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ACID PHOSPHOMONOESTERASE OF HUMAN PROSTATE

MOLECULAR WEIGHT, DISSOCIATION AND CHEMICAL COMPOSITION

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SUMMARY

Acid phosphomonoesterase I (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2), isolated in highly purified form from human prostate glands, was shown to be homogeneous by sedimentation equilibrium analysis. This was true at pH near 5.5, when the mol. wt. was found to be 102 000, but not in higher or lower pH ranges, where dissociation occurs. Dissociation is favored by extremes of pH, and is accompanied by aggregation of the, presumably unfolded, subunits. Sodium dodecyl sulfate promotes the same pair of effects. Evidence was found for dissociation into two subunits without cleavage of any of the disulfide bonds.

The sedimentation and diffusion coefficients of the native molecule were also measured, and the amino acid composition was determined. The molecule has 16 half-cystine residues, of which 2 appear to be present as cysteine. This enzyme is a glycoprotein, containing 13 residues of neutral sugars, 10 of hexosamine and 6 of *N*-acetylneuraminic acid, per molecule.

INTRODUCTION

Although several versions of the purification of the acid phosphomonoesterase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) of human prostate gland have been reported (for references see ref. 1), a detailed characterization of the molecule has not yet been made. The mol. wt. has been estimated by means of sucrose density centrifugation[1] or gel filtration[2–4], yielding values in the range 96 000–130 000.

Two forms can be separated by DEAE-cellulose chromatography[4–6]. One of these, prostatic acid phosphatase I, has been obtained in a form that is homogeneous in chromatography and gel filtration[1,7]. We have wished to complement catalytic studies on this enzyme in these laboratories by a physical characterization of the protein. We report here ultracentrifugal studies to determine several parameters of

Abbreviations: M_{app}, apparent molecular weight; $\bar{v}$, partial specific volume.

the molecule, together with an analysis of its amino acid and carbohydrate composition. The dissociation behavior of the enzyme has also been studied, in relation to its possible subunit composition.

MATERIALS AND METHODS

Acid phosphomonoesterase I from hypertrophic human prostate glands was purified as described previously[7]. The chromatographically homogeneous material from the final step in the purification[7] was employed for these studies. Sodium dodecyl sulfate was the twice-recrystallized Matheson, Coleman and Bell product. Reagents for polyacrylamide gel electrophoresis[7] were purchased from Canalco Co. β-Mercaptoethanol was freshly distilled from the Eastman product. *N*-Acetylneuraminic acid was from Koch-Light (Colnbrook, England). Other reagents were Certified grade (Fisher) or as specified elsewhere[6,7]. All buffers were made from the reagents indicated, in concentrations such as to contribute a total of 0.02 to the ionic strength, with the remainder of the ionic strength quoted being due to NaCl added. Dialysis tubing (Visking) was extracted in 0.5% $NaHCO_3$ solution at 90°, 30 min, to remove contaminants that adhere to the protein.

Ultracentrifuge measurements

Sedimentation measurements were made in a Spinco Model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm. Double sector cells (12 mm optical path) were used routinely for velocity experiments. Sapphire windows were used in experiments at higher speeds and quartz windows at lower speeds. The plates were analyzed using a Nikon Model 6 micro-comparator, which we have equipped with 6-inch diameter micrometer drums (Shardlow Micrometers, Ltd., Sheffield 4, England) giving readings at 2 μm on each division.

Sedimentation coefficients were determined with a rotor speed of 50 740 rev./min, and were corrected to values in water at 20° ($s_{20,w}$). For the calculation of $s_{20,w}$, the displacement of the maximum ordinate of the schlieren peak as a function of time was determined. Sedimentation equilibrium experiments utilized the 3 mm column technique of VAN HOLDE AND BALDWIN[8].

Densities of solutions employed were determined by pycnometry, in a bath maintained at 20 $\pm$ 0.01°. Protein concentrations were determined in a Hilger Model M154 refractometer with a water-jacket maintained at 20 $\pm$ 0.05°; a constant value for the specific refractive increment of 0.190 ml·g^{-1} for the protein was assumed. This instrument was calibrated using a series of standard KCl solutions in the range 1.00 to 20.00 mg/ml (1- and 2-cm paths) assuming a specific refractive increment of 0.140 ml·g^{-1} at 20°. The protein was dialyzed to equilibrium against the buffer solution for each measurement.

