phys. Acta 80, 441.
Rushizky, G., and Sober, H. A. (1963), *J. Biol. Chem. 238*, 371.
Sarin, P. S., Zamecnik, P. C., Bergquist, P. L., and Scott, J. F. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 579.
Tener, G. M., Gillam, I., von Tigerstrom, M., Millward, S., and Wimmer, E. (1966), *Federation Proc. 25*, 519.
Weiss, G. B., and Legault-Demare, J. (1965), *Science 149*, 429.
Yoshida, M., and Ukita, T. (1965a), *J. Biochem. (Tokyo) 57*, 818.
Yoshida, M., and Ukita, T. (1965b), *J. Biochem. (Tokyo) 58*, 191.
Zamir, A., Holley, R. W., and Marquisee, M. (1965), *J. Biol. Chem. 240*, 1267.

Yeast Hexokinase. I. Preparation of the Pure Enzyme*

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ABSTRACT: A method is described for the preparation of baker's yeast hexokinase, which can be adapted for the preparation of yeast enzymes located at sites similar to that of hexokinase. It has been demonstrated by studies of the contaminating proteases that it is possible to isolate such an enzyme from yeast without apparent proteolytic degradation. This is achieved by rigorous maintenance of conditions ensuring the inhibition, including organophosphate inhibition, of the proteolytic enzymes. The procedure is then superior to previous hexokinase preparations in respect to the lack of degradation, the yield, the specific enzymic activity, the stability, and the preparation time. Two hexokinase species appear to be present under conditions of isolation, hexokinase A (in equal or larger amount) and hexokinase B. Each is a homogeneous protein by a number of criteria. Hexokinase B, as judged by criteria of homogeneity, purity, and stability, is a proteolytically undegraded enzyme having a specific activity of 800 units/mg at 25°; this is the highest reported for yeast hexokinase. Hexokinase A has a lower specific activity. Substrate specificity, chromatographic elution position, and electrophoretic mobility differences exist between these two species.

For active center and subunit studies on a kinase enzyme, baker's yeast hexokinase (ATP[1]:D-hexose 6-phosphotransferase, EC 2. 7. 1. 1) offers some advantages, but it is desirable for such studies to secure the enzyme in a homogeneous and completely undegraded form. Present methods of preparation, which involve a deliberate autolysis at 37° for its release from the yeast, create difficulties in this respect. We have examined other methods for this release, and describe here a new procedure for hexokinase purification that throughout minimizes proteolytic attack. The enzyme thus obtained is stable and homogeneous.

Yeast hexokinase was first partially purified by Meyerhof (1927) and later by N. van Heyningen;[2] crystallization was performed by Cori *et al.*[3] (Bailey and Webb, 1948). Later Kunitz and McDonald (1946), using ammonium sulfate fractionation for the first time in the hexokinase purification procedure, also isolated and crystallized the enzyme. The yields in the Kunitz and McDonald (1946) method and the Bailey and Webb (1948) modification of this procedure did not encourage large-scale studies of the enzyme. More recently, Ågren *et al.* (1963) and Darrow and Colowick (1962) have described improved methods of purification. The former workers, using commercially available enzyme, purified it by chromatography on CM-cellulose followed by chromatography on DEAE-cellulose. A sedimentation analysis of the enzyme, in 0.5% glucose at pH 7.0, yielded a single boundary with a sedimentation coefficient of 4.0 S. A determination of the molecular weight by the Archibald method gave a value of 50,000. This discrepancy with the molecular weight of 96,000 as reported by Kunitz and McDonald (1946) was thought to be due to cleavage of the molecule by glucose as had previously been

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[1] Abbreviations used in this work: ATP, adenosine 5′-triphosphate; TCA, trichloroacetic acid; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; BAEE, *N*-benzoyl-L-arginine ethyl ester; TPCK, *N*-tosyl-L-phenylethyl chloromethyl ketone; PMB, *p*-mercuribenzoate.

[2] Cited by M. Dixon, in Report to Ministry of Supply, No. 10, London, 1942.

[3] C. F. Cori, S. P. Colowick, L. Berger, and M. W. Slein (1942) cited by Bailey and Webb (1948).

noted by Schachman (1960) and Ramel *et al.* (1961). Agren *et al.* further state that the specific activity of their preparation is not as high as that obtained by the Darrow and Colowick procedure. The material from the Darrow and Colowick procedure, though repeatedly recrystallized, exhibits upon column chromatography six active forms, designated A, B, C, D, E, and F in order of elution from DEAE-cellulose (Trayser and Colowick, 1961). It has, furthermore, been shown that some of these chromatographic forms are produced during the isolation procedure by the action of yeast proteolytic enzymes (Ramel, 1964; Kenkare *et al.*, 1964). Even the five-times recrystallized enzyme can be shown to carry proteolytic contamination.

The heterogeneous nature of these various products naturally raises questions regarding changes suffered by the enzyme during isolation. We have directed our attention to this problem. We have also sought to simplify the purification procedure as well as to increase the purity and the yield of the enzyme. In the succeeding paper (Derechin *et al.*, 1966), the enzyme thus obtained is characterized physically.

