 6.3, ionic strength 0.10, where the highest degree of asymmetry was apparent. The latter conditions were selected for a more detailed study and the effect of temperature and total concentration on $\bar{s}_{20,w}$ for the system is presented in Table V. It is evident from Table V that $\bar{s}_{20,w}$ is a function of total protein concentration, but not of temperature. In the last column of Table V values are given of $\Delta_s$, a quantity defined by Nichol and Bethune (1963) as the concentration at which the minimum occurs in a reaction boundary. In this study estimates of $\Delta_s$ were obtained from enlarged tracings of the schlieren patterns by dropping a perpendicular from the maximum ordinate to the base line and subtracting the area of the fast peak symmetrical about this perpendicular from the total area. The area so obtained was converted to a concentration on the g/100 ml scale and it is evident that the values are

TABLE IV: Dependence of the Weight-average Sedimentation Coefficient of Sulfatase A on pH in 0.10 Ionic Strength Buffers.

| Sample | pH | Concentration (g/100 ml) | $\bar{s}_{20,w}$ (S) |
|---|---|---|---|
| 3 | 5.0 | 0.45 | $14.0_6$ |
| 5 | 5.0 | 0.35 | $14.1_0$ |
| 2 | 5.5 | 0.10 | $14.1_4$ |
| 6 | 5.7 | 0.61 | $12.1_0$ |
| 3 | 6.0 | 0.74 | $10.5_3$ |
| 4 | 6.1 | 0.52 | $9.3_2$ |
| 7 | 6.3 | 0.61 | $8.8_0$ |
| 1 | 6.6 | 0.31 | $6.2_3$ |
| 3 | 7.5 | 0.87 | $5.9_2$ |
| 3 | 7.5 | 0.61 | $6.0_3$ |
| 3 | 7.5 | 0.52 | $6.1_1$ |

TABLE V: Effect of Temperature and Total Concentration on the Weight-average Sedimentation Coefficient of Sulfatase A at pH 6.3, Ionic Strength 0.10.

| Sample | Temperature of Run (deg) | Concentration (g/100 ml) | $\bar{s}_{20,w}$ (S) | $\Delta_s$ (g/100 ml) |
|---|---|---|---|---|
| 7 | 21 | 0.61 | $8.8_0$ | 0.18 |
| 7 | 11 | 0.60 | $9.0_3$ | 0.16 |
| 7 | 4 | 0.61 | $8.6_7$ | 0.13 |
| 6 | 20 | 0.81 | $9.1_7$ | 0.16 |
| 7 | 20 | 0.60 | $8.8_8$ | 0.14 |
| 5 | 20 | 0.42 | $8.6_2$ | 0.14 |
| 4 | 20 | 0.14 | $8.1_3$ | 0.14 |

constant within experimental error at all concentrations and insensitive to temperature variation.

Discussion

Fundamentally the Archibald and equilibrium ultracentrifugation methods both provide a measure of $s/D$, where $s$ and $D$ are sedimentation and diffusion coefficients (LaBar and Baldwin, 1963). In equilibrium ultracentrifugation, $\sigma_w(r)$ is the ratio $\omega^2 s/D$, and in Archibald experiments $(dn/dx)_m/(\omega^2 x_m c_m)$ is equal to $s/D$. The value of $s/D$ at pH 7.5 was $1.27 \times 10^{-6}$ sec$^2$ cm$^{-2}$ and at pH 5.0 was $4.89 \times 10^{-6}$ sec$^2$ cm$^{-2}$. The large change in this ratio is the primary index that in the sulfatase A system there operates a polymerization reaction rather than a shape change. Indeed, as the molecular weight is obtained by multiplying $s/D$ by $RT/(1 - \bar{V}\rho)$ it is clear that $\bar{V}$ at pH 7.5 would have to be much greater than at pH 5.0 if the molecular weight were to remain constant. The data in Table II are of

sufficient accuracy to exclude the latter possibility, and when these are used to calculate the molecular weights shown in Table I the results definitely confirm that a monomer exists at pH 7.5 and a tetramer at pH 5.0. The similarity of the optical rotatory dispersion of the enzyme at pH 5.0 and 7.5 (Figure 3) also suggests that the polymerization is not accompanied by a large shape change. The failure to obtain linear plots of the optical rotatory dispersion may be related to the disruption of the helical structure by the high proline content. In this connection it might also be noted that poly-L-proline, form 1, similarly does not give linear plots (Blout and Fasman, 1958).

Yphantis (1964) has shown that the point value of $\sigma_w(r)$ at the base of the solution column, normally obtained by extrapolation, is the $z$-average effective reduced molecular weight averaged over the whole cell. The point values of $\sigma_w(r)$ at pH 5.0 are shown in Figure 2 to be essentially constant in the concentration range 0.01–0.10 g/100 ml and are therefore not functions of $r$. It is clear then that the weight-average and $z$-average molecular weights are identical for the tetramer, indicating that in the specified concentration range the equilibrium lies almost entirely in favor of the tetramer at pH 5.0. Previous boundary analyses (Nichol and Roy, 1964) and the time independence of the quantity $(dn/dx)_m/(\omega^2 x_m c_m)$ found in Archibald experiments also show that tetramer exists essentially as the single homogeneous form under these conditions. In these circumstances the term $\sigma_w(r)$ and $\sigma$ are equivalent. At pH 7.5, ionic strength 0.1, it is similarly evident that the equilibrium strongly favors monomer. Moreover, as the point values of $\sigma$ found at different initial concentrations but at the same angular velocity all lie on a straight line parallel to the $x$ axis, as shown for pH 5.0 in Figure 2, it may be concluded that any nonidealities inherent in studying sulfatase bearing a net charge at finite concentrations are small. It is of interest that the use of commercially available wedge-shaped centerpieces to run simultaneously three experiments at pH 5.0 does not appear to affect the results, although the special centerpiece designed by Yphantis (1964) is preferable in principle.

The marked dependence of the weight-average properties of sulfatase A on pH which has been discussed, and is further illustrated by data in Table IV, suggests at first sight that ionization of particular groups on the protein is essential for polymerization. However, the effect of increasing the ionic strength at pH 7.5 and 5.0 leads to an alternate hypothesis, namely, that the net charge at pH 7.5 is sufficiently large to prevent polymerization in 0.10 ionic strength buffers, but it can be masked either by lowering the pH or by increasing the ionic strength. At pH 7.5 in 2.0 ionic strength buffer a single peak was observed (Figure 5a), and its $\bar{s}_{20,w}$ of 8.72 S was close to the average value for dimer calculated on the basis of models representing extremes in shape. The appearance of aggregated material in solutions of sulfatase A at pH 5.0, ionic strength 2.0, further suggests that polymerization is extensive in an environment where repulsive forces are minimized.

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No evidence for the actual mechanism of this polymerization has been obtained at this time, but it should be noted that polymerization of carboxypeptidase A has also been induced by markedly increasing the ionic strength (Bethune, 1963).

The design of the Sephadex chromatography experiments to include a plateau region not only permits the specification of the concentration of the experiment but also provides an elution profile which is directly analogous to the concentration versus distance curve obtained by the integration of electrophoretic schlieren patterns. The reciprocal of the elution volume is the analog of velocity in other transport processes and therefore in a rapidly interacting mixture of polymeric species it is, on the ascending side, a weight-average property (Gilbert, 1959). In this study the activity of the enzyme, rather than the protein concentration, was found as a function of the volume of the eluate and accordingly no detailed analysis of the profile shape has been attempted. However, all plots showed a single steep gradient on the ascending and descending sides, and thus $1/V_e$ may be taken as a measure of the size of the species, assuming that no shape change occurred at the low concentrations employed. The latter assumption seems to be valid as $V_e$ for the monomer remained constant on dilution (Figure 4). The data in Figure 4 at pH 5.0 show that tetramer dissociates in the concentration range $0.3 \times 10^{-3}$–$0.3 \times 10^{-4}$ g/100 ml but do not permit the evaluation of the single or successive equilibrium constant(s) pertaining to the system. It is clear, however, that the apparent association equilibrium constant is several orders of magnitude greater than those usually reported for polymerizing systems of proteins. It should nevertheless be kept in mind that the use of enzymic methods of frontal analysis to study quantitatively other systems at extreme dilution may show that the behavior of sulfatase is not unique.

The possibility that several polymers of sulfatase may coexist in equilibrium at relatively high concentrations is shown by consideration of the weight-average data found at pH 6.3 in 0.10 ionic strength buffers (Table V). The values of $\bar{s}_{20,w}$ are seen to be insensitive to temperature in the range 4–20°, the slight scatter being attributed to the large temperature correction necessary to obtain results at 20° in water. The observation implies that if polymeric species do exist in equilibrium, then $\Delta H°$ for the reaction at pH 6.3 is essentially zero; it may be quite different at other pH values. Accordingly, small temperature corrections may be applied with confidence to $\bar{s}$ data found at pH 6.3 and this has been done to obtain $\bar{s}_{20,w}$ values at a series of concentrations. The decrease in the weight-average sedimentation coefficients with decreasing concentration (Table V) clearly indicates that two or more polymeric forms coexist in equilibrium, dilution favoring the polymers of lower molecular weight. The peak shown in Figure 5b may therefore be regarded as a reaction boundary in the sense that it represents a refractive index gradient in all polymeric species present. As the refractive index gradient does not fall to zero at any position in the highly asymmetric boundary, even after long periods

of time, a rapid equilibrium between species is suggested. Further confirmation of this point comes from the theory of Gilbert (1959) which will be discussed here.

The peak presented in Figure 5b may be interpreted simply as a single peak asymmetric on the trailing side. On this basis, and following the predictions of Gilbert (1959), it would be concluded that the system was either an equilibrium mixture of monomer and dimer or of dimer and tetramer. The existence of polymers higher than the tetramer may be reasonably excluded in the sulfatase A system on the basis of the molecular weight results found at pH 5.0. In addition, the largest $\bar{s}_{20,w}$ value, of 9.17 S, found at 0.81 g/100 ml shows that significant amounts of higher polymers ($s_{20,w} > 14$ S) cannot exist in the concentration range studied. For each of the above-mentioned models (monomer-dimer and dimer-tetramer), an apparent association equilibrium constant, $K'_{app}$, may be calculated from the $\bar{s}_{20,w}$ values reported in Table V, by employing equation (8) presented by Nichol et al. (1964). The latter is a definition of $\bar{s}$ and requires for its application the value of $s_{20,w}$ for each polymeric species at the concentration specified for a particular value of $\bar{s}_{20,w}$. The $s^0_{20,w}$ values for monomer and tetramer were found experimentally from the data in Table IV, while that for the dimer was taken as 9.0. It was assumed that the concentration dependence found for the monomer applied to other polymeric species. For the monomer-dimer model the calculated concentration of the monomer was negative at the total concentrations 0.42, 0.60, and 0.81 g/100 ml. To obtain meaningful values for $K'_{app}$ it would be necessary to assume that $(s_{dimer})^0_{20,w} > 9.2$ S, and even in this case $K'_{app}$ would be markedly concentration dependent. The model may therefore be excluded. The second model involving dimer and tetramer resulted in values of $K'_{app}$ of 0.001, 0.017, and 0.034 liter g$^{-1}$ at the concentrations 0.42, 0.60, and 0.81 g/100 ml, respectively. Better agreement with the experimental data would be obtained if $8.1 < (s_{dimer})^0_{20,w} < 9.0$ S. Thus a system in which a stable dimer coexisted with tetramer would be compatible with the experimental findings, provided the peak in Figure 5b is regarded as a single peak. It should be stressed, however, that the variation in $K'_{app}$ does indicate that the model may be too simple. Alternative models consisting of only two polymers (i.e., monomer-trimer and monomer-tetramer) seem unlikely as $\bar{s}_{20,w}$ reaches the value of 8.1 S at the lowest concentration studied, which is considerably higher than that of the monomer.

The final possibility is that monomer, dimer, trimer, and tetramer coexist in a series of rapid equilibria, the relative amounts being determined by the successive equilibrium constants $K_2$, $K_3$, $K_4$. For similar systems it has been shown by numerical example (cf. Figure 1a and Figure 3 of Gilbert, 1959) that the schlieren pattern is either a bimodal boundary with a distinct minimum or a single peak with a shoulder on the trailing side. Inspection of Figure 5b reveals that the peak could be interpreted in these terms, especially as diffusional spreading neglected in the theory may be expected to

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obscure any minimum in the experimental curve. It is noteworthy that values of $\Delta_s$ reported in Table V remain constant as the total concentration is decreased until a point is reached when only slow peak is evident. As there is no distinct minimum in the curve, the values of $\Delta_s$ are to this extent uncertain but their apparent constancy lends support to the hypothesis that several polymers coexist in rapid equilibrium at $pH$ 6.3. In previous studies with rapidly polymerizing systems (Massey *et al.*, 1955; Nichol and Bethune, 1963; Townend *et al.*, 1960) it has been shown that the $s$ of the slow peak is only slightly greater than that of the monomer. In this study the $s$ of the slow peak was approximately 8.1 S, which is considerably higher than that of the monomer and closer to that of the dimer. Thus, even with the more complicated model it appears that the dimer is the relatively stable species under these conditions.

A more detailed characterization of the system must involve the evaluation of the successive equilibrium constants. The latter may be estimated from weight-average data obtained with great precision over a wide concentration range (Steiner, 1952). Only weight-average molecular weights together with the concentration-independent molecular weights of the individual species are suitable for this purpose, and even here the accuracy required to determine $K_3$ and $K_4$ is very great (Rao and Kegeles, 1958). As different values of the equilibrium constants must pertain at each $pH$ (Table IV) the detailed study was not attempted with the small quantities of sulfatase presently available.

In summary, it is clear that sulfatase A, like most polymerizing systems, exhibits a wide range of behavior depending on environmental conditions. If the various polymeric forms of the enzyme exhibit different activities the enzyme activity will not be a linear function of the concentration in the range where two or more forms coexist in significant amounts and the kinetic results may appear anomalous. A nonlinear dependence has in fact been observed with sulfatase A at $pH$ 5.0 (Roy, 1953). There are, of course, several other factors which may contribute to complicated kinetic results and these have previously been discussed in detail for the sulfatase A system (Roy, 1957; Baum and Dodgson, 1958; Baum *et al.*, 1958), but their presence emphasizes more strongly the necessity of studying the enzyme under conditions where the polymerization is not a further complication. Studies with monomeric sulfatase are feasible over a wide concentration range at $pH$ 6.6–7.5 provided the ionic strength of the medium is not too high. In the $pH$ region near 6.0 the extreme dilution usually employed in kinetic studies would probably result in almost complete dissociation to monomer, but the possibility of a stable dimer cannot be excluded even at low ionic strengths. Most kinetic studies have been performed at $pH$ 5.0, the $pH$ optimum, and probably with enzyme concentrations below $0.3 \times 10^{-4}$ g/100 ml where only monomer is anticipated at ionic strength 0.1. In future kinetic studies at $pH$ 5.0 it would appear advisable to employ concentrations less than $0.3 \times 10^{-4}$ g/100 ml or greater than $0.3 \times 10^{-3}$ g/100 ml to ensure that a single polymeric form (either monomer or tetramer) was present.

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# Activation of Phosphoglucomutase[*]

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ABSTRACT: The specific activity of phosphoglucomutase may be increased 2- to 6-fold by preincubation in $Mg^{2+}$ and imidazole. Neither alone is effective. The rate of activation is a function of temperature reaching a maximum in about 5 minutes at $0°$ and in 30 seconds at $30°$. The process is readily reversed upon lowering the concentration of $Mg^{2+}$ and imidazole by dilution or by dialysis. The rate of deactivation is comparable to that of activation. Activation is immediately arrested but not reversed by the addition of substrate and is assumed to result from the association of a $Mg^{2+}$-imidazole complex with the enzyme which stabilizes an active configuration. The $Mg^{2+}$-imidazole-induced activation has an activation energy $E$ of 4.8 kcal which contrasts with a value of 19.3 kcal for that of catalysis.

It has been reported from this laboratory that the observed specific activity of phosphoglucomutase (PGM)[1] may be considerably increased by preincubating the enzyme with $Mg^{2+}$ and imidazole. This process will be referred to, henceforth, as activation and the enzyme so treated as activated enzyme (Robinson and Najjar, 1960, 1961). This induced activation does not obviate the necessity for the continuous presence of both $Mg^{2+}$ and imidazole during the catalytic action of the enzyme. Thus it is clear that the phenomenon of activation differs from the usual type of catalytic stimulation of enzyme activity that has been observed with metal ions and metal binding agents (Cori et al., 1937; Sutherland, 1949). A detailed study of the effect of $Mg^{2+}$ and imidazole on the catalytic stimulation of PGM is the subject of an accompanying report (Robinson et al., 1965). In this paper, a study of the factors affecting the time-dependent activation is reported. This includes the influence of temperature, hydrogen ion, $Mg^{2+}$, and imidazole concentrations, as well as the effect of imidazole derivatives and other complexing agents. The data reported here support the hypothesis that a specific complex composed of magnesium and imidazole is involved in this type of activation.

## Experimental Procedure

Crystalline PGM, prepared either from fresh or frozen rabbit muscle (Pel-Freez, Rogers, Ark.) was used throughout this study. A slight but important modification of the earlier procedure (Najjar, 1948) was found to be useful in removing heat-denatured proteins from solution which are of gelatinous consistency and not readily sedimented by centrifugation. After the first heat step of the aqueous muscle extract, ammonium sulfate is added to 0.20 saturation and the precipitated protein is removed by centrifugation. The rest of the procedure remains unaltered.

The glucose 1-phosphate (glucose-1-P) (Nutritional Biochemicals Corp., Cleveland, Ohio) used in all these experiments contained a sufficient quantity of the coenzyme glucose 1,6-diphosphate (glucose-1,6-diP) (Leloir et al., 1948) for optimal enzyme activity.

The imidazole (Aldrich Chemical Co., Milwaukee, Wis.) used throughout showed no activating effect unless $Mg^{2+}$ was added. Occasional batches exhibited some activation. These were presumed to be contaminated with traces of activating metal ions and were not used further. Water was first distilled, then passed through a Barnstead ion-exchange demineralizer. This was found necessary to remove trace amounts of inhibitory metals. The same assay procedure was used throughout all these studies and was essentially similar to that described earlier (Najjar, 1948). Unless otherwise indicated, the standard reaction was carried out at $30°$ and pH 7.5 in a mixture that contained the following components expressed as $\mu$ moles: imidazole, 20; glucose-1-P, 2.0; $MgCl_2$, 0.5; in a final volume of 0.5 ml. The reaction was followed by estimation of the appearance of acid-stable phosphate. The specific

[1] Abbreviation used in this work: PGM, phosphoglucomutase.