For the calculation of mol.wt., a plot was made of $1/r \cdot dc/dr$ *versus* c. Here, c is the protein concentration in arbitrary units, obtained by trapezoidal integration of the area between the concentration-gradient curve and the base line, and r is the distance from the center of rotation. The mol. wt. was related to the slope of the straight line resulting from the plot, according to the equation (with the usual symbolism):

$$\frac{1}{r} \cdot \frac{dc}{dr} = \frac{M_z(1 - \bar{v}\varrho)\,\omega^2 c}{RT} \qquad (1)$$

The diffusion coefficient was calculated from the rate of approach to equilibrium as described by VAN HOLDE AND BALDWIN[8]. For this purpose a plot of log ε *versus* time, t, was made and the diffusion coefficient, D, calculated, on the basis of the equation[8]:

$$\log \varepsilon = -\frac{D^2 U(\alpha) t}{2.303\,(b-a)^2} + \text{const.} \qquad (2)$$

$$\text{where } \varepsilon = \frac{\Delta c_{eq} - \Delta c_t}{\Delta c_{eq}}$$

$$U(\alpha) = \frac{1}{4\pi^2\alpha^2} + 1$$

$$c = c_b - c_a$$

$$\text{and } \alpha = \frac{c_0}{c_{eq}}$$

Here, b and a, respectively, indicate the distances of the base and meniscus of the solution from the center of rotation, and the subscripts 0, t and eq indicate values at times 0, t and after attainment of equilibrium, respectively. The plot of log ε *versus* time employed values of ε starting with a suitably early photograph (60–90 min) and continuing for 3 to 4 h. The value of log ε at zero time was calculated[8] from Eqn. 3:

$$\log \varepsilon_0 = \log \frac{4\left[1 + \cosh \frac{1}{2\alpha}\right]}{\pi^2 [U(\alpha)]^2} \qquad (3)$$

which corresponds to the constant term on the right-hand side of Eqn. 2.

The value of c_0, the initial protein concentration, was obtained in each case from the area under the schlieren peak, by numerical integration of the pattern from a double sector synthetic boundary cell, after layering the solvent on the solution. A value of $\bar{v} = 0.735$ for the protein was used, obtained from amino acid composition data (see RESULTS).

All the computations indicated above for the ultracentrifuge measurements were carried out making use of an IBM computer 7044 for which a suitable program was devised. This yielded also the standard deviation of the slopes, the mol. wt. and the diffusion coefficient.

The partial specific volume of sodium dodecyl sulfate was taken[9] to be 0.885. Uniform binding of sodium dodecyl sulfate to the protein molecules was assumed and, by implication, a constant specific refractive index increment for the complex.

Amino acid analysis

The protein solution was dialyzed against deionized water (free of O_2) for about 70 h at 4°, and the final concentration was determined precisely (as above) with the refractometer (assuming complete equilibration). To each sample of the protein (0.4–0.8 mg) was added an equal volume of concentrated HCl (using metal-free "ultra-pure" HCl, E. Merck, Darmstadt, Germany, obtained from Brinkmann Instruments, N.Y.) in a Pyrex tube, which was degassed thoroughly at an oil pump

and sealed under vacuum. Hydrolysis was in an oil bath in an oven at 110° ± 1°. After rotary evaporation to dryness, the residue was at once dissolved in the sample buffer for the amino acid analyzer[10] and stored frozen. Analysis was by standard methods[10] using a Beckman Spinco amino acid analyzer. The hydrolyses were conducted for 24, 48 and 72 h for the analyses reported.

The sum of cysteine and half-cystine was determined as cysteic acid by similar hydrolysis and chromatography on the analyzer, on 1 mg samples of the protein oxidized by performic acid following the procedure of HIRS[11], but using a temperature of −5°. Two oxidation periods, 4 and 8 h, were employed on parallel samples. Since the values obtained for cysteic acid in these two cases did not differ significantly, it was deduced that oxidation was complete.

Tryptophan was determined by a version[12] of the colorimetric method of SPIES AND CHAMBERS[13]. Triplicate analyses, in the range of 2 mg of protein, were made, using the modification[14] of the method employing an enzymic predigestion, to obtain the maximal yield of tryptophan color at 590 nm: it was found that 5 h digestion gave this maximum. The tryptophan determination was calibrated using L-tryptophan (Calbiochem, analytical standard grade), for which a calibration plot was constructed and found to be linear in the range used.

Analysis of neutral sugars

The protein (3 mg) in 1 ml of 0.5 M H_2SO_4 was hydrolyzed in a sealed glass tube at 100° for 10 h, and then neutralized by Dowex 2-X8 (bicarbonate form)[15]. The resin was removed by filtration under suction, washed with distilled water, and the total filtrate was evaporated *in vacuo*. The dry residue was dissolved in 0.1 ml water and assayed with the aniline–acetate–phosphate reagent as described by WALBORG AND CHRISTENSON[16], measuring the absorbance at 365 nm. Separation of the individual sugars was performed by descending chromatography on Whatman No. 1 filter paper, using *n*-butanol–pyridine–water (5:3:2, by vol.) as solvent system, and detection with an aniline–phosphate reagent spray[17], followed by densitometry of the paper strip. Identification and quantitation of the peaks was by comparison with samples of the pure sugars treated identically.

Hexosamine and N-acetylneuraminic acid determination

The protein (3.4 mg) was heated at 110° for 16 h in 20 ml of water with 6 g of Dowex 50 (H^+ form) in a sealed glass tube[18]. The resin was removed by filtration and washed several times with water, and then 5 times with small portions of 2 M HCl. The combined HCl washings were evaporated to dryness, and the hexosamine content determined thereon using Ehrlich's reagent[19].

Five samples (~ 2 mg) of the phosphatase were each heated in 2 ml of 5% trichloroacetic acid solution at 100° for 15 min. The released *N*-acetylneuraminic acid was estimated by the thiobarbituric acid method of WARREN[20], using *N*-acetylneuraminic acid as the standard. Ampholine interfered and was absent.

Electrophoresis

Cellulose acetate electrophoresis was carried out on 12 cm × 5 cm Cellogel membranes (Oxo Ltd., London). Each sample was dialyzed against the buffer to be used, and contained dextran (6%), used as a marker of electroosmotic flow in meas-

urements of the electrophoretic mobility[21]. The buffer solutions used were: pH 3.6–5.5, acetate; pH 6.0–6.6, Tris-HCl; pH 6.9–8.3, veronal-HCl; all of ionic strength 0.05. Runs were at 8.35 V/cm, 3 mA per strip, for 3 h at room temperature. For enzyme staining, strips of Whatman No. 1 filter paper were soaked with substrate solution (0.02 M *p*-nitrophenyl phosphate in 0.1 M citrate buffer, pH 5.0) and applied to the wet cellulose acetate strips. After a few minutes, the paper was removed and both strips were sprayed with 0.1 M NaOH. The enzyme zone was marked by a yellow band on both the cellulose acetate and the filter paper strip.

Polyacrylamide gel electrophoresis, and isoelectric focussing, were performed as described elsewhere[6].

RESULTS

Electrophoretic characterization of prostatic acid phosphatase I

The chromatographically pure acid phosphatase I preparation described in the preceding paper[7] was examined, firstly, in cellulose acetate electrophoresis. The

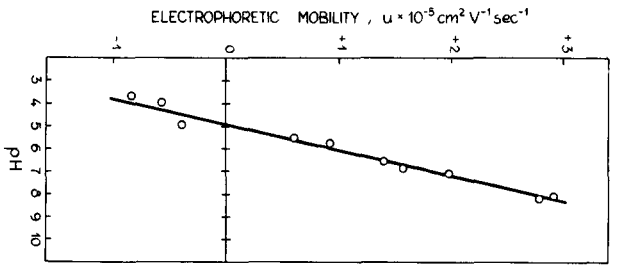


Fig. 1. Electrophoretic mobility of acid phosphatase I on cellulose acetate. The migration of the single band revealed by enzyme activity staining was measured as a function of pH.

enzyme migrated as a single, sharp, active band, at a series of pH values from 3.6 to 8.3. A plot of the anodal and cathodal mobilities as a function of pH (Fig. 1) showed an isoelectric point (at $I = 0.05$) of pH 4.9. In polyacrylamide disc gel electrophoresis, at pH 8.5 or pH 4.0, apparent homogeneity was again seen. However, upon isoelectric focussing[6,22] of the phosphatase I, two overlapping peaks of enzyme activity and (closely corresponding) protein could be discerned, with isoelectric points of pH 4.80 and 4.90. The profile obtained in this pH region has been illustrated previously[6] for a rather less pure preparation, the additional minor peaks seen there being absent here (except for a very minor active component at pH 5.15). Since the two major peaks are roughly equal in amount, the uniform behavior in gel filtration[7] shows that they have very similar molecular weights.

Carbohydrate content

N-Acetylneuraminic acid, neutral sugars and amino sugars were each determined on the acid phosphatase I sample (Table I). Considerable numbers of each are present. The individual neutral sugars were identified by paper chromatography and densitometry (Fig. 2). By elution and estimation[16] of the sugar in each band, the proportions of the four neutral sugars present were determined (Table I).

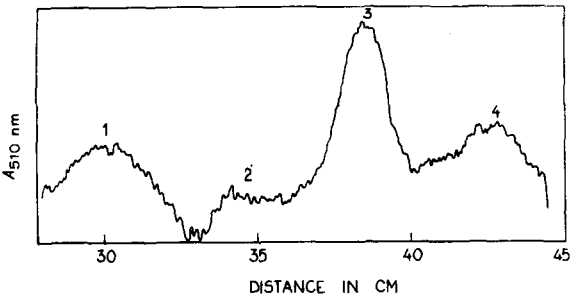


Fig.2. A typical tracing of the absorbance at 510 nm ($A_{510\ nm}$) of a developed[17] chromatogram after separation of the neutral sugars in a hydrolyzate of acid phosphatase I. 1, galactose; 2, glucose; 3, mannose; 4, fucose.

Amino acid composition

The consistent results of 4 series of analyses of the protein were used in calculating the mean amino acid composition (Table II). These values include the corrections applied for the destruction of threonine and serine; the zero-time values were deduced from the decreases from 26 to 48 to 72 h of hydrolysis, assuming a first-order rate of loss. For isoleucine, the values increased slightly with the period of hydrolysis, as is generally found, and the 72-h hydrolysis value was taken as the best approximation. No other corrections were applied. The sum of cysteine and half-cystine was determined separately by a performic acid oxidation and measurement of cysteic acid content. Tryptophan was determined by a colorimetric procedure[12] on an enzymic digest[14] of the protein.

TABLE I

CARBOHYDRATE ANALYSIS OF PROSTATIC ACID PHOSPHATASE I

Carbohydrate	*Content (g/mole)*	*Residues per molecule*
N-Acetylneuraminic acid	1825	5.9 ± 0.9
Hexosamine	1837	10.2
Neutral sugars:	2416	13.0
Mannose 39%		
Glucose 9%		
Fucose 30%		
Galactose 22%		
Total	6078	29.1

A total of about 764 amino acid residues was found to be present (Table II), giving a molecular weight of about 89 000. The difference of 13 000 between this value and the higher molecular weight found by sedimentation equilibrium measurement (see below), is accounted for (within the accuracy of these various measurements) by the weight of the carbohydrate present (Table I).

TABLE II

AMINO ACID COMPOSITION

Expts. I and II each comprised a time series (see text) of hydrolyses. The corrections (see text) to the values for serine, threonine and isoleucine were applied, the mean (or corrected mean) of the values in each experiment being recorded, and the overall mean is rounded to the nearest integer (4th column). Two further replicate analyses of samples (without cysteine or tryptophan determinations) were also performed, giving results within the range shown here for Samples I and II. Cysteine represents the mean of two determinations of cysteic acid after oxidation. W_i, the total weight contribution of the residue of type i, and V_i, its specific volume[23], were used to calculate $\bar{v}$, using $\bar{v} = \Sigma V_i W_i / \Sigma W_i$. Aspartic acid and glutamic acid are each the sum for the acid and its amide. W_i values were calculated separately for the amides (for the $\bar{v}$ calculation) taking arbitrarily the amide contribution to be equal to the acid contribution.