Experimental Section

Materials. All chemicals unless otherwise specified were Fisher certified reagent grade. Tris was Fisher primary standard reagent. Glycylglycine (free base), ATP–disodium salt[1] (99–100% assayed and corrected for 2–3 H_2O/mole), and the DFP were obtained from Sigma Chemical Co. The DFP was stored at −20° as approximately 1 M solution in anhydrous propanediol (1 g of DFP to 4 ml of solvent).[4] Trypsin (two times crystallized, salt free, 180 units/mg) and α-chymotrypsin (three times crystallized, salt free, 45 units/mg) were purchased from Worthington Biochemical Corp., Freehold, N. J. BTEE and BAEE (grade M.A.) were products of Mann Research Labs. Inc., New York, N. Y., and TPCK (grade I) was from Cyclo Chemical Corp., Los Angeles, Calif. Casein (Hammersten quality) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. 3-Deoxy-D-glucose and 3-deoxy-D-mannose were kindly supplied by Dr. Daniel H. Murray, School of Pharmacy (State University of New York, Buffalo, N. Y.), and were samples of those characterized by Murray and Prokop (1965).

The water used throughout this procedure was doubly deionized by running distilled water through two hose-type mixed-bed cartridges. The dialysis tubing was purchased from Union Carbide Corp. Tubing (1-in. width) was acetylated to reduce permeability, in a solution of 20% (v/v) acetic anhydride in glacial acetic acid for 24 hr at room temperature. The tubings were washed free of reagent with excess of deionized water, and then stored in deionized water to which 1 mg/l. of EDTA was added. For small volumes, narrow (3/8 in.) tubing, which is less porous, was used without acetylation, but was pretreated in a solution of 5% $NaHCO_3$ at 80° for 30 min (to remove ultraviolet-absorbing plasticizers) and then thoroughly washed.

Fleischmann's baker's yeast was used. The yeast was either immediately processed, or stored at 4°. Storage was never longer than 1 week.

DEAE-cellulose was Selectacel standard grade no. 70 (lot no. 1595) of 0.91-mequiv/g capacity from Schleicher and Schuell Co., Keene, N. H.

TEAE-cellulose was Cellex T from Bio-Rad Laboratories, Richmond, Calif. Samples of the following batches gave successful results: 2593, 0.53 mequiv/g; 2612, 0.54 mequiv/g; 2690, 0.55 mequiv/g. No resolution was obtained with batches 3017, 0.41 mequiv/g or 3212, 0.50 mequiv/g.

Centrifugations. High-speed, small-volume centrifugations were performed on Spinco Model L using the type 40 rotor. All other centrifugations were performed with an International refrigerated centrifuge, Model HR-1. Larger volumes were centrifuged with the six-place angle-head 858 in 250-ml tubes. Smaller volumes with head 856 (eight place) in 50-ml tubes.

Protein concentrations were determined by the method of Lowry *et al.* (1951), as modified by Layne (1957), and spectrophotometrically at 280 mμ, assuming a specific absorptivity of 1 ml mg^{-1} cm^{-1} at lower purification levels. The specific enzymic activities at these levels were calculated on this basis. At high levels of purification, the exact value we have determined for hexokinase of 0.92 ml mg^{-1} cm^{-1} was used. A Cary Model 15 was used for all spectrophotometry.

Preparation of the Exchanger. The dry exchanger is uniformly dispersed in water. After the coarse material has settled the supernatant with the fines is removed by decantation. This procedure is repeated three times. The material is then sucked to "dryness" on a coarse sintered glass funnel, resuspended in 0.2 N NaOH, filtered on the funnel, resuspended in 0.2 N HCl, filtered, and washed to near neutrality on the funnel with deionized water. Finally it is sucked to dryness and stored at 4° in tightly closed containers.[5]

Preparation of Columns

(a) DEAE-cellulose (60 g), prepared as described above, is suspended in 400 ml of 0.1 M sodium phosphate of pH 6.75. The suspension is poured into a column of 50-cm height, and 2-cm diameter, and allowed to settle with open outflow. The loosely packed exchanger bed is then compressed by a packing rod to a height of 30 cm. The exchanger is equilibrated at 4° with 4 l. of 5 mM succinate–1 mM EDTA, at pH 6.75. (b) TEAE-cellulose (30 g), pretreated a sdescribed above, is resuspended in 200 ml of 0.1 M sodium phosphate of pH

[4] The freezing of material to which DFP has been added results in precipitation of the enzyme with concomitant loss of activity. It is recommended, therefore, that dialysis follow the addition of DFP.

[5] Recycling of used DEAE-cellulose by this procedure, however, has been repeatedly shown to be unable to regenerate original performance. The TEAE-cellulose can be recycled repeatedly with no loss in performance.

