2-deoxy-2-amino-D-glucose-6-P, and D-galactose-6-P to their respective inositols using the same system that is active toward D-mannose-6-P. Whether these negative results support the idea that a second cyclase is present in the preparations, which is specific for D-mannose-6-P, or whether the D-glucose-6-P–L-*myo*-inositol-1-P cyclase is nonspecific only with respect D-mannose-6-P cannot be determined with the data at hand.

Acknowledgment

We acknowledge the help of Mr. W. H. Holland in obtaining some of the mass spectra used in this work.

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Yeast Hexokinase. IV. Multiple Forms of Hexokinase in the Yeast Cell*

Armin H. Ramel,† Youcef M. Rustum, J. Gareth Jones,‡ and Eric A. Barnard§

ABSTRACT: Using improved methods for the preparation of hexokinase from baker's yeast without autolysis by any of the yeast proteases, the extract was shown to contain four hexokinase isoenzymes, A, A′, B, and C, and a specific glucokinase. The latter is extracted in a form having molecular weight about one million. Other active hexokinase species, arising by proteolysis of native forms, are distinct from these, and are suppressed when initial removal of proteases is efficient. Conditions are described for obtaining hexokinases A, B, and C in pure form, each homogeneous in starch gel electrophoresis. Hexokinases A and B are the previously described forms, and differ in that A has a greater relative activity on fructose, a lower specific activity, and a higher negative charge (at pH above 5) than B. Their measured activity differences are essentially a reflection of differences in the Michaelis–Menten maximum velocities on glucose and on fructose. Hexokinase C is a newly described isoenzyme, identical with B in most properties but having a different mobility in anion-exchange chromatography. It can be converted to B when a high ionic strength is applied at pH 4.6 or 8, followed by gel filtration. Immediately upon rupture, yeast cell lysates contain most or all of their hexokinase B in the form of C. Also present in the fresh lysates is hexokinase A′, which appears to be a hybrid of A and B. This hybrid splits to give A and B in the conditions of purification. The course of release of the hexokinases was followed in the melting of frozen yeast. Sixty per cent of the total activity was readily liberated as soluble forms, these being characterized as A, A′, and C. The remainder is probably osmotically trapped in the cell debris, and is not bound to the cell walls.

In part I of this series (Lazarus *et al.*, 1966) a procedure was described for the preparation of hexokinase from yeast under conditions designed to protect the enzyme against degradation by the proteases readily available in cell homogenates of baker's yeast. This was achieved by liberating the enzyme from the cells by a freeze-thawing method, by inactivating the

* From the Departments of Biochemistry and Biochemical Pharmacology, State University of New York, Buffalo, New York 14214. *Received January 6, 1971.* This work was supported by Grant GM-16726 from the U. S. Public Health Service, and (for supporting services) by the National Aeronautics and Space Administration Grant NGR 33-015-016. It was taken in part from a dissertation submitted by Y. M. R. to the State University of New York at Buffalo in partial fulfillment of the requirements for the Ph.D. degree.

† Present address: Chemical Research Department, Hoffman-La Roche Inc., Nutley, N. J.

‡ Present address: Department of Biochemistry, University College, Cathays Park, Cardiff, Wales.

§ To whom correspondence should be addressed.

yeast proteolytic activity with DFP, and by performing all operations under conditions of pH and temperature where proteolysis was shown to be minimal. The previously reported multiplicity of six proteins having hexokinase activity (Trayser and Colowick, 1961) could be reduced to two forms, designated A and B in part I of this series; the results indicated that those six forms must have been proteolytic artifacts of the usual isolation procedures, which include an autolysis at 37° at pH 8 (Darrow and Colowick, 1962) to release the enzyme. These findings essentially concur with others reported by Schulze *et al.* (1966) and Schulze and Colowick (1969).

Hexokinases A and B differ (Lazarus *et al.*, 1966) in their F:G ratio,[1] chromatographic elution position, and electrophoretic mobility. Their molecular weights (when measured near the isoelectric point, pH 5.0) are, however, about identical, being about 102,000 (Derechin *et al.*, 1966; Lazarus *et al.*, 1968). A third species, hexokinase C, has since been obtained, as is reported below.

The question has remained as to whether these three forms are native or arise from initial proteolytic attack on a native hexokinase. The latter possibility has seemed plausible, because a fraction of the yeast proteolytic activity had originally been found to be resistant to DFP (see Figure 5 of Lazarus *et al.*, 1966), and since a subsequent isolation of the proteases from cell homogenates of baker's yeast by Hata *et al.* (1967) showed that, indeed, one of the major proteases is DFP-resistant. The latter enzyme is maximally active at pH 4.5 (whereas the other two major proteases found have optima at 6 and 9 and are inactivated by DFP). In the hexokinase preparations of Lazarus *et al.* (1966) and of Schulze *et al.* (1966) most of the purification steps are necessarily carried out at pH 4.5–6, albeit at 4°.

In the present work we have developed means to capture the enzyme immediately upon release and to eliminate during the purification steps even a brief exposure to the proteases, including the DFP-insensitive acid protease. These investigations, as well as resulting in an improved procedure for the isolation of hexokinases A and B (described in detail as a preparative method elsewhere: Rustum *et al.*, 1971b), have led to the recognition of additional yeast hexokinase isoenzymes, which are characterized here. In addition, a specific glucokinase of the yeast cell has been discovered.[2] The interrelationship of the various hexokinases becomes clearer in the light of information on their subunit structures, considered in the succeeding paper (Rustum *et al.*, 1971a).

Materials and Methods

The yeast and all materials and methods were as described previously (Lazarus *et al.*, 1966), except where specified here. Ammonium sulfate was Enzyme Grade from Mann. Agarose gel was Bio-Gel A-0.5 m (Bio-Rad) (100–200 mesh). Starch was from the Electrostarch Co. (Madison, Wis.).

Sucrose gradient centrifugation was performed using the swinging-bucket rotor SW39 in the Spinco Model L ultracentrifuge, as described by Martin and Ames (1961).

Yeast protoplasts, and the digested cell wall fraction, were prepared by the method of Gascón and Lampen (1968), using the snail gut enzyme preparation of L'Industrie Biologique Francaise (Gennevilliers, France). Lysis of the washed protoplasts was in hypotonic medium (Gascón and Lampen, 1968), with agitation in a Waring Blendor for 30 sec at medium speed. These stages were checked for apparent completion by phase-contrast microscopy.

Electrophoresis. Vertical starch gel electrophoresis employed the Buchler Instrument Co. apparatus, and the EDTA–borate–Tris buffer system of Boyer *et al.* (1963) for the gel. The electrode buffer was 0.013 M Tris–0.08 M sodium borate–0.003 M EDTA (pH 8.5). Gels were prepared (Massaro, 1967) using 13.7% starch. A voltage gradient of 12 V/cm was applied for 15–18 hr at 4°. Following electrophoresis, the gels were removed from the molds and sliced horizontally. The upper half was stained for protein at room temperature with a 0.3% solution of coomassie blue (or Amido Black) in a 5:5:2:2 (v/v) mixture of methanol–water–acetic acid–glycerol. The lower half was stained for hexokinase activity by a modified version of the method of Eaton *et al.* (1966): staining was for 15 min in the dark at 37° in 100 ml of 0.1 M Tris-HCl buffer (pH 8.3), containing 5×10^{-3} M glucose, 3.3×10^{-3} M $MgCl_2$, 5.4×10^{-3} M ATP, 1.8×10^{-4} M TPN, 0.1 mg of glucose 6-phosphate dehydrogenase (Boehringer), and 6 mg each of phenazine methosulfate and nitro blue tetrazolium. Electrophoresis on cellulose acetate was by methods detailed by Rustum *et al.* (1971a).

All experiments unless otherwise specified were carried out at 4°. All pH values are stated as read at 4° without temperature correction. At all stages where DFP was added, this was to a final concentration of 1 mM. All buffers specified below contained 1 mM EDTA.

Preparation of Crude Extract. The crude extract, *i.e.*, the supernatant obtained upon centrifugation of the slurry of disrupted yeast cells at 23,000*g* for 1 hr, was prepared by one of three methods. (i) Freezing of the yeast in toluene–Dry Ice and thawing at 4°, as described by Lazarus *et al.* (1966), but with the improvements reported by Rustum *et al.* (1971b). (ii) The yeast was homogenized thoroughly with ground Dry Ice for 10 min at top speed in a 1-gal Waring Blendor. Melting of the frozen batch (from 1.4 kg of yeast) was conducted on a 40° water bath, with stirring such that the whole batch melted at 0–4° within about 2 hr. DFP was added to the medium immediately after melting. (iii) The yeast cells were suspended in 0.2 M sodium phosphate (pH 7.0) (200 ml/100 g wet weight of yeast) at 4° and disintegrated in a precooled French press (American Instrument Co.) at a pressure of 15,000–20,000 psi. The slurry was chilled in ice as it was formed and DFP was added to the medium both before and after the disintegration.

Processing of Crude Extracts. Gel filtration of the crude extract (about 40 ml at a time) was on a column (70 × 5 cm) of Bio-Gel A-0.5m, equilibrated and eluted (at 50 ml/hr) with 5 mM succinate (pH 5.8). The collected material was used without delay for chromatography on DEAE-cellulose (see below). DFP was added at pH 7 before and after gel filtration.

When ammonium sulfate fractionation was instead employed, the crude extract was given an acid treatment (at pH 4.6) and the fraction precipitating between 0.42 and 0.60 ammonium sulfate saturation was collected, using the procedure described by Lazarus *et al.* (1966) with some modifications (Rustum *et al.*, 1971b). Ammonium sulfate was completely removed by gel filtration on a Sephadex G-25 column

[1] F:G designates the ratio of the rate of enzymic phosphorylation of fructose to that of glucose at pH 8.3, 25°, in the conditions of routine assay (27 mM sugar–3 mM ATP·Mg).

[2] Since we noted that finding in an abstract (Rustum and Ramel, 1969), a fuller study has been made (Maitra, 1970) of this glucokinase. A form of mol wt ~230,000 predominated in 2 mM glucose–1 mM mercaptoethanol medium. The findings are compatible with ours, since it is clear that some dissociation of our higher molecular weight form occurs in our starch gel electrophoresis.

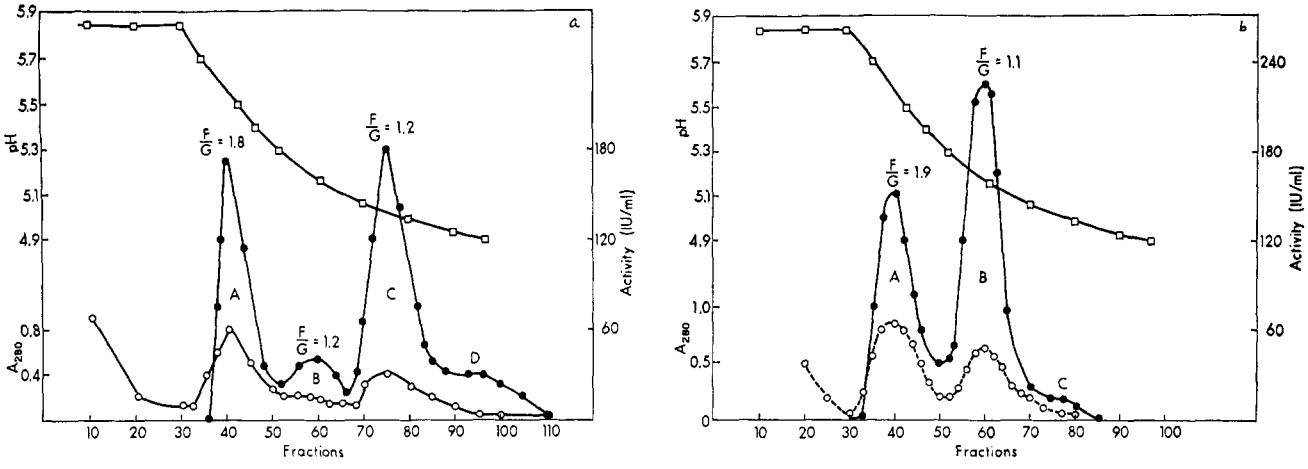

FIGURE 1: Chromatography of hexokinase preparations on DEAE-cellulose. The pH gradient in the effluent is shown (□). (○) Protein concentration (by A_{280}); (●) hexokinase activity in International units per milliliter of the effluent. F:G ratios for peak tubes are marked. The processed crude extract was prepared for the chromatography (a) by dialysis at pH 5.8, 4° (Lazarus *et al.*, 1966), or (b) by the conversion treatment with 0.8 M ammonium sulfate and rapid gel filtration. When gel filtration replaced the lengthy dialysis in part a, peak D was absent.

(70 × 6 cm) in 5 mM succinate (pH 5.8) at 800 ml/hr, and the pH of the pooled enzyme solution was readjusted to 5.8.

This extract was chromatographed on a DEAE-cellulose column using a concave pH gradient (Figure 1) generated as described by Lazarus *et al.* (1966) for TEAE-cellulose chromatography. This gave higher yields and improved purification compared to the salt displacement chromatography on DEAE-cellulose used at this stage previously (Lazarus *et al.*, 1966). Further chromatography here was on a column (17 × 1.5 cm) of microgranular DEAE-cellulose, type DE-52 (Whatman), with a linear pH gradient (pH 5.8–4.6) using 10 mM succinate buffer (250 ml in each chamber). Further details of chromatographic procedures are given by Rustum *et al.* (1971b).

The purified enzyme was finally concentrated by displacement (Lazarus *et al.*, 1966) on a 2-ml DE-52 column. The pure enzyme was stored at 4° in solution, without activity loss, using 1 mM EDTA as preservative when necessary.

Results

Isolation of Hexokinases A and C. The method used to liberate hexokinase from yeast cells in bulk was a form of that used previously (Lazarus *et al.*, 1966), involving rapid freezing of the yeast in a toluene vehicle at −75° and thawing at 4°. After acid (pH 4.6) treatment and ammonium sulfate fractionation, excess salt was removed and the hexokinases were separated by chromatography on a DEAE-cellulose column (Figure 1a). Two major peaks of hexokinase activity, A and C, were obtained, and a minor peak, B. The other minor peak, D, was present only when the material received certain pretreatments, and is discussed below.

The enzyme in peaks A and C (when D was absent) was, in each case, rechromatographed in the system used in Figure 1, and the enzyme in the non-overlapping region of the A or C peak was further chromatographed twice in succession in a higher resolution system on DE-52 DEAE-cellulose (as used in Figure 2). Hexokinase A was obtained thus (not shown here) as a homogeneous peak with constant specific activity and F:G value throughout the peak, whereas hexokinase C (Figure 2a) still showed some evidence of heterogeneity (discussed below). Neither isoenzyme was improved in specific activity upon a further rechromatography of the central two-thirds of the peak. Hexokinase C had the F:G value of 1.1–1.2 characteristic (Lazarus *et al.*, 1966) of hexokinase B.

Formation of Hexokinase B. The yield of hexokinase C in

TABLE I: Conversion of Hexokinase C into B.

Treatment[a]	Starting Material	% Present[b] Initially B	Initially C	Finally B	Finally C
pH 4.6, 5 mM succinate–1 M NaCl	Pure C	0	100	73	27
pH 4.6, 5 mM succinate–0.8 M ammonium sulfate	Pure C	0	100	100	0
pH 4.6, 5 mM succinate	Dialyzed enzymes[c]	17	83	17	83
pH 4.6, 5 mM succinate–0.8 M ammonium sulfate				100	0
pH 8.0, 5 mM succinate–Tris				19	81
pH 8.0, 0.8 M ammonium sulfate				100	0

[a] The starting material was placed in the medium stated, at 4° (except where noted); any precipitate was collected and discarded, and the solution was gel filtered on G-25 in the same medium. [b] Parallel samples of the solution were analyzed with and without the treatment noted in the first column, by DEAE-cellulose chromatography. The percentage of hexokinase B and of C protein, in the total protein present in the peaks of B and C, is recorded. [c] This is the material obtained after the standard ammonium sulfate fractionation of the crude extract: it was redissolved in 5 mM succinate (pH 5.8) and dialyzed (24 hr) against that medium.

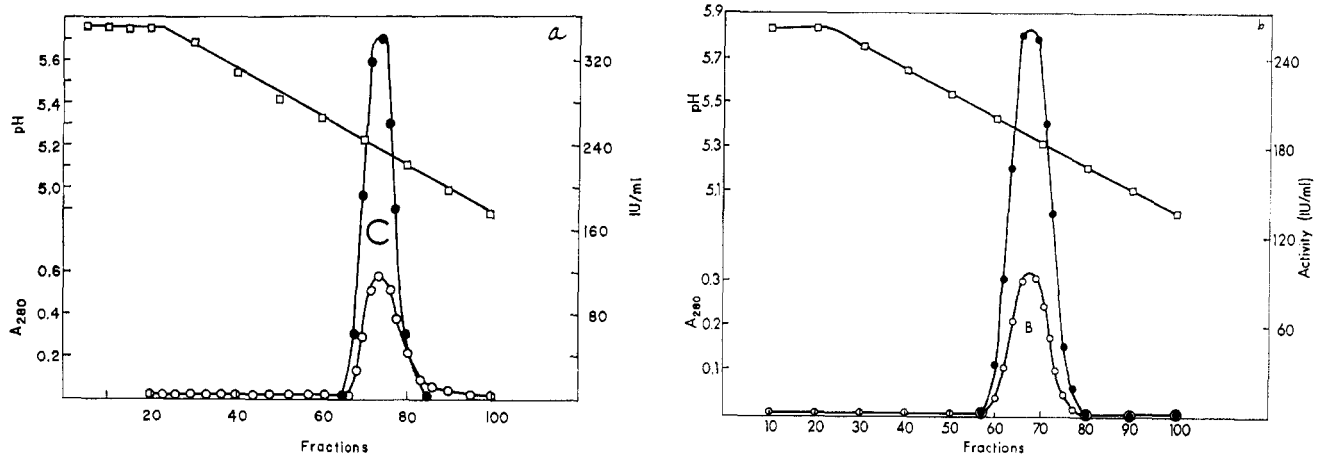

FIGURE 2: Second rechromatography of hexokinases. The enzyme (from peak C or peak B of Figure 1b or 1a) was rechromatographed twice, on a DE-52 column (pH in effluent, □); (●) hexokinase activity; (○) protein (as A_{280}).

the initial chromatography was observed to vary inversely with that of B (the total being about constant), depending upon the ionic strength and pH of the enzyme solution prior to the gel filtration. Pure hexokinase C was, accordingly, exposed to high ionic strength at several pH values, followed by chromatographic analysis: an experiment in 1 M NaCl medium is illustrated in Figure 3. Conversion of most of the hexokinase C into B has occurred there. Results obtained in various conditions are summarized in Table I. For complete conversion, the ionic strength of 0.8 M ammonium sulfate or above was required, together with a pH as low as 4.6 or as high as 8.0.

To achieve this conversion of C into B, rapid gel filtration (see Methods) must be applied after the high ionic strength treatment. This step need not be on a gel column maintained at the same ionic strength (which would be inconvenient for further purification); equivalent results to those shown in Table I could be obtained when the gel column was eluted with 5 mM succinate buffer, so long as a rapid flow rate (800 ml/hr, on a 70 × 6 cm G-25 column) is used in the latter case. The observations suggest that some ligand is released from hexokinase C (to form B) in the high ionic strength conditions.

For the preparation of hexokinase B, therefore, a conversion step was inserted. The extract was exposed to 0.8 M ammonium sulfate at pH 4.6 and then desalted by rapid gel filtration on the G-25 column in 5 mM succinate, just prior to the first DEAE-cellulose chromatography. The profile of Figure 1b was then always obtained, *i.e.*, with a high yield of hexokinase B, and not C. Hexokinase B could be brought to maximum specific activity by the further chromatographic purifications used (see above) for hexokinase A, when a homogeneous peak was obtained (Figure 2b).

It should be noted that the precise pH of elution in the chromatography of the various peaks varies with the amount of protein applied to the column, as well as with the profile of the pH gradient applied. The values shown in the chromatograms are only for the conditions specified. In other conditions, however, the relative separations indicated for the peaks are still maintained. Characteristic values are shown in Table II.

Hexokinase C. The material in the hexokinase C peak (Figure 2a) gave a distinctly yellow solution when highly concentrated. The visible absorption spectrum was very similar to that of FMN, with a maximum at 450 mμ. Hexokinase B

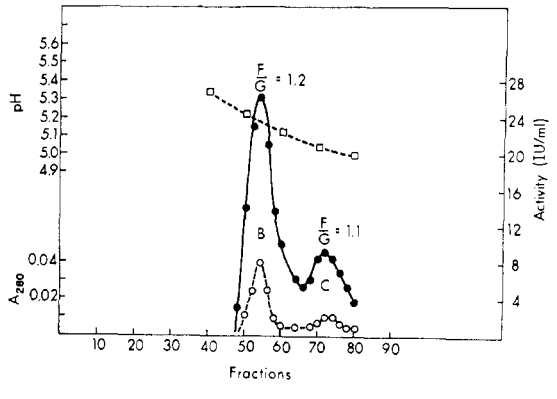

FIGURE 3: Chromatography of partly converted hexokinase C. Pure hexokinase C was treated with 1.0 M NaCl (pH 4.6), 4°, followed rapidly by G-25 gel filtration. Chromatography and notation as in Figure 1a.

TABLE II: Characteristics of the Yeast Hexokinases in DE-52 Chromatography.

Property	A	B	C
F:G	2.0[a]	1.1	1.1
pH of elution[b]	5.6	5.4	5.2
Specific activity[c]	275	900	750

[a] The F:G value of the A peak in the initial chromatographic steps usually lies in the range 1.8–2.0, due to the presence of some B present in the A peak. The value for A rechromatographed on DE-52 with special precautions for final purification of A (Rustum *et al.*, 1971b) is 2.0–2.3. [b] pH values measured in the eluate at 4° (without temperature correction). The values vary (±0.05 unit) between chromatograms, and are only valid for the pH gradient of the type shown in the relevant figures. Somewhat different pH values are involved in the chromatography on fibrous DEAE-cellulose (Rustum *et al.*, 1971b). [c] After repeated rechromatography to constant specific activity (measured on glucose at 25°, pH 8.5) in International units per milligram.

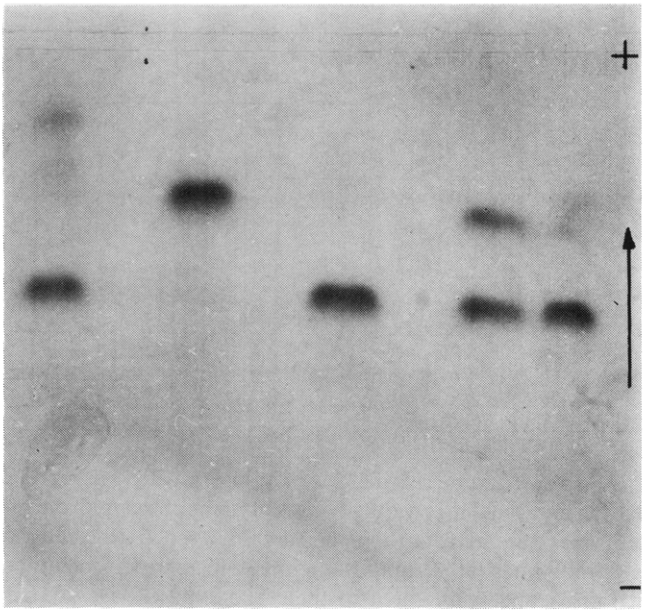

FIGURE 4: Starch gel electrophoresis (EDTA–borate–Tris buffer, pH 8.6), with staining for hexokinase activity, of (right to left): (1) hexokinase B; (2) hexokinases A + B; (3) hexokinases B + C; (4) hexokinase A; (5) hexokinase B (gel filtration step omitted and slight autolysis occurring, in the initial stages of the preparation). The origin (not shown) is below: only a segment of the gel (about one-quarter of the whole) that contained all the detectable bands is shown.

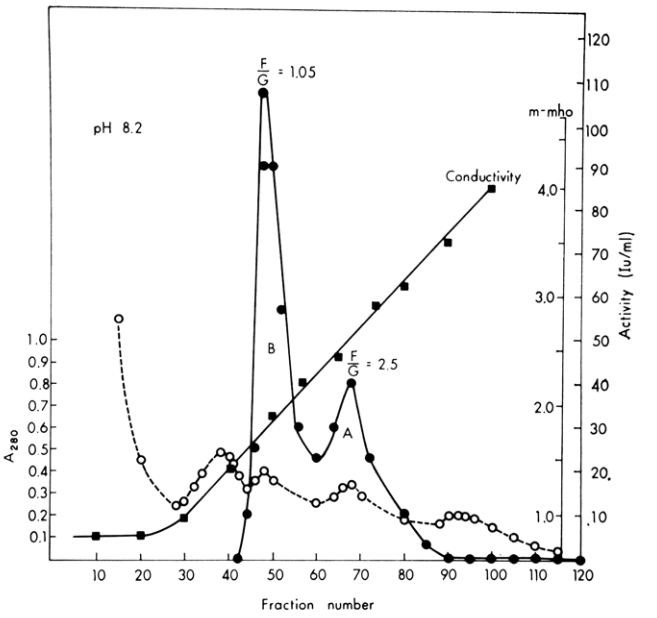

FIGURE 5: Chromatography on DEAE-cellulose at pH 8.2 in a Tris·HCl gradient (10–200 mM), shown by the measured conductivity of the effluent (■). Other notation as in Figure 1. A standard crude enzyme preparation (as used in Figure 1) was applied to this column. The peak marked B contains both hexokinases B and C, as was shown by its resolution in a rechromatography in the conditions used in Figure 2.

(Figure 4), however, was always devoid of visible absorption, even when concentrated to >10 mg ml, and had a normal protein absorption spectrum, as recorded by Lazarus *et al.* (1966) for hexokinase B. Addition of a large excess of FMN or of FAD to hexokinase B did not produce any binding or reconversion, as seen in a subsequent rechromatography. It was observed that in conversions of C to B the total enzymic activity was conserved quantitatively, but the specific activity rose: that of pure B is about 20% higher than that of C which has been rechromatographed to constant specific activity (Table II). These observations, and the electrophoretic analysis of C (see below), indicate that the heterogeneity detectable (Figure 2a) in the C peak is due to a foreign flavoprotein which is eluted with hexokinase C in the chromatography, but which does not affect its activity other than by dilution. The flavoprotein is not required for the integrity of hexokinase C, since the latter can be obtained without it by a conversion back from B in the presence of substrates,[3] as will be reported elsewhere.

Hexokinase D. The minor peak D (Figure 1a) was observed here when the removal of excess salt prior to the chromatography was accomplished by dialysis to equilibrium at 4° against 5 mM succinate buffer, as used by Lazarus *et al.* (1966). When this procedure was replaced by gel filtration on a Sephadex G-25 column, as specified in the Methods section, the same chromatographic profile as in Figure 1a was obtained but with the absence of peak D. This difference is attributed to the occurrence of slight proteolysis of hexokinase B or C during the long exposure to pH 5.5 in the dialysis at 4°, since the hexokinase D (i) had the same F:G value as hexokinases B and C, (ii) was always of lower specific activity than B, or C, and (iii) increased progressively in relative amount when much longer exposures of the extract to pH 5–6 (at 4°) were permitted. A similar peak in the D region is also found in these chromatograms when the DFP treatment of the extract is omitted, suggesting that several forms of slightly proteolytically modified, active hexokinase can chromatograph at this position. The occurrence of such a peak in TEAE-cellulose chromatography of extracts deliberately unprotected by DFP was illustrated by Lazarus *et al.* (1966). (That peak was then labeled C, a designation that should now be changed to D in view of the separation (Figure 1a) of the hexokinase C, eluted immediately after the B peak and prior to the region where these proteolytically produced forms emerge.)

Electrophoretic Characterization. Hexokinases A, B, and C, each purified to constant specific activity by DE-52 chromatography, were examined by high-resolution electrophoresis in starch gel. At pH values around 8.5 in 0.1 M Tris-HCl, Tris-succinate, or Tris-borate buffers, each was a single, anionic species (Figure 4), hexokinase A migrating distinctly faster than hexokinase B or C. Hexokinase C showed a mobility identical with that of B (Figure 5) when stained for hexokinase activity. Staining of C for protein, however, revealed a faint inactive band in all preparations, travelling faster anodally. This corresponds to a yellow protein contamination in samples of C (discussed above). A single band was found by activity staining in electrophoresis of mixed samples of B and C at pH 8 or 8.5. The true mobility of C is not actually determined by electrophoresis, since other experiments have shown that it is converted to B in the conditions of running used in these gels.[3] Electrophoresis of A or B was also performed at pH 4.6–8.2 on cellulose acetate (Table III), when a single band was again present in each case, as evidenced by enzymic activity (tested after electrophoresis at pH 6.7, and 8) and by protein staining. In electrophoresis below pH 4.9, in 30 mM succinate buffer (conditions comparable to the DEAE-cellulose elution medium for hexokinases A and B), both species

[3] Y. M. Rustum and E. A. Barnard, unpublished results.

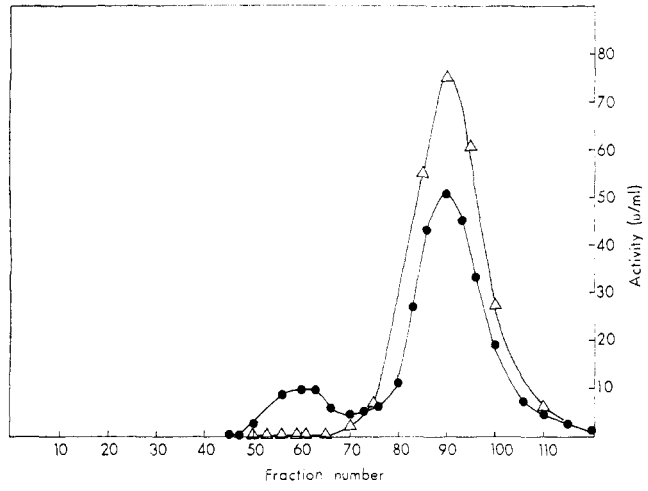

FIGURE 6: Gel filtration on Agarose of the DFP-protected crude extract. The same profile was obtained if either the French press or homogenization with solid CO_2 was used to make the crude extract. Activity (International units per milliliter) was read (at pH 8.3) on fructose (△) and on glucose (●), in the 5-ml fractions.

TABLE III: Electrophoretic Differences between Hexokinases A and B.

pH	Buffer	Mobility Ratio (A:B)[b]
4.6	30 mM succinate[a]	1.6[c]
5.0		0.5[d]
5.6		0.9
6.0		1.0
7.0		1.2
8.0		1.2
8.5		1.1
8.2	25 mM phosphate	1.1
8.2	100 mM Tris-HCl	1.2

[a] 30 mM sodium succinate was adjusted with Tris or HCl to the indicated pH value. [b] Mobility was measured on cellulose acetate at room temperature, at 2.5 mA; the ratio of the mobilities of A and B on the same strip is recorded. [c] Both A and B migrated to the cathode here. [d] At this and all higher pH values, both A and B migrated to the anode.

became cationic, A more so that B (Table III). In this medium, the isoelectric points were about pH 4.95 (A) and 4.85 (B) in such electrophoresis. A became more anionic than B above pH 6.0, the difference reaching a maximum of 20% (Table III), an increase that persisted in several types of buffer. It has previously been noted (Lazarus *et al.*, 1966; Gazith *et al.*, 1968) as an anomaly that, in contrast to the known relative electrophoretic mobilities of A and B, on DEAE- or TEAE-cellulose columns B behaves as the more anionic of the two species (see Figure 1). However, the chromatography is at pH near 5, and the electrophoresis was at pH near 8. Therefore, chromatography on DEAE-cellulose was also performed at pH 8.2 (Figure 5), a suitable salt gradient being applied. The order of elution of A and B was now reversed, consistent with the electrophoretic results. All of the reported chromatographic behavior of hexokinases A and B can be understood in terms of the relative net charges upon these proteins in the conditions used, as summarized in Table III.

Forms of Hexokinase Present in Initial Extracts. Steps were taken to prevent the attack of a DFP-insensitive, acid-optimum protease in the initial stages of the preparation. In the first preparative method used above, the melting of the frozen bulk yeast necessarily occupies 15–20 hr in the toluene procedure, a period which, although at −4° to +4°, might give some opportunity for autolysis. Disintegration was, therefore, carried out by a high-pressure French press, which can give an immediate cell lysate at 0° without freezing. An alternative method employed high-speed homogenization of the yeast cells with solid CO_2 and subsequent rapid thawing.[4] DFP was added at once to all the extracts. Further, these "native" extracts were at once gel filtered on an Agarose (A-0.5m) column, since the proteases of yeast extracts, analyzed by Hata *et al.* (1967), are all of much lower molecular weight than the 100,000 of the hexokinases. Tests of the hexokinase peak in the gel filtrate for free protease activity (Lazarus *et al.*, 1966) showed that the latter was all removed, within the limits of the detection, by these procedures.

In the gel filtration (Figure 6), total activity recovery was 100%, and the hexokinase activity all emerged at a position corresponding to a globular protein of molecular weight about 100,000. An additional peak having glucokinase and not fructokinase activity always appeared, near the exclusion volume of the gel column. This glucokinase is further described below.

The protein in the hexokinase peak (Figure 6) was then chromatographed directly on DEAE-cellulose, *i.e.*, the treatment at pH 4.6 and ammonium sulfate precipitation steps, used for convenience in preparing large amounts of material, were omitted. In such cases (Figure 7), the A and C peaks are distinct, but B is relatively small and a large new peak[5] (A′) appears, only partly resolved from A. The hexokinase in the A′ peak has an F:G ratio intermediate between that of A and B. The results obtained were identical whether the French press method or the CO_2 homogenization method was used for making the initial extract, so long as each extract was at once protected with DFP and gel filtered to remove proteases. Peak C was then invariably very much larger than peak B, the latter often being scarcely detectable.

[4] An alternative version which we have recently adopted for preparative work employs a larger scale mechanical disintegrator, the Manton-Gaulin Model 15M-8TA Disperser with Cell Rupture Assembly. The yeast, in the thickest compatible suspension in 0.1 M phosphate buffer (pH 7), is broken at 8000 psi in double-pass operation at 4°. The DFP-protected crude extract is processed on an Agarose column (as described in the text, but larger in proportion to the volume used) and then directly by DEAE-cellulose and appropriate further chromatographies. This gives maximal protection with higher yields and speed.

[5] The term hexokinase A′ has already been used by Easterby and Rosemeyer (1969) for a protease-modified form of yeast hexokinase A. However, no evidence was reported that would establish that form as a new species, rather than the equivalent of hexokinase S-I already described by Colowick and colleagues (Schulze *et al.*, 1966; Gazith *et al.*, 1968). Each is obtained after omission of the same protease inhibitor in the preparation, and the pH dependence of molecular weight reported for the A′ of Easterby and Rosemeyer (1969) is very similar to that already shown to be characteristic of the hexokinase S forms (Gazith *et al.*, 1968; Schulze and Colowick, 1969). If any of the hexokinase S forms were to occur in our preparations, they would not correspond to any of the peaks designated here, since the S forms are not eluted by the pH gradient used, but require excess salt for displacement (Schulze *et al.*, 1966).

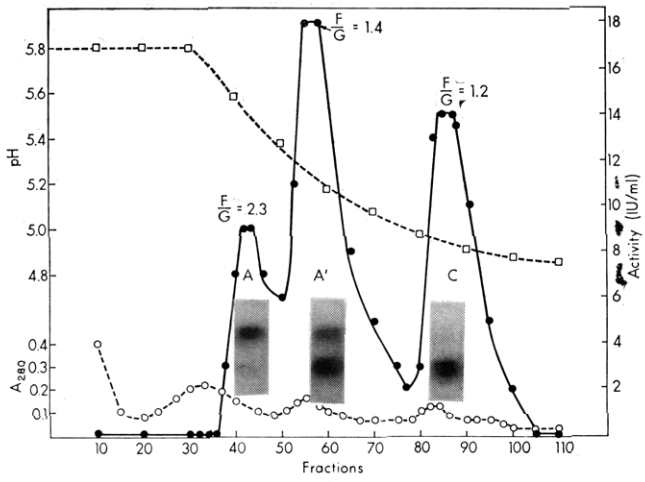

FIGURE 7: Chromatography on DEAE-cellulose of the DFP-protected yeast crude extract. Elution was by a pH gradient (upper curve) in 5 mM succinate. Measurements were made of protein by A_{280} (○), of activity on glucose in International units per milliliter of effluent (●), and of the ratio of activities on fructose and glucose (F:G). Starch gel electrophoretograms of samples taken from the centers of the peaks in such a chromatogram are shown beneath those peaks. Only one segment of the gel is shown, containing all the detectable bands, taken after electrophoresis as in Figure 4; the strips are aligned with respect to the electrophoretic origin below. Electrophoresis of ten samples taken from points throughout the chromatogram showed that only the two species seen here were present.

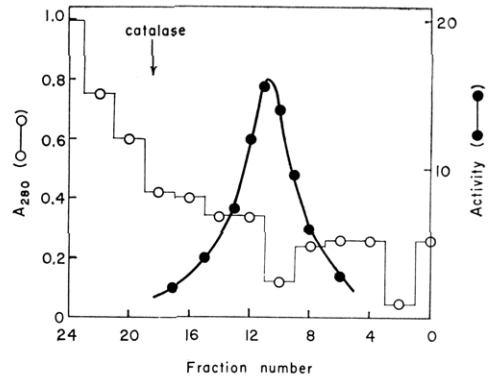

FIGURE 8: Zone sedimentation of yeast glucokinase. The pellet of the enzyme prepared as described in the text and dissolved in 0.2 ml of 5 mM succinate–0.5 mM EDTA (pH 5.8) was layered on a sucrose gradient (5–20%, 4.4 ml) and centrifuged in a SW-39 rotor for 3 hr at 38,000 rpm 12°. Twenty-four fractions of twelve drops each were collected and analyzed for glucokinase activity (in arbitrary units). $s_{20,w}$ of the enzyme, calculated (Martin and Ames, 1961) from the peak position with reference to the catalase marker, was 28.5 S, and the molecular weight is ~1,000,000.

The hexokinase C of Figure 7 produced hexokinase B by the same conversion treatment used above. After purification by repeated chromatography as before, identity was established of the A, B, and C forms produced under these conditions of maximum protection with hexokinase A, B, and C, respectively, obtained in the routine procedure without gel filtration, by cochromatography of each pair on DE-52, when the corresponding sharp peaks coincided precisely. It was concluded that neither the A nor the B nor the C form arises by proteolysis occurring during the preparation.

Although the position of the A peak is fairly close to that of the B peak, A′ is not an artifact of mere overlap of A and B but a distinct peak, lying between the latter two; this was shown by addition of pure B to an extract containing A′, when the separate peak B was augmented, while A′ was unchanged. On rechromatography on DEAE-cellulose of the central part of the A′ peak this species proved to be unstable, in that only A and B peaks were obtained (as confirmed by a subsequent cochromatography with A or B), in the ratio of about 3 parts of B to 1 of A (by protein content), indicating that A′ is a hybrid. However, since A′ is only partly resolved from A and B, the ratio of the subunits of A and B in the hybrid form A′ is not reliably obtained by this chromatography.

Samples were taken for electrophoresis (Figure 7) from each of the chromatographic peaks present. The A peak produced only the expected hexokinase A band, the C peak the expected hexokinase B band (*cf.* Figure 4). The second peak, A′, yielded bands of hexokinases A and B, as shown in Figure 7. It appears that hexokinase A′ is not stable at the pH (5.3) in which it is eluted in the first chromatography. As yet, it cannot be further purified. Other evidence is given by Rustum *et al.* (1971a) to show that a hybrid of A and B forms only in 1:1 ratio, and this is taken to be the constitution of the A′ form seen here.

Glucokinase. The high molecular weight fraction of the gel filtrate of a crude extract (Figure 6, first peak) was found to be active in phosphorylating D-glucose, no detectable activity (at pH 8.5) being found on D-fructose, D-mannose, or on D-galactose (with each sugar at 27 mM concentration). In sucrose gradient centrifugation this glucokinase activity sedimented with material having a molecular weight of about one million (Figure 8).

Electrophoresis of Hexokinases in Initial Extracts. The occurrence of the hexokinases in the yeast cells was further analyzed by starch gel electrophoresis. For this purpose, the extract obtained immediately upon breaking the cells in a French press, with full protection by DFP, was employed. Six bands with hexokinase activity (Figure 9) were identified (anode to cathode) as A (the fastest migrating band), A′, B (or C), and glucokinases I and II. The A and the B (or C) bands were identified by comparison to the pure samples of hexokinases A, B and C (Figure 4). It was not possible to identify the slowest migrating band, denoted as X. This was a minor band and appeared only in certain preparations. Glucokinases I and II were identified by electrophoresis (not shown on the zymogram) of samples taken from the peak of glucokinase activity in the gel filtration chromatogram of Figure 6. The glucokinase molecular weight is ~10^6, and molecules of this size cannot enter the starch gel. Thus, they must have become dissociated under the conditions of the electrophoresis. These bands are not, therefore, a guide to the amount or the identity of the glucokinase in its intracellular form.

The band (2) migrating between A and B (Figure 9) can be compared with the A′ peak in DEAE-cellulose chromatography of the crude extract (Figure 7). Since A′ has a chromatographic mobility intermediate between that of A and B, and since no other major active form was found in the chromatography that could account for band 2, the assignment of band 2 as a hybrid, A′, appeared reasonable.

Hexokinase A′ yields only A and B when examined by electrophoresis after chromatography at pH 5.3 (Figure 7), but appears to be stable (or, more probably, partly stable) in the electrophoresis of the entire crude extract at pH 8.5. It is known (Rustum *et al.*, 1971a) that the hybrid of A and B

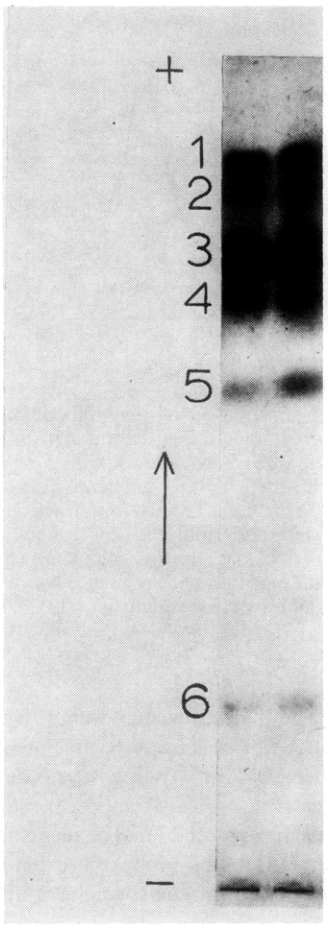

FIGURE 9: Starch gel electrophoresis of the DFP-protected yeast crude extract. The bands are assigned as 1, hexokinase A; 2, hexokinase A′; 3, hexokinase B or C; 4, glucokinase I; 5, glucokinase II; 6, hexokinase X. Staining is for activity on glucose. The top quarter of the gel (devoid of bands) is not shown. Band 3 is always the heaviest; in electrophoresis of smaller quantities it appeared to be a single band.

varies greatly in its stability, depending on the ionic strength and pH of the medium.

Stability of the Hexokinases. After the homogeneous preparations of A or B have been stored frozen in 5 mM sodium succinate (pH 5.5) solution at −20° for about 2 months, several additional bands with hexokinase activity appear in electrophoresis at pH 8.5, all moving somewhat faster anodally. When A, B, or C is frozen even briefly at −20° in 0.1 M sodium phosphate–1 M NaCl (pH 5.5) similar new forms appear upon thawing and 60–80% of the enzymic activity is lost. However, when stored without freezing in these media at 4° (with 10^{-3} M EDTA present as preservative), there is no loss of activity or change in composition up to at least 3 months, so that the changes noted above must be consequent upon freezing-induced dissociation or degradation.

Electrophoretograms obtained as in Figure 9 showed that if the crude extract is maintained at 4° for further periods up to 40 hr (with DFP protection), the glucokinases I and II completely disappear, as does hexokinase X, while bands more anionic than A appear and become more prominent. If the acidification or ammonium sulfate fractionation procedures of the routine preparative method are applied, the glucokinases I and II also disappear.

Characteristics of the Hexokinases. The maximum specific activities of the purest forms isolated, and some F:G values, are listed in Table II.

TABLE IV: Some Kinetic Parameters of Pure Hexokinase Isoenzymes.[a]

Parameter	Values		
	A	B	C
K_m (fructose) (mM)	4.1	2.5[b]	3.3[b]
K_m (glucose) (mM)	0.3[c]	0.6[b]	0.6[b]
F:G (sugar at 0.5 mM)	0.60 (0.53)[d]		
F:G (sugar at 1.0 mM)	0.65 (0.77)[d]		
F:G (sugar at 10 mM)	2.2 (2.2)[d]		
F:G (sugar at 27 mM)	2.6 (2.6)[d]	1.1	1.1
V_{max} (fructose)/ C_{max} (glucose)	3.0	1.3[b]	1.3[b]

[a] Except where noted, all measurements were at 25.0° in 0.0083 M glycylglycine, at pH 8.5, using the cresol red assay method as specified by Lazarus *et al.* (1966). K_m and V_{max} values were obtained by least-squares fitting of $1/v$ *vs.* 1/(S) plots over the range (S) = 5.0×10^{-4} to 2.7×10^{-2} M. ATP was at 3.0 mM and $MgCl_2$ at 13 mM. [b] These values were obtained similarly, but using pH-Stat titration assays, with the Radiometer TTIA Autotitrator with scale expander, in a similar medium at pH 8.0, 25.0°, with $MgCl_2$ 6 mM, over the range (S) = 6.0×10^{-5} to 1.0×10^{-2} M. [c] For the comparable hexokinase P-I of Schulze and Colowick (1969), under slightly different conditions, K_m for glucose has been reported to be 0.17×10^{-3} M at pH 8.5 and K_m for ATP (using 5 mM glucose) 0.3×10^{-3} M at pH 8.5 and 8.0 (Gazith *et al.*, 1968; Kosow and Rose, 1970). [d] Values in parentheses are the theoretical F:G ratios calculated for these conditions from the K_m and V_{max} values listed for the two sugar substrates.

Elementary kinetic comparisons were made using glucose or fructose as the varied substrate, with total ATP·Mg constant at 3.0 mM. The Michaelis–Menten equation was obeyed for A, B, and C enzymes, with linear reciprocal plots, giving K_m and V_{max} values for the sugars (Table IV). It is apparent that the difference in F:G values is not due to a higher degree of saturation with fructose in the case of hexokinase A, since this enzyme actually has the highest K_m for fructose. When F:G is measured in our standard assay conditions all substrates are near saturation, and in practice F:G is close to the ratio of V_{max} values for the enzyme on the two sugars (Table IV). In confirmation of the Michaelis–Menten behavior, F:G is seen to vary with initial sugar concentration in the manner predicted thus.

It is also seen (Table IV) that the K_m values on glucose of hexokinases A, B, and C are quite close, so that the observed differences in specific activity of the isoenzymes are essentially measures of different V_{max} values.

Release of Hexokinase in Various Conditions. Conditions for the maximal release of the various forms of hexokinase were investigated. After the freezing in toluene at −75° and melting at 4°, the hexokinase activity present in soluble form increased steadily (Figure 10). The measured pH of the slurry was 5.6; addition of sodium phosphate (to 0.2 M) gave a sharp increase in the rate of release. The maximal release of hexokinase activity from the total cellular material occurred

at 35° and was unaffected by the presence of glucose or DFP (Figure 10). This, and the very sharp increases seen in each case on raising the temperature, suggest that the release of hexokinase from the cellular material is not due to an enzymic attack, but only to a rupture of residual osmotic barriers that trap the soluble enzyme.

In a separate experiment, intact yeast protoplasts were prepared by means of snail gut enyzmes, releasing the digested cell wall and extracellular fraction (Gascón and Lampen, 1968). After separation of the protoplasts and their lysis and assay, it was found that 100% of the activity in phosphorylating glucose that was present in the yeast cells was retained in the isolated protoplasts, none being detectable in the fraction representing the cell wall and exterior. Hence, the slowly released fraction seen in Figure 10 is not attributable to enzyme bound to the cell walls. Further, Nurminen *et al.* (1970) have reported that when baker's yeast is disintegrated by the thorough grinding of small quantities with glass beads and lightly centrifuged (1000*g*, 10 min) and the cell debris is washed, all of the hexokinase activity is present in the soluble fraction, providing support for the interpretation made here of the release shown in Figure 10.

The hexokinases liberated at various stages of the thawing process (Figure 10) as routinely used, but extended up to 62 hr, were analyzed. The actual melting process (at 4°) is complete after about 20 hr of this period. In all cases hexokinases A, A′, and C were present (Table V), only the total amount of each increasing with time, showing that the forms isolated after the routine 40-hr thawing are not formed from others during this period. However, when further release is forced at 35° (as in Figure 10), evidence for some change in the hexokinase species is obtained (Table V). It was found by chromatography of such extracts that hexokinase C disappears with progressive incubation at 35° in 0.2 M phosphate solution, and the A and B peaks are replaced by a peak in the position of hexokinase A′ (*cf.* Figure 7) having intermediate enzymic properties. When the latter peak was rechromatographed on TEAE-cellulose (Lazarus *et al.*, 1966) it was split as the A′ species (see above), but to form the A peak and a species similar but not identical with B, in that it is eluted at 0.1 pH unit earlier in the chromatogram, and never achieves a specific activity greater than 500 IU/mg despite repeated rechromatography. It is considered that some attack by DFP-resistant proteases occurs in the extract at 35° to give damaged subunits that form an active species.

The insoluble cellular material sedimented at the stage of crude extract preparation contained 17% of the original total activity, and this residual activity can be fully and rapidly released at 35° (Figure 10). However, no species of hexokinase is preferentially lost (Table V) when this residue is discarded, as in the routine preparation.

When the cells were ruptured in the French press, the initial extract contained 60% of the total hexokinase activity present (the potential maximum being estimated from data of Figure 10). When protected and analyzed, the usual proportions of A, A′, and C were present in the initial extract (Table V). Autolysis at 35° or 4° increased the yield in all types of preparation, but, due to the problems just noted, the initial conditions of release at 4°, although necessitating a lower yield, are preferable.

Discussion

The present results extend the previous findings (Lazarus *et al.*, 1966; Schulze and Colowick, 1969) on the preparation

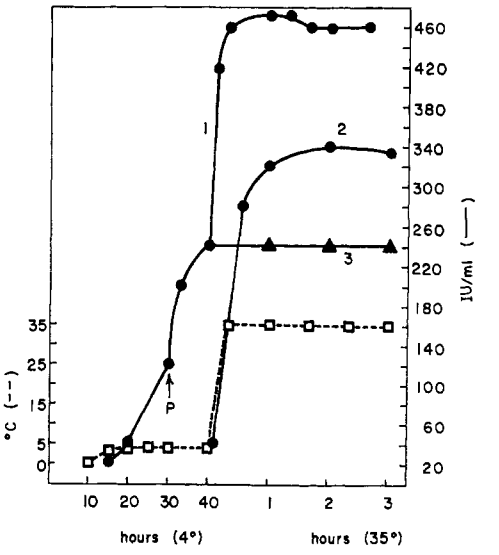

FIGURE 10: Release of hexokinase activity (filled symbols) from thawed yeast cells in various conditions. The slurry was prepared by the toluene–CO_2 procedure and allowed to melt at 4° ambient temperature. The measured temperature of the slurry is shown (□). After 30 hr (point P) the slurry was made 0.2 M in sodium phosphate (pH 5.8). At 40 hr the pH was adjusted to 7.0, and the stirred slurry was divided into three portions (100 ml each); incubations at 35° were made of: (1) the total slurry, in 0.1% glucose–0.01 M DFP solution; (2) the debris of the centrifuged slurry (resuspended in 100 ml of 0.2 M phosphate, pH 7.0); (3) the supernatant of the centrifuged slurry. All the values are expressed per 4-lb initial quantity of yeast. For analysis of the hexokinase species present, see Table V.

TABLE V: Release of Hexokinase in Various Conditions.

Treatment[a]	Activity of Species Present (%)[b] A	A′	B	C
Crude extract, fully protected	18	33		49
Crude extract (toluene)				
30 hr at 4°	16	23		61
62 hr at 4°	19	17		64
Crude extract, 40 hr at 4°, then 3 hr at 35°	20	80		
Slurry, 30 hr at 4°, then 0.5 hr at 35°	22		50	28
Slurry, 30 hr at 4°, then 3 hr at 35°		100		
Debris from crude extract (30 hr at 4°), incubated at 35°, 3 hr, pH 5.6	30	70		

[a] See Figure 10 for further details, except for the first line, where the fully protected crude extract refers to the French press procedure with DFP additions. In all cases, the extract was gel filtered and chromatographed. All samples were at pH 5.6 without added salt. [b] In the analysis by DEAE-cellulose chromatography, the contribution is recorded of the *activity* (on glucose) of each peak to the total hexokinase activity in the chromatogram.

of pure, intact forms of hexokinase (A and B, or P-I and P-II) from yeast, in several respects. These include (i) the separation of hitherto undetected, major, native components (hexokinase C and particulate glucokinase); (ii) elucidation of a number of characteristic features of the various hexokinase forms; (iii) the absence of dependence upon proteolysis or freeze-thawing in the liberation of the major forms from yeast cells; and (iv) revisions in the preparative method in the light of these observations.

The results show that hexokinases A and B produced by the procedure of Lazarus *et al.* (1966) are, in fact, identical chromatographically and in enzymic activity with respectively the A and B forms produced in the most cautious modified version of that procedure. However it has been shown (Rustum *et al.*, 1971) by electrophoresis that slight damage is suffered by the subunits when the earlier procedure is used, although this change is not detectable when they are recombined into the active forms of molecular weight about 100,000. If some autolysis is deliberately permitted, new hexokinase species are produced that *are* detectable by chromatographic (Figure 1a; Table V) and electrophoretic (Figure 4) analysis. Classical preparative procedures for yeast hexokinase start with an autolysis at 37° (Darrow and Colowick, 1962), and, in view of these findings, any version in which that is retained may reasonably be viewed with some reserve until a comparison of the type described here is made. Our original preparative procedure (Lazarus *et al.*, 1966) involved thawing of the frozen yeast at 0–4° for 40 hr, and even this might be, in fact, an opportunity for slow autolysis. Neither the use of DFP (Lazarus *et al.*, 1966) nor of phenylmethanesulfonyl fluoride (Schulze *et al.*, 1966) or similar inhibitors would be a guarantee against alteration during mild autolysis, since proteases not of the serine active-center type may contribute thereto and, indeed, such proteases have been shown to be well represented in yeast cell lysates (Hata *et al.*, 1967). The electrophoretic evidence on the intact enzymes (Figure 4) and on their subunits (Rustum *et al.*, 1971a) shows that a gel filtration—taking advantage of the smaller size of all the known or detectable yeast proteases—is efficient (when combined with the other precautions described earlier) in preventing any such proteolysis.

Since the same results are achieved after rapid cell rupture at 4° by the French press, neither the freezing and thawing nor an enzymic process occurring during the long thawing is necessary for release of any of the species finally purified. Similarly, the acidification and ammonium sulfate fractionation steps in the purification procedure are seen to be without effect on hexokinases A and B, nor on their relative amounts (although the preparation in bulk is more convenient when these steps are retained). Ammonium sulfate treatment can, however, bring about one change, namely the conversion of the native form C to an isoenzyme of it, B. This depends on a high ionic strength (>1 M) at a pH value well removed from neutrality (Table I), and upon a subsequent separation of the protein by gel filtration. All of the evidence so far obtained on hexokinase C is compatible with the proposal that it is a conformational isoenzyme of B, stabilized by a ligand. Further analysis will be reported elsewhere.

Some of the hexokinase B is also initially present in another form, A′, a hybrid (Rustum *et al.*, 1971a) of hexokinases A and B. A′ was shown to be a quite distinct species by co-chromatographic experiments. A band corresponding to A′ was found in the electrophoresis of the initial extract (Figure 9). A question posed by Lazarus *et al.* (1966), enquiring whether the forms finally purified are modified by some enzymic release from intracellular sites of hexokinase binding, is now answered. The entirely soluble nature of all of the activity released upon yeast cell lysis is shown by data in Figure 10, and by the total activity estimations of lysed yeast cells reported by Nurminen *et al.* (1970). When the cells are ruptured, part of the activity is initially insoluble, but this is most probably merely trapped by osmotic barriers in the cell debris. No enzymic cleavage seems to be necessary to release into solution any of the hexokinases of the yeast cell. From all of the evidence obtained (Figures 6, 7, and 9; Table V, line 1) it is concluded that the forms of hexokinase that exist in the yeast cell are A, A′, and C and, in much smaller amount, the high molecular weight specific glucokinase.

Acknowledgments

We thank Dr. Mary P. Bell for her assistance with some of the preparations of hexokinase described here.

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