Amino acid	*Residues found per molecule*		*Nearest integral number of residues*	*Weight contributions*	
	I	*II*		W_i	$V_i W_i$
Aspartic acid	52.8	56.0	54	3107	1864
Asparagine				3107	1926
Threonine	49.4	49.7	50	5056	3539
Serine	57.1	51.6	54	4703	2962
Glutamic acid	97.6	102.1	100	6456	4261
Glutamine				6456	4326
Proline	49.4	50.7	50	4856	3691
Glycine	41.0	42.3	42	2397	1534
Alanine	26.9	28.0	27	1919	1420
Valine	32.9	35.2	34	3371	2899
Methionine	19.2	21.5	20	2604	1953
Isoleucine	25.7	28.4	27	3056	2750
Leucine	90.1	96.8	93	10525	9472
Tyrosine	42.6	44.0	43	7017	4982
Phenylalanine	31.2	33.7	32	4710	3627
Lysine	44.4	46.5	45	5769	4731
Histidine	26.8	26.5	26	3566	2389
Arginine	32.7	33.4	33	5155	3608
Tryptophan	17.9	18.1	18	3352	2480
Cysteine	15.9		16	1634	997
Total			764	88 816	$\bar{v} = 0.735$

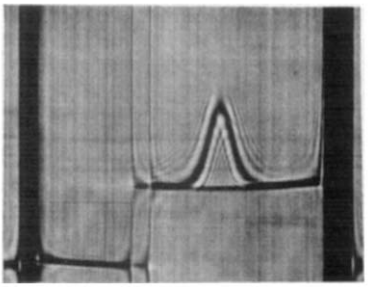
The partial specific volume, $\bar{v}$, was calculated[23] from the amino acid composition data, giving a value of 0.735. This does not take into account the effect of the carbohydrate.

Sedimentation studies

Sedimentation at pH 7.4. In phosphate buffer, pH 7.4, acid phosphatase I showed a single and very symmetrical boundary (Fig. 3). The sedimentation coefficient decreased with increasing protein concentration (c, in mg/100 ml), and from a series of such experiments (Fig. 4) it was found that the values could be fitted by a linear relationship:

$$s_{20,w} = 5.65\ (1 - 0.13c)\quad \text{S} \tag{4}$$

This small dependence of sedimentation velocity upon concentration indicates that the molecule is a compact globular protein. The negative slope shows that dissociation of the protein is absent or very minor under these conditions.



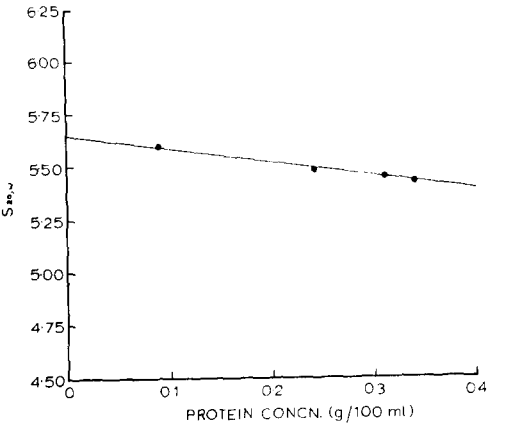


Fig. 3. Representative schlieren pattern of prostatic phosphomonoesterase I sedimented at 52 640 rev./min in phosphate buffer at pH 7.4, $I = 0.1$, 8°. Photograph was taken with a bar angle of 45° after 68 min. The initial protein concentration was 0.34 g/100 ml.

Fig. 4. Plot of $s_{20,w}$ *versus* initial protein concentration for prostatic phosphomonoesterase I in phosphate buffer, pH 7.4, $I = 0.1$.

Four different preparations of the enzyme were employed in nine sedimentation equilibrium experiments conducted at pH 7.4 (phosphate buffer, $I = 0.1$). These gave apparently linear plots (Fig. 5A) throughout almost the entire column, but with an upward curvature in the region corresponding to the base of the cell. The mol. wt. calculated for the linear range for each of these preparations gave consistent values, in the range 97 000 ± 3500.

Dissociation at pH 10.4. Sedimentation equilibrium experiments at pH 10.4

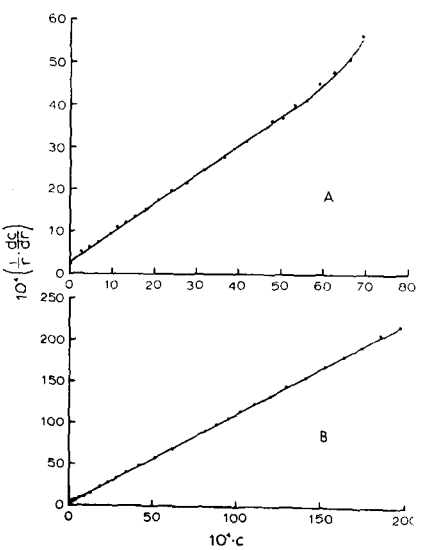


Fig. 5. Sedimentation equilibrium plots for prostatic phosphomonoesterase I at pH 7.4 (A) and at pH 5.5 (B), at 5°.

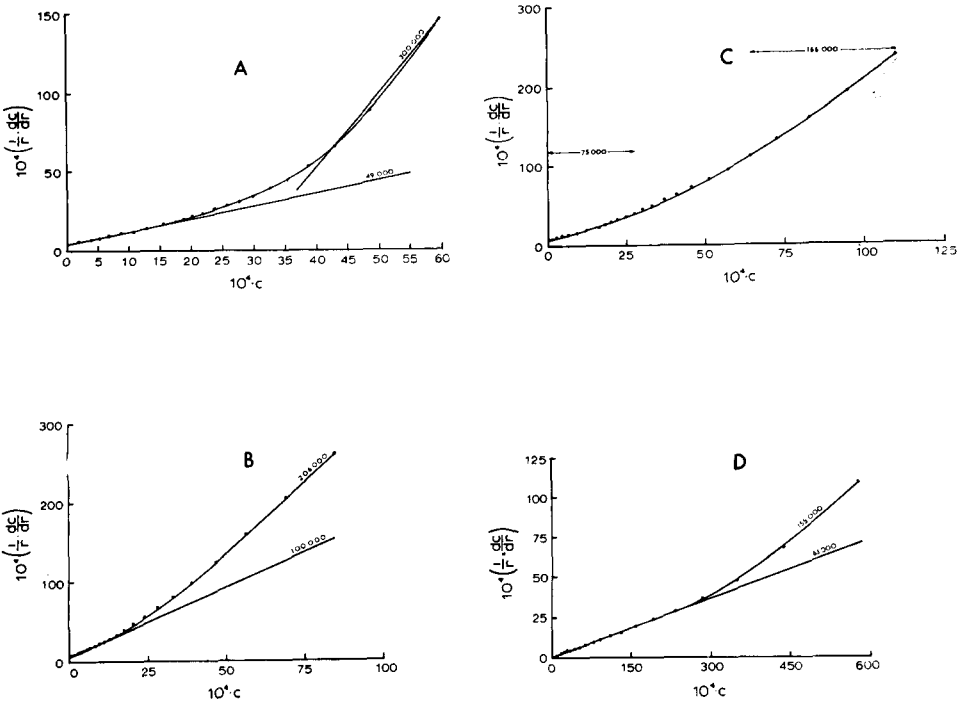


Fig. 6. Sedimentation equilibrium plots of prostatic phosphomonoesterase I (at 5°) in 0.01 M HCl (A); or glycine–NaOH–NaCl buffer, pH 10.4, $I = 0.4$ (B) or $I = 0.1$ (D); or after treatment with sodium dodecyl sulfate at pH 7.0 in phosphate buffer (C). In A only, $5 \cdot 10^{-2}$ M mercaptoethanol was present.

indicated that the protein had undergone extensive dissociation. In an appropriate plot (Fig. 6D), in addition to a linear section extending from the meniscus region through at least three-fourths of the solution column, there was a sharp increase in the slope corresponding to the region at the base of the cell, giving an upward curvature. The mol. wt. calculated from the slope of the linear section gave a value of $M_{app} = 63$ 000, whereas the mol. wt. calculated from the slope corresponding to the base of the cell indicated a value of $M_{app} = 155$ 000.

The behavior of the protein at pH 10.4, $I = 0.4$, was similar to that at $I = 0.1$ (Fig. 6) but the apparent molecular weights at corresponding levels of the solution were substantially higher than at $I = 0.1$. Thus, the M_{app} in the top half of the cell was approx. 100 000 and near the base of the cell it was 206 000.

Dissociation at pH 2.0. An equilibrium experiment in 10^{-2} M HCl resulted in a plot showing an upward curvature (Fig. 6A). The initial, nearly linear section of the plot corresponded to less than half the length of the liquid column. The slope of this section of the plot indicated an average molecular weight for this region of the cell of about 49 000, whereas the molecular weight near the base of the cell was $M_{app} =$ 300 000. Hence, in acid there apparently occur simultaneous dissociation and aggregation. Mercaptoethanol ($5 \cdot 10^{-2}$ M) was present, in this experiment only.

Influence of sodium dodecyl sulfate. One experiment was carried out to investigate the possibility of dissociating the protein completely by the binding[25] of the

detergent, sodium dodecyl sulfate. For this purpose a 2:1 (w/w) ratio of protein to sodium dodecyl sulfate was taken at pH 7.4 (phosphate buffer) at 5°, and a sedimentation velocity run was first made to establish the proportion of unbound sodium dodecyl sulfate. Since no indication was seen of the presence of a boundary corresponding to the sodium dodecyl sulfate micelles (as is obtained in a control sedimentation of the sodium dodecyl sulfate alone), it was assumed that all the sodium dodecyl sulfate was bound to the protein. This assumption was used in the subsequent sedimentation equilibrium analysis to correct the effective mass of the sedimenting unit according to the equation:

$$M_3 (1 - \bar{v}_3\varrho) = M_1 [(1 - \bar{v}_1\varrho) + 0.5 (1 - \bar{v}_2\varrho)] \quad (5)$$

where the subscripts 1, 2 and 3, respectively, indicate the free protein, sodium dodecyl sulfate and the protein–sodium dodecyl sulfate complex. Thus, the buoyancy term in Eqn. 1 was replaced by the term in square brackets on the right hand side of Eqn. 5.

The effect of adding sodium dodecyl sulfate was to produce some dissociation in addition to some aggregation (Fig. 6C). In the meniscus half of the solution column M_{app} was 75 000, whereas near the bottom of the solution M_{app} was about 166 000. Thus, it can be seen that although the effect of sodium dodecyl sulfate in this experiment was small in comparison with its strong dissociating effect on many other proteins[25,26], nevertheless it was in line with the results obtained at acid and at alkaline pH.

Molecular weight of the undissociated protein. Since the protein had shown a tendency to dissociate on increasing the pH from pH 7.4 to pH 10.4, the molecular weight obtained at pH 7.4 could not be taken with certainty as the molecular weight of a fully associated and monodisperse system. Further study was carried out at pH 5.5 and 6.58 in acetate and phosphate buffer ($I = 0.1$), respectively. At these two pH values the equilibrium plots (in two experiments in each buffer) were linear from

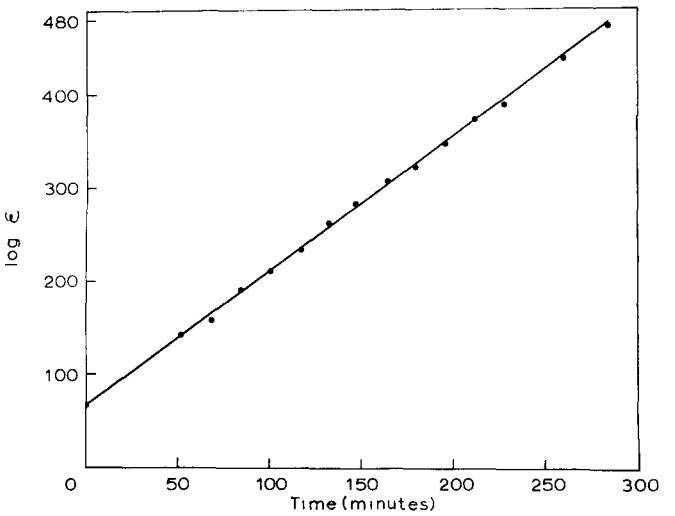


Fig. 7. Plot of log ε *versus* time, used to obtain the diffusion coefficient from the approach to sedimentation equilibrium, at 5°, pH 5.5 (acetate, $I = 0.1$).

the meniscus to the base of the solution (Fig. 5B), and the molecular weight (to the nearest 1000, calculated from the slope of the straight lines) was 102 000 (pH 5.50) or 105 000 (pH 6.58). Since no heterogeneity was detectable here, and since the method[8] used for molecular weight determination accentuates the contribution of very small amounts of higher aggregates, it was concluded that the true molecular weight is close to 102 000, and that at pH 6.6 there is the commencement of the aggregation discerned clearly at pH 7.4 (Fig. 5).

Two determinations of the diffusion coefficient (Fig. 7) at pH 5.50 gave, respectively, $5.45 \cdot 10^{-7}$ and $5.38 \cdot 10^{-7}$ $cm^2 \cdot sec^{-1}$. The frictional coefficient was calculated using standard equations[24] based on the molecular weight and either $s^0_{20,w}$ or $D_{20,w}$, which gave values of 1.34 or 1.29, respectively. This corresponds to an axial ratio which, depending upon hydration and shape factors, would be in the range 4–6.

DISCUSSION

The results indicate that prostatic phosphomonoesterase I exists in solution as a species with molecular weight 102 000, at pH values about 5–6. It is a glycoprotein, with slight heterogeneity in the carbohydrate fraction. This heterogeneity does not appreciably affect the molecular weight of the components, but gives rise to the slight charge difference between the two major components. Since the phosphatase activity closely corresponded to the protein profile in the two peaks partly resolved by isoelectric focussing[6], the two components of different carbohydrate content have essentially the same specific enzymic activity.

The large amount of *N*-acetylneuraminic acid accounts for the low isoelectric point, 4.9. 80% of it can be removed by a neuraminidase, removing the heterogeneity and not affecting the enzymic activity[4,6]. It is not yet clear if the resistant fraction of *N*-acetylneuraminic acid and other carbohydrate plays any role in the catalytic activity.

We have found that this protein tends to dissociate into smaller molecular species on increasing the pH to pH 10.4 or on lowering it to pH 2.0. In addition, a tendency to form aggregates of the original molecule is well apparent at both extreme pH values. This tendency towards aggregation is enhanced by increasing the ionic strength. Thus, at pH 10.4, by increasing the ionic strength from 0.1 to 0.4, the M_{app} in the meniscus half of the solution was raised from 63 000 to 100 000, and near the bottom of the solution M_{app} increased simultaneously from 155 000 to 206 000. Results at pH 2.0 lead to the same conclusion as at pH 10.4, that is, there are concomitant tendencies to dissociation and to aggregation (Fig. 6).

These tendencies to dissociation and to aggregation are difficult to explain as resulting from the same primary cause, *e.g.* increase in the net charge of the protein molecule leading to increased repulsion. It appears more likely that there is an unfolding of the tertiary structure as the charge increases, eventually permitting aggregation by hydrophobic bonding of the newly exposed groups. The results of the experiment with sodium dodecyl sulfate lend support to this interpretation, since the binding of sodium dodecyl sulfate produced a restricted dissociation and a more pronounced aggregation. The increase in aggregation when the ionic strength is higher, *i.e.* when charges are more screened, is also consistent with this view. The

unchanged result with mercaptoethanol suggests that SH oxidation is not the cause of the aggregation.

The amino acid composition reveals no particular abnormality. The value of 16 half-cystine residues per molecule by analysis for cysteic acid, can be compared with the value of 20 per molecule estimated from amperometric titration (after sulfitolysis) by DOMANSKI *et al.*[27]. Those authors found that two SH groups could be (after denaturation and without sulfite) titrated directly, the others being apparently in disulfide bridges. Since most of our dissociations were observed in the absence of mercaptoethanol, our results show that a separation at least to the stage of approximate half-molecules can occur without breakage of any of the disulfide bonds. The occurrence of both SH and SS groups in the same protein molecule is uncommon, and the subunit structure of this enzyme merits further investigation.

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