6.0 and poured into a column of 25-cm height and 1.6-cm diameter. In pouring the column a hydrostatic pressure head of 145 cm is convenient. This is achieved by extending the column with a glass tube of 120-cm length and 1.6-cm diameter. The 30 g of cellulose packs to a column height of about 25 cm, when allowed to settle under the conditions described. The cellulose is then compressed to a height of 15–18 cm. The striations observed after compression have no influence on the quality of the chromatogram. They can be avoided if the resin is slowly compressed with the outflow valve closed. Equilibration is accomplished by perfusing the column with 250 ml of 0.2 M succinate–0.5 mM EDTA, at pH 6.0, followed by 1 l. of 5 mM succinate–0.5 mM EDTA, of the same pH. The columns, prepared in the way described, are used both for the second chromatographic step and the rechromatography.

Assay for Hexokinase. Our procedure follows closely the method of Darrow and Colowick (1962). A few modifications are introduced for convenience. Stock solutions are: 0.1 M ATP–disodium salt, 0.1 M glycylglycine, 1.8% $MgCl_2 \cdot 6H_2O$–0.006% cresol red, and 0.2 M glucose. For the assay solution (stored in the cold), 2 ml of ATP and 5 ml of glycylglycine are mixed with 10 ml of the $MgCl_2$–cresol red solution. The pH is adjusted to 8.5 with 0.1 N NaOH (about 7.4 ml), and the volume to 50 ml with deionized water. Standardization of the assay mixture: in a cuvet of 1-cm light path, 2.5 ml of assay solution is mixed with 0.4 ml of 0.2 M glucose. The absorbance at 574 mμ at 25° should read between 0.6 and 0.8, depending on the pH and the purity of the cresol red used. HCl (0.1 N) (100 μl) is then added to give a change of absorbance in the region of 0.3–0.5. If the reading does not fall within these limits, and if the pH is correct, it can be concluded that the amount of cresol red has to be adjusted. The exact value for each particular assay solution is of no importance provided that this value is established anew for each batch. The standard value does not change, unless the assay solution remains at room temperature for several hours. Assay procedure: to the mixture of 2.5-ml assay solution with 0.4 ml of 0.2 M glucose 100 μl of enzyme solution is added, and the change in absorbance is recorded with time. An amount of enzyme is used such that the tangent at zero time can be conveniently drawn. One unit is defined as that amount of enzyme which catalyzes the formation of 1 μmole of acid/min at 25° under these conditions. The temperature correction factor between 25 and 30° is 1.5, derived from a measured Q_{10} of 2.2.

Assay for Proteolytic Contamination. Contaminating proteases present at various stages of the hexokinase purification process were determined by three methods. In the first, the casein digestion method of Kunitz (1947) was used. The version described by Laskowsky (1956) was followed, but with the conditions of temperature, pH, and exposure time stated below for each case. The extent of proteolysis was expressed either by the absorbance increment at 280 mμ due to formation of TCA-soluble material during proteolysis, or by comparison with a trypsin standard run under the same conditions. The second method, self-digestion, was used when the conditions under study were such as to make the casein digestion method inapplicable. The degree of proteolysis was then expressed by the amount of TCA-soluble material formed per mg of sample protein due to self-digestion. In the third method esterase activity was assayed against BTEE by the spectrophotometric method of Hummel (1959), and against BAEE by the method of Schwert and Takenaka (1955).

Purification Procedure.[6]

Preparation of Crude Extract. Yeast (8 lb) is crumbled into a container holding 8 l. of toluene, previously cooled by the addition of excess Dry Ice. The temperature is kept below −20° for a period of 6 hr either by continued addition of Dry Ice or by placing the container in a −20° room. The toluene is then decanted and the frozen yeast is divided equally between two 4-l. beakers. It is allowed to melt in a 4° refrigerator over a period of 36 hr. Once the toluene is decanted the frozen yeast can be kept indefinitely at −20°. *All* subsequent steps must be carried out between 0 and 4°. The slurry, obtained after melting, is centrifuged at 15,000 rpm for 30 min in the refrigerated centrifuge and the supernatant is collected.

Acid Fractionation. The crude extract is acidified with acetic acid (3 N) to pH 4.5, keeping the temperature compensator of the pH meter (*e.g.*, radiometer Model TTTlC, as we have used) at 25°. The acid must be added carefully to avoid high local concentrations. The extract must be stirred continuously, preferably with a magnetic stirrer, but without formation of foam. Continuous monitoring of the pH is necessary to ensure that it does not drop below 4.5, because lower values cause loss of activity. The acidified material is then held at 4° for 24 hr, during which time copious precipitation occurs.

Ammonium Sulfate Fractionation. The precipitate is removed by centrifugation at 14,000 rpm for 30 min, and the clear supernatant (about 900 ml) is adjusted to 0.4 saturation by gradually adding 24.3 g of ammonium sulfate/100 ml of supernatant. The precipitate is removed by centrifugation at 14,000 rpm for 30 min, and the salt saturation of the supernatant is adjusted to 0.55 by the further addition of 9.7 g of ammonium sulfate/100 ml. The precipitate so formed is collected by centrifugation at 14,000 rpm for 30 min, and taken up in 40 ml of ice water. The green-brown enzyme solution is adjusted in an ice bath to pH 7 by slowly adding 2 N NaOH, with magnetic stirring. DFