# Yeast Hexokinase. II. Molecular Weight and Dissociation Behavior*

Moisés Derechin, Armin H. Ramel,† Norman R. Lazarus, and Eric A. Barnard

ABSTRACT: A highly purified native hexokinase preparation from yeast has been studied. In acetate buffer at pH 5.5, it appears to exist as a homogeneous dimer of molecular weight 99,000. Complete dissociation of the dimer into two monomers (50,000) was achieved in glycine–NaOH–NaCl buffer at pH 10.4, $I = 0.1$. At pH values intermediate between 5.5 and 10.4, partial reversible dissociation of the dimer into monomers takes place, and no resolution into separate boundaries was observed except after an attack on the protein by proteolytic enzymes from the original yeast extract. A complex hydrodynamic behavior has been observed at pH 2.1, due to charge effects and ion binding, but dissociation to monomers there, also, was deduced. No species smaller than a monomer was formed from the pure enzyme at pH values from 2.1 to 10.4.

The first paper of this series (Lazarus *et al.*, 1966) described a new procedure for preparation of hexokinase from baker's yeast, in conditions designed to avoid the proteolytic partial degradation of this enzyme that is otherwise incurred. Thus it was found that two forms (designated A and B) of hexokinase are isolated. Each was shown to be distinct and homogeneous, by the criteria of chromatographic behavior and electrophoreses on cellulose acetate and on polyacrylamide gel. For the purposes of active center studies on this enzyme (which are now in progress), as well as for the intrinsic physicobiochemical interest of the system, it is important first to characterize the material by physical analyses. A number of questions raised by the new preparation need to be answered. (i) What is the molecular weight of native hexokinase? Does it contain subunits, and if so, are they held together by purely noncovalent bonds? (ii) What is the association–dissociation behavior of the protein across the pH range in which active center studies might be conducted? (iii) Does hexokinase A (with about one-quarter of the specific enzymic activity of hexokinase B) differ in size, or in subunit composition, from hexokinase B? (iv) Are these species truly homogeneous by other criteria? (v) How does this material differ from yeast hexokinase isolated by previous methods (which involve considerable autolysis)?

For this last question, we should note that some such differences have already been demonstrated (Lazarus *et al.*, 1966). Previously reported preparations have, in contrast to the present material, lower specific enzymic activities, markedly lower stability, a multiplicity of chromatographically separable active forms (Trayser and Colowick, 1961),[1] and a different carbohydrate spectrum of substrate specificity. As regards the size of the hexokinase molecule, previous preparations characterized have shown (at pH 5–7) material in one of two narrow molecular weight ranges: (a), 93,000 (Ramel *et al.*, 1961, 1963), or 95,000 (Kenkare and Colowick, 1965), or about 96,000 (Kunitz and McDonald, 1946); (b), 46,000 (Kenkare and Colowick, 1965), or 47,000 (Ramel *et al.*, 1963), or 50,000 (Ågren *et al.*, 1963). Hexokinases of each of these two types actually coexist (as the main active species present, at neutral pH and low ionic strength) in preparations made by the standard (Darrow and Colowick, 1962) method, as has been shown by ultracentrifugal and chromatographic analysis of such material (Ramel *et al.*, 1963).[2] It should be noted that the latter studies established that these two forms are not in a mutual equilibrium there; they are present as two distinct, noninterconvertible species. The smaller can be formed (irreversibly) from the larger by treatment with proteases (Kaji *et al.*, 1961; Ramel, 1964) and it appears to arise similarly by partial proteolysis during hexokinase preparation by the methods previously in use.

Hence, it is important in any isolation of hexokinase to determine if any multiple forms present are due to proteolytic attack, and to distinguish such a case from that of association–dissociation equilibria. It is shown in the present report that the former case is absent in the new preparation, but that a single species of un-

* From the Molecular Enzymology Unit, Departments of Biochemistry and Biochemical Pharmacology, State University of New York, Buffalo 14, New York. *Received August 18, 1966.* This work was supported by Grant GM-11603 from the National Institutes of Health, U. S. Public Health Service.

† On leave of absence from the Physical Chemical Institute, University of Basel, Basel, Switzerland.

[1] Our designation of hexokinases as A and B is made anew for the present preparation; these species are not equivalent to those named similarly (Trayser and Colowick, 1961; Ramel *et al.*, 1963) as forms present in previous preparations.

[2] A fuller account of this characterization of hexokinase species present in material prepared by a modification of the method of Darrow and Colowick (1962) will be presented elsewhere (A. H. Ramel and H. K. Schachman, in preparation).

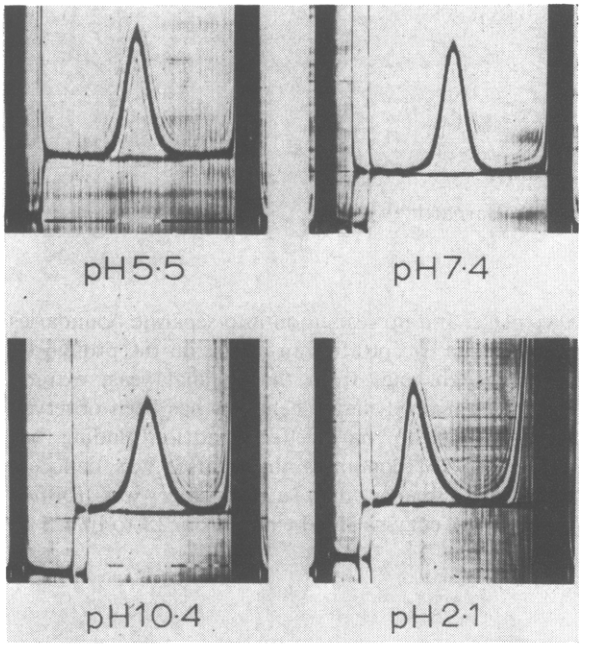


FIGURE 1: Representative schlieren patterns of hexokinase B sedimented at 50,740 rpm at the pH values shown, all at an ionic strength of 0.10 except the pH 2.1 medium. The media were: sodium acetate–sodium chloride, pH 5.5, 6°, 0.74 g of protein/100 ml; sodium phosphate–sodium chloride, pH 7.4, 20°, 0.79 g of protein/100 ml; glycine–NaOH–NaCl, pH 10.4, 5°, at 0.76 g of protein/100 ml; and 0.01 M HCl, pH 2.1, 7°, 0.63 g of protein/100 ml. Photographs were taken with a bar angle of 45° after (in the same order) 136, 88, 176, and 241 min at 50,740 rpm.

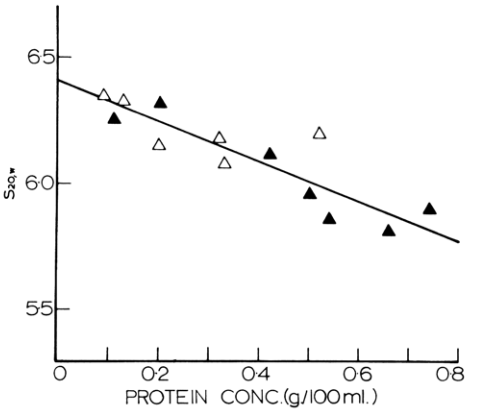


FIGURE 2: Plot of $s_{20,w}$ *vs.* initial protein concentration, for hexokinase B in sodium acetate–sodium chloride, pH 5.5, $I = 0.10$. Open symbols, runs at 20°; filled symbols, runs at 5–7°.

degraded hexokinase B which is present can, however, undergo reversible dissociation to half-molecules. An approach to each of questions i–v above is initiated in the present report.

## Materials and Methods

Hexokinase preparations employed were those described in the preceding paper (Lazarus *et al.*, 1966). The material used was rechromatographed and had a specific enzymic activi y in the range 700–800 units/mg of protein (measured at 25°), and was completely stable (giving no activity loss) when stored for at least 1 week at 4°. Adjustment of the protein to each of the media reported upon was by dialysis (24 hr or more) at 4° against several changes of solvent.

β-Mercaptoethanol was the Eastman product. Other reagents were certified grade (Fisher).

Sedimentation measurements were made in a Spinco Model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm and a rotatable light source for Rayleigh interference optics. 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 microcomparator, which we have equipped with 6-in. diameter micrometer drums (Shardlow Micrometers Ltd., Sheffield 4, England) giving readings to 2 μ on each division. Sedimentation coefficients were determined with a rotor speed of 50,740 rpm, and were corrected to values in water at 20° ($s_{20,w}$).

For the calculation of the sedimentation coefficient, the displacement of the maximum ordinate of the schlieren peak as a function of time was estimated. Sedimentation equilibrium experiments utilized the 1-mm column technique of Yphantis (1960) *or* the 3-mm column technique of Van Holde and Baldwin (1958). Molecular weights were obtained from graphs of $1/r \cdot dc/dr$ *vs.* $c$, where $c$ is the protein concentration (in arbitrary units, obtained from numerical integration of the area between the concentration–gradient curve and the base line) and $r$ is the distance from the center of rotation, assuming $\bar{v}$ to be 0.740. For all graphical determinations referred to in the text, the slope and intercepts were obtained by least-squares fitting.

Densities of solutions employed were determined by pycnometry, in a bath maintained at 20 ± 0.01°. Protein concentrations were determined in a Hilger Model M154 refractometer with a water jacket maintained at 20 ± 0.05°; a 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 0.10–1.50 (10-cm path) and 1.00–20.00 mg/ml (1- and 2-cm paths) assuming a specific refractive increment of 0.140 ml $g^{-1}$ at 20°. The same methods were used in determining the molar extinction coefficient of the protein, in conjunction with spectrophotometry on a Cary Model 15 instrument.

Enzyme assays and other methods were as described previously (Lazarus *et al.*, 1966). In the buffers used, unless otherwise noted, 80% of the ionic strength

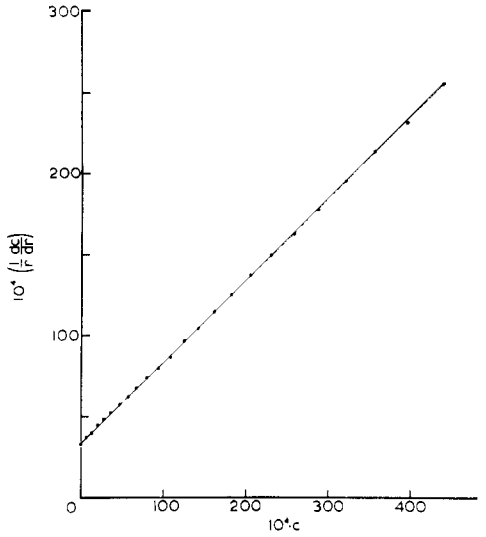


FIGURE 3: A typical sedimentation equilibrium plot for hexokinase B in sodium acetate–sodium chloride, pH 5.5, $I$ = 0.10, 6°, in a 3-mm solution column.

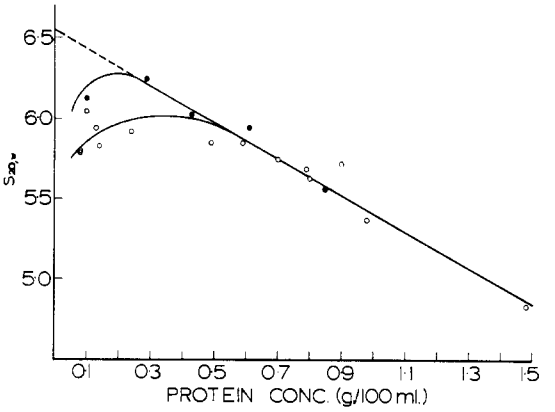


FIGURE 4: Plot of $s_{20,w}$ *vs.* initial protein concentration, for hexokinase B in sodium phosphate–sodium chloride, pH 7.4, $I$ = 0.10. Open symbols, runs at 20°; filled symbols, runs at 5–7°. The extrapolation of the linear segment (fitted to both sets of points for the appropriate sections) gives $s^0_{20,w}$ = 6.54 S.

stated is always contributed by NaCl. In buffers at pH 2.1 and 10.4, β-mercaptoethanol was always present, at $10^{-3}$ M concentration.

## Results

*Sedimentation at pH 5.5.* At pH 5.5 ($I$ = 0.10) hexokinase B in solution showed a single sedimenting boundary (Figure 1). The schlieren patterns were symmetrical, except at the edges of the curve, a behavior to be expected (Fujita, 1956) due to self-sharpening in a protein concentration-dependent sedimentation. The sedimentation coefficient decreased with increasing protein concentration (Figure 2) and can be represented closely by the equation $s_{20,w}$ = 6.41 S (1 − 0.12$c$), where $c$ is the protein concentration in g/100 ml. The slope of $s_{20,w}$ *vs.* $c$ was negative within the range of $c$ studied (0.09–0.9 g/100 ml), both in the cold (5–7°) and at 20°, thus excluding the presence of association–dissociation equilibria.

The sedimentation equilibrium plot (Figure 3) gave a value for the molecular weight of hexokinase B of 99,000 and showed that the preparation was homogeneous.

*Sedimentation at pH 7.4.* In phosphate buffer at pH 7.4 ($I$ = 0.10), hexokinase B showed (Figure 1) a single sedimenting boundary. The values of the sedimentation coefficient were similar to those at pH 5.5 when the initial protein concentration was above 0.5 g/100 ml at 20° or above 0.2 g/100 ml at 5–7° (Figure 4). Below these concentrations, a decrease in the sedimentation coefficient on dilution was observed, indicating the presence of an association–dissociation equilibrium.

The dependence of sedimentation coefficient with concentration (Figure 3) calculated for enzyme solutions above 0.2 g/100 ml at 5–7° and above 0.5 g/100 ml at 20° could be represented closely by the equation $s_{20,w}$ = 6.54 S (1 − 0.17$c$). Thus, the extrapolated value at infinite dilution is very similar to that obtained at pH 5.5 and a slightly greater dependence of $s$ on concentration was found.

The molecular weight (Table I) obtained by means of the Archibald (1947) procedure was 93,500. A sedimentation equilibrium plot, in an experiment at 0.67 g/100 ml of protein concentration, was linear throughout most of the liquid column but showed a slight upward curvature near the bottom of the cell. The molecular weight, calculated from the slope of the linear portion in this experiment, was 90,300. Hence some dissociation appears at pH 7.4 at these protein concentrations.

TABLE I: Molecular Weights Obtained for Hexokinase B under Various Conditions.[a]

| pH | Protein Concn (g/100 ml) | Method | Mol Wt |
|---|---|---|---|
| 5.5 | 0.44 | SE[b] | 99,000 |
| 7.4 | 0.67 | SE[b] | 90,300 |
| 7.4 | 0.90 | Archibald | 93,500 |
| 10.4 | 0.70 | SE[b] | 49,000 |
| 10.4 | 0.57 | SE[c] | 47,000 |

[a] Ionic strength was always 0.1, and temperature 5–7°; buffer compositions as given for Figure 1. [b] SE = sedimentation equilibrium; 3-mm solution column. [c] 1-mm solution column.

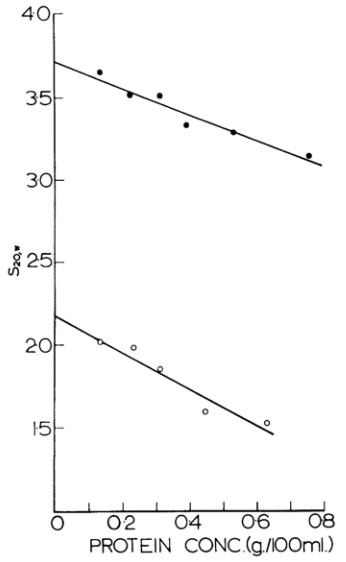


FIGURE 5: Plot of $s_{20,w}$ *vs.* initial protein concentration, for hexokinase B at pH 10.4 (filled symbols; glycine–NaOH–NaCl buffer, $I = 0.10$) or 2.1 (open symbols; 0.01 M HCl, without added salt).

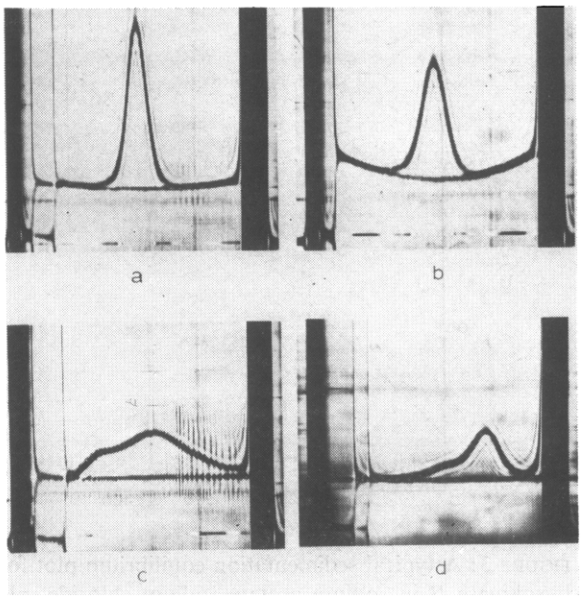


FIGURE 6: Schlieren patterns of hexokinase B sedimented under various conditions: (a) sodium phosphate, pH 7.0, $I = 0.10$, 5°; (b) medium as in a, but with sodium sulfate added to give $I = 0.40$, 5°; (c) HCl solution at pH 2.8, containing sodium sulfate to give $I = 0.03$; 6°; (a–c) each with 0.74 g of protein/100 ml; (d) a hexokinase B preparation that gave initially normal behavior (at pH 7.4) but retaining some uninhibited protease (in an amount not detectable in the original schlieren pattern for this sample) is shown, after 4-days storage at 4° in sodium phosphate–sodium chloride, pH 7.4, $I = 0.10$, in the third of three sedimentation velocity runs made at 20° during this period. The protein in d is at 0.59 g/100 ml. Photographs were taken after 128, 160, 48, and 112 min at 50,740 rpm, in a–d, using bar angles of 45, 50, 45, and 50°.

*Sedimentation at pH 10.4.* In glycine–NaOH–NaCl buffer, pH 10.4 ($I = 0.10$), hexokinase B showed a single symmetrical boundary (Figure 1). The sedimentation coefficient decreased with increasing protein concentration (Figure 5) according to the relation $s_{20,w} = 3.71\,(1 - 0.21c)$.

Molecular weight determinations (Table I) by the short-column equilibrium method of Yphantis (1960) at pH 10.4 gave values between 46,000 and 50,000 (at protein concentrations in the range 0.30–0.80 g/100 ml) while a value of 49,000 was obtained with the 3-mm column equilibrium method of Van Holde and Baldwin (1958) (at a protein concentration of 0.7 g/100 ml). Further, the plot of $1/r \cdot dc/dr$ *vs.* $c$ was strictly linear, indicating the homogeneity of the preparation.

*Effects of Proteolysis.* Kenkare and Colowick (1965) showed that, at pH 7 ($I = 0.1$) and protein concentration 0.8 g/100 ml, their preparation of hexokinase had two distinct components in the ultracentrifuge, with sedimentation coefficients $s_{20,w}$ of 5.3 and 3.2 S, respectively. A similar behavior, with the existence of two species not in equilibrium, has been noted by Ramel *et al.* (1963; *cf.* footnote 2) for material prepared essentially by the procedure of Darrow and Colowick (1962). In a second experiment at pH 7, Kenkare and Colowick observed the complete transformation by higher ionic strength of the faster peak into a slower one having $s_{20,w} = 3.3$ S. When the hexokinase B preparation used in the present work was tested in the conditions and with the reagents used by Kenkare and Colowick (1965), it showed a single boundary sedimenting with $s_{20,w} = 5.85$ S, with no indication of any slower component (Figure 6a, b). This sedimentation coefficient falls within the range to be expected from our other studies at pH 5.5 and 7.4.

It is of interest to enquire whether hexokinase B can give rise to a similar slower sedimenting species (which, in the other material, had appeared to be about half the original molecular weight), under the influence of the yeast proteases. It was found that a sample of hexokinase B for which, atypically, DFP treatment had been omitted at stages after the first chromatographic step (see Lazarus *et al.*, 1966) and which therefore contained a small amount of firmly held proteolytically active material, gave information on this point. When sedimented at pH 7.4 ($I = 0.1$), after remaining in solution for 1–4 days at 4°, this sample showed two components, with $s_{20,w} = 5.75$ and 3.9 S (Figure 6d). It is interesting to note that the specimen that gave this pattern contained, when examined immediately after preparation, a single boundary, with $s_{20,w} = 5.7$ S, and progressively developed the slower component while being reexamined in several sedimentation velocity experiments at 20°,

with storage at 4° between these. After the 4th day of this treatment, the slow component amounted to about 30% of the whole. Such a behavior has never been observed in the other hexokinase preparations, which were (Lazarus *et al.*, 1966) apparently free from proteolytic contamination.

In a further test of the effect of the yeast proteases, a small amount of the crude extract obtained in the first stages of the preparation (Lazarus *et al.*, 1966) before DFP treatment, was added to a normal, stable sample of purified hexokinase B. This addition introduced a relatively insignificant amount of protein, but a strong potential proteolytic activity. Sedimentation velocity experiments were then performed at pH 7.0, 20° ($I$ = 0.1), at 0.59 g of protein/100 ml. The hexokinase, which orginally migrated as a peak with $s_{20,w}$ about 5.8 S, gave after 2-hr incubation at pH 7.0 at 30° a smaller area under that peak, together with a continuum of more slowly sedimenting material, extending to the cell meniscus. A similar experiment with incubation with a smaller amount of the same protease-containing mixture to a point where the lightweight material produced was only just discernible also showed only that continuum and the original 5.8-S peak. At no time was any new, distinct sedimenting peak formed in addition to the original one. Hence no intermediate half-molecule can be detected as a stable form as a result of the action of the combined attack of the proteases present in the initial extract containing hexokinase. It appears, however, that a small protease fraction with affinity for hexokinase exists in that extract, and can be purified with the hexokinase if this protease is not inactivated by DFP treatment. This fraction, from this and the previous observations cited on the standard preparations, appears to be able to give a limited cleavage to partly active forms.

*Sedimentation in HCl Solutions.* Experiments using hexokinase B in 0.01 N HCl (with no added salt) showed a single, slowly sedimenting boundary (Figure 1), with $s_{20,w}$ = 1.5 S (at 0.63 g of protein/100 ml). The normal addition of neutral salt to damp out the charge effects that complicate hydrodynamic studies, itself led to complications in this case. However, it should be noted that for such a case, with a protein of isoelectric point near pH 4.6, in this acid medium a high positive net charge must be present on the molecule, and the very low value of $s_{20,w}$ is no guide to the size of the species present.

On increasing the ionic strength (by addition of NaCl) at a constant pH of 2.1, sedimentations produced schlieren patterns showing faster sedimenting, multiple boundaries (Figure 7). Despite the poor resolution of these boundaries, approximate sedimentation coefficients were calculated for them, in the series of experiments at about 0.6 g of protein/100 ml. Thus, at $I$ = 0.09, three boundaries with $s_{20,w}$ = 5.7, 4.0, and 2.1 S, respectively, were obtained; at $I$ = 0.07, two boundaries with $s_{20,w}$ = 3.9 and 2.6 S were present, as well as a small amount of faster moving material (the major proportion of the total material being contained in the 2.6-S peak). At $I$ = 0.05, one major boundary with $s_{20,w}$ = 2.06 S and a small unresolved boundary on the leading edge of the pattern could be seen. At $I$ = 0.03 and 0.01, a single boundary with $s_{20,w}$ = 1.5 S was seen, with apparent homogeneity being attained near $I$ = 0.01.

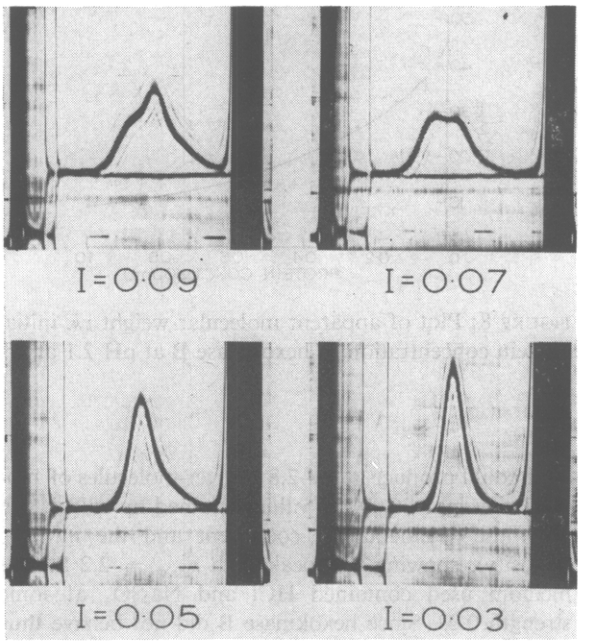


FIGURE 7: Schlieren patterns of hexokinase B (at 0.96 g/100 ml) sedimented at 5°, in 0.01 N HCl containing NaCl to give the ionic strengths indicated. Photographs were taken with a bar angle of 45° after 224, 192, 288, and 288 min at 50,740 rpm, for $I$ = 0.09, 0.07, 0.05, and 0.03 runs, respectively.

The forms present in these media were interconvertible. Thus, after a sedimentation at pH 2.1, $I$ = 0.09, producing a pattern identical with that in Figure 7 (top left), the material was on one occasion dialyzed against 0.01 N HCl (without salt) and recentrifuged. The normal pattern for the latter medium (Figure 7, bottom right) was then obtained.

The dependence of the sedimentation coefficient upon protein concentration (Figure 5), obtained in a series of experiments in 0.01 N HCl (with no added salt), could be represented approximately by a linear relationship, the straight line of best fit giving $s_{20,w}$ = 2.17 S (1 − 0.51$c$), down to 1.3 mg/ml of protein. However, since in this medium a marked anomalous concentration dependence of the molecular weight was concurrently seen in the low protein concentration region (see below), a linear extrapolation cannot be assumed from the lowest point measured in the $s_{20,w}$ plot to the $c$ = 0 axis. In this low salt medium, it is quite uncertain whether the value of $s$ = 2.17 S can be taken as $s^0_{20,w}$ here.

Kenkare and Colowick (1965) reported (while this work was in progress) that the hexokinase prepared by the standard (Darrow and Colowick, 1962) proce-

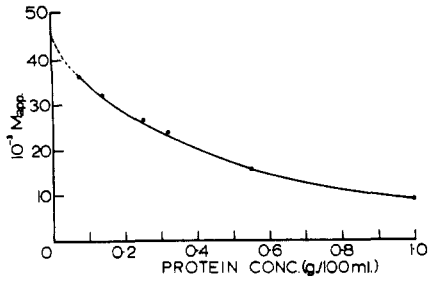


FIGURE 8: Plot of apparent molecular weight *vs.* initial protein concentration of hexokinase B at pH 2.1 at 5°.

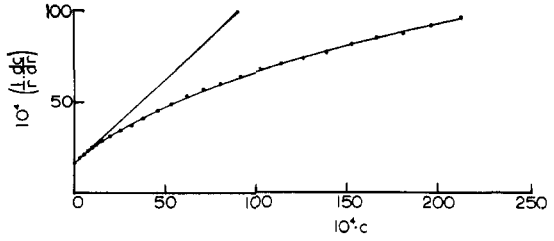


FIGURE 9: Sedimentation equilibrium plot of hexokinase B (at 0.19 g/100 ml) at pH 2.1, 5°. The slope obtained from the points near the meniscus, as shown, was used to give an approximation to $M$- at infinite dilution.

dure could produce at pH 2.8 quarter-molecules of molecular weight 26,000 (a value obtained by calculation from the sedimentation coefficient and the intrinsic viscosity), showing one peak with $s^0_{20,w}$ = 2.2 S. The medium used contained HCl and $Na_2SO_4$ at ionic strength 0.03. Since hexokinase B did not behave thus even at pH 2.1, this particular medium was also employed, for a direct comparison. A typical sedimentation pattern in this pH 2.8 sulfate-containing medium (at 5°) is shown in Figure 6c. In all cases the pattern was complex, showing marked aggregation, together with convectional disturbances, attributed to fast reequilibration of heavy aggregates. At higher concentrations of sodium sulfate at pH 2.8, visible precipitation occurred.

Molecular weight determinations on hexokinase B at pH 2.1 (without added salt) revealed a complex behavior. Sedimentation equilibrium measurements by the short (1 mm) column technique yielded values of the molecular weight that increased with decreasing protein concentration (Figure 8). Thus, the apparent molecular weight[3] increased from $M_{app}$ = 9000 at 1.0 g/100 ml of protein to $M_{app}$ = 36,000 at 0.07 g/100 ml, a behavior suggesting a nonideal solution of the protein. If extrapolation of the plot of $M_{app}$ *vs.* concentration is made to the $c$ = 0 axis, this gives a molecular weight value between 40,000 and 50,000. Equilibrium experiments using the longer (3 mm) columns resulted in a plot of $1/r \cdot dc/dr$ *vs.* $c$ that was concave downwards (Figure 9), again indicating nonideality under these experimental conditions. Such a behavior was observed at all concentrations studied, the lowest being 0.08 g/100 ml. If we take the slope at the initial region (*i.e.*, the first 3 or 4 points from the meniscus of this plot (Figure 9) to give an approximation to $M_w$ at infinite dilution, we obtain a value of about 50,000. This provides some justification for the extrapolation of $M_{app}$ to $c$ = 0, which also gave about 50,000, in Figure 8.

*Sedimentation of Hexokinase A.* The second form of hexokinase, separated chromatographically in our preparation (Lazarus *et al.*, 1966), hexokinase A, is enzymically active, to the extent of about one-quarter that of hexokinase B. A specimen of hexokinase A, from the TEAE-cellulose chromatographic step, was rechromatographed as described (Lazarus *et al.*, 1966) for hexokinase B. Sedimentation of this at pH 7.4 ($I$ = 0.1), 20° (at 1.17 g protein/100 ml) gave a single symmetrical peak with $s_{20,w}$ = 6.0 S. Sedimentation at pH 5.5 ($I$ = 0.1), 20° (at 1.07 g of protein/100 ml), also gave a single peak with $s_{20,w}$ = 5.8 S. Only these preliminary studies are available as yet on hexokinase A, but they are taken to indicate that this is a species of about the same molecular weight as hexokinase B (but apparently with a greater sedimentation coefficient).

*Determination of Specific Absorptivity.* To enable protein concentrations of hexokinase B solutions to be accurately determined in the future conveniently by spectrophotometry, the absorptivity of this protein was measured. A hexokinase B solution was dialyzed against 5 mM sodium succinate, pH 5.0 (adjusted to $I$ = 0.05 with NaCl), and was passed through a fine-porosity glass sinter to remove any suspended material. The concentration was determined in the Hilger refractometer at 20.0°, using the 10-cm path cell, after calibration of the instrument with standard KCl solutions. Samples of this solution were used for absorbance measurements in the Cary spectrophotometer (1-cm path cuvet). The absorption spectrum of hexokinase B has been given previously (Lazarus *et al.*, 1966). Such concentration determinations are best performed at 278 mμ since there is a maximum in the spectral curve there. $A^{1\%}_{1cm}$ at 278 mμ was found to be 9.20 (mean of three determinations, from data at 0.81–0.92 mg of hexokinase B/ml). For chromatographic analyses, $A^{1\%}_{1cm}$ at 280 mμ (which will be less accurate) had previously been assumed to be 10.0. It was found here to be 9.16.

## Discussion

*Forms of Hexokinase B.* The pH 5.5 medium, near the isoelectric point of the protein, is seen to be a suitable reference point for the physical behavior of hexokinase B. It is a homogeneous species there, with a molecular weight of 99,000. The sedimentation velocity behavior is consistent with this, and linear with

[3] Abbreviations used: $M_{app}$, apparent molecular weight; $M_w$, weight-average molecular weight.

concentration with $s^0_{20,w} = 6.41$ S. There is no tendency apparent to dissociate either with changing temperature (4–20°) or with dilution.

As the pH is removed from 5.5, dissociation can occur. At pH 10.4, the molecular weight is 49,000, *i.e.*, dissociation is complete to half-molecules (which we can call monomers, without assumption of identity of the two halves, regarding the original species as a dimer). Here, the material is homogeneous physically, and shows simple linear sedimentation velocity behavior, with $s^0_{20,w} = 3.71$ S.

At pH 7.4, we take the rather lower molecular weight to be due to a partial, freely reversible, dissociation to the monomer. This dissociation is greatly favored by low protein concentration, or by higher temperature, and the sedimentation velocity behavior (Figure 4) is not simple but is fully consistent with a partial dissociation.

At intermediate pH values between 8 and 10, behavior intermediate between that at pH 7.4 and at pH 10.4 was seen, consistent with increasing dissociation in alkali, but has not yet been studied in detail. Mercaptoethanol was not essential for the dissociation.

At pH 2.1, dissociation is again seen but here the behavior is complex. We assume that the boundary having $s_{20,w} = 5.7$ S at $I = 0.09$ represents some of the original dimer form, and that the boundaries of lower mobility represent dissociated forms. The complications appear to be largely due to extensive ion binding, which can be expected here where the charge on the molecule will be about maximal. Such binding is evidenced by the much more pronounced effect of sulfate ion than chloride ion, at the same low ionic strength, in giving complex boundaries; binding will be greater with the divalent ion, and intermolecular bridging is possible with it. It cannot be concluded from the sedimentation patterns themselves in the acid media just which molecular species are present and why several distinguishable boundaries form. A number of situations involving interacting components and ion binding could give such behavior (*cf.* Longsworth and McInnes, 1942; Cann, 1958) but do not merit speculation in relation to the present data. The significant feature here is that in acid a peak having $s_{20,w}$ close to that of the native dimeric molecule is progressively transformed as the ions present are removed, to a peak having a very low $s_{20,w}$ value. We presume, from the evidence of Figure 8, that this latter peak represents the form of the monomer existing at this pH. That is, the extrapolated value of about 50,000 at $c = 0$ (Figure 8) is taken to be a good estimate for the true $M_w$, overcoming the nonideal behavior at finite protein concentrations, which is presumably due to extreme charge interactions. Justification for this comes from the similar value obtained from the lowest protein concentration region of the 3-mm solution column at equilibrium (Figure 9). The $s_{20,w}$ value of this monomer is reduced to an exceptionally low figure, due in part to the complicating effects of the high charge on the molecule, which we cannot relieve in the usual way, and, quite probably, in part to an unfolding or expansion. Such an expansion is quite likely in this pH region where the molecule carries a high net positive charge. In any case, errors would probably be incurred if the $s^0_{20,w}$ value were to be deduced from a linear extrapolation from the accessible concentration range of $s$ determination, and in the face of the other evidence for nonideal behavior in these media.

Hence, it is concluded that hexokinase B undergoes progressive dissociation to two monomers, as the net charge on the molecule increases (in either direction), and that there is equilibrium between these and the dimeric form, with dissociation favored also by decreasing protein concentration and increasing temperature. This equilibrium is reversible. Thus, after a centrifugation in 0.01 N HCl to produce the pattern shown in Figure 7 and being held for 10 days at pH 2.1 at 4°, the material, when neutralized and then dialyzed against the pH 7.4 ($I = 0.1$) buffer, gave largely one peak with the expected $s_{20,w}$ value for hexokinase B in that medium (together with some very aggregated material). Concurrent enzyme assay of this latter solution (at the usual assay pH) showed that the final activity was about 420 units/mg (from an original level of about 650 units/mg, both at 25°). However, full investigation of the course of reactivation will be of interest.

*Comparison of Hexokinase B with Previous Preparations.* We have noted in the introduction to this paper the molecular weights previously reported for preparations of hexokinase from baker's yeast. For all those specimens, the isolation from the yeast cells and the subsequent treatment were essentially by the method that has become the standard procedure (Darrow and Colowick, 1962), although the terminal purification steps varied in each case. There are four main features present in those methods, collectively, and absent in the present one, which can be expected to introduce changes in the molecule of the hexokinase produced: (a) the initial gradual drying of the yeast cells at room temperature, which requires about 10 days for good yields (and which is certainly accompanied by enzyme actions); (b) the autolytic incubation at 35° at pH near 8 for 4 hr, which is essential for liberation of soluble hexokinase in those methods; (c) the uninhibited protease activity present in the solution of hexokinase at subsequent stages; and (d) the use of final purification procedures which do not fully remove contaminants. For the last point, we can note that repeated recrystallization (Darrow and Colowick, 1962; A. H. Ramel, unpublished observations) does not increase the specific enzymic activity above one-half of that which can be attained by the alternative preparation (see footnote 8 of Lazarus *et al.*, 1966). Careful choice of conditions in chromatographic steps is necessary to remove adhering proteolytic contamination (Lazarus *et al.*, 1966). In view of these features, there is ample opportunity for some proteolytic attack to have been suffered by the fraction of active hexokinase that is isolated in the usual methods. Hexokinase may, of course, be stable throughout the isolation to the

TABLE II: Comparison of Behavior of Hexokinase Specimens.[a]

| pH | $I$ | Observation | This Work | Kenkare and Colowick (1965) |
|---|---|---|---|---|
| 5.5 | 0.1 | $s^0_{20,w}$ | 6.41 S | — |
| 5.0 | 0.1 | $s^0_{20,w}$ | — | 5.5 S |
| 5.5 | 0.1 | $M_w$ | 99,000 | — |
| 5.0 | 0.1 | $M_w$ | — | 95,000 |
| 7.0 | 0.1 | $s_{20,w}$ | 5.8 S | 5.3 + 3.2 S |
| 7.0 | 0.4 | $s_{20,w}$ | 5.7 S | 3.3 S |
| 2.8 | 0.03 | $s_{20,w}$ | Aggregation | 2.1 S |
| 2.1–2.2 | 0.01 | $s^0_{20,w}$ | 2.17 | 2.2 |
| 2.8 | 0.03 | $M_w$ | Undetermined | 29,000 |
| 10.4–10.5 | 0.10 | $M_w$ | 49,000 | 27,000 |

[a] The property denoted is compared for hexokinase B examined here and for the hexokinase preparation reported upon by Kenkare and Colowick (1965).

proteases present (as, *e.g.*, pancreatic ribonuclease is to the pancreatic proteases), but the higher specific activity and higher yields shown when the yeast proteases are inhibited (Lazarus *et al.*, 1966) suggests that such attack does otherwise occur. The observations in the preceding paper and in the present one on the loss of hexokinase activity and the decline of molecular weight when allowed to stand in the presence of these proteases, even in residual amounts, strongly support this conclusion.

We interpret, therefore, the quite large physical differences between hexokinase B and the previous preparations as being due to partial proteolytic change in the latter. These differences are best documented by comparison with the data of Kenkare and Colowick (1965) who have now provided a physicochemical study of baker's yeast hexokinase prepared by the Darrow and Colowick (1962) procedure. (Commercially available hexokinase from Boehringer and from Sigma, after two recrystallizations, was also used.) These differences are mostly summarized in Table II.

At pH 5–5.5 ($I$ = 0.1) both preparations appear to be homogeneous by sedimentation velocity patterns. The molecular weight of our preparation is slightly larger, but the value of 95,000 of Kenkare and Colowick (1965) was obtained by use of $s^0_{20,w}$ and intrinsic viscosity, with an assumed value of the $\beta$ function (Scheraga and Mandelkern, 1953). The $s^0_{20,w}$ values can be compared more exactly, however (Table II); our specimen (at pH 5.5) has a significantly higher value, suggesting a somewhat larger or more compact molecule.

However, our material has been shown to be homogeneous by additional criteria, *i.e.*, sedimentation equilibrium studies, column chromatographic analysis, and electrophoretic mobilities. The material reported upon by Kenkare and Colowick (1965) was only recrystallized, and Trayser and Colowick (1961) have already shown that this recrystallized hexokinase can be resolved by column chromatography into three major and three minor species (all active). Ramel *et al.* (1963) obtained two major active peaks from comparable material, and note that (at pH 7) one has a molecular weight of 93,000 and the other 47,000. This explains the two distinct sedimenting boundaries shown by Kenkare and Colowick, at pH 7.0 ($I$ = 0.1), having $s_{20,w}$ = 5.3 and 3.2 S. These were interpreted there as showing a partial dissociation of an original 95,000 molecules into half-molecules, this process continuing further to the half-molecule completely at higher ionic strength or higher pH (Kenkare and Colowick, 1965). However, the two distinct boundaries would, in fact, indicate that this equilibrium is not easily reversible. Our hexokinase B, in contrast, never shows two boundaries when dissociation occurs between pH 5.5 and 10.4. Hexokinase B is in free equilibrium with the half-molecules, dissociation being favored by alkalinity. The species in the previous preparations having $s_{20,w}$ about 3.2 S at pH 7 and low ionic strength can independently dimerize on lowering the pH to near 5, as has been noted by Ramel *et al.* (1963) for this species when chromatographically isolated from such a preparation. We have also described above the production from hexokinase B of such a species with $s_{20,w}$ about 3.9 S by a yeast protease. Hence, we conclude that the material giving the peak seen at pH 5 in the Kenkare and Colowick case must be heterogeneous; part of it readily dissociates to the 3.2S form seen at pH 7 ($I$ = 0.1) while part remains dimeric up to higher pH (or $I$). Our hexokinase B has little physical resemblance to the first of these forms, which has a molecular weight at pH 7 of about one-half that of hexokinase B. That form also appears to be the one examined in another study using chromatographed commercial yeast hexokinase (Ågren *et al.*, 1963), where a major species of about 50,000 molecular weight was purified.

The second species present in the preparation of Kenkare and Colowick, which remains dimeric about pH 7, has more resemblance to hexokinase B. However, important differences are present, here, too. Their material dissociates much more readily than hexokinase B, in a variety of conditions. Thus, at pH 7 ($I$ = 0.4) their material is completely dissociated to half-molecules, whereas ours is essentially dimeric (Figure 6b). At pH 10.4 ours is a stable monomer whereas theirs gives mostly quarter-molecules. At pH 2.8, their material again gives mostly quarter-molecules, whereas ours is monomeric even at pH 2.1. The effect of added salts is also strikingly different in the two cases. The sulfate medium used by Kenkare and Colowick for dissociation to quarter-molecules at pH 2.8 led to aggregation of hexokinase B. Even small amounts of sodium chloride gave aggregation of the hexokinase B monomer at pH 2.1.

In conclusion, hexokinase B differs from previous preparations in being a homogeneous dimeric molecule which dissociates to two monomers with much less ease than the equivalent species in the mixtures present in previous preparations. It is deduced that the proteolytic attack inherent in the latter destroys some hexokinase, but leaves the rest in a fairly stable form, which is heterogeneous, and which has a distinctly looser structure than the native molecule.

The essential molecular assembly of the present and the previously described types of hexokinase seems to be similar, in that both eventually give half-molecules, and further, it is presumed, subunits of quarter-molecules. Subunit studies on hexokinase B are in progress.

*The Nature of Hexokinase A.* Hexokinase A is a major component in the extracts of yeast made by our procedure. It has only about one-quarter of the specific enzymic activity of hexokinase B, and differs from B in substrate specificity (Lazarus *et al.*, 1966). It is not equivalent to the second major component referred to above, that is present in the conventional preparations, since it has roughly twice the molecular weight of the latter. It is obtained as a homogeneous species, which has unchanged behavior on rechromatography. The proteolytic experiments in the ultracentrifuge referred to above show that hexokinase A is not derived from hexokinase B by an initial action of yeast proteases. We have also found that a TEAE-cellulose chromatography, by the method already described, of the initial yeast extract (with proteases inhibited by DFP), while less efficient than the full procedure of Lazarus *et al.* (1966), still gives the characteristic pattern of A and B peaks, showing that A is present from the start. Further, when another cultured strain (National Collection of Yeast Cultures, No. 77) of *Saccharomyces cerevisiae* was grown in the presence of glucose, hexokinase isolated by the procedure of Lazarus *et al.* (1966) showed the same active peaks of A and B (Y. Rustum and E. A. Barnard, unpublished data), indicating that A and B probably do not arise selectively from different strains in the commercial baker's yeast. It is tentatively concluded that hexokinase A is another form that also exists as such in the yeast cell, perhaps in another location to that of B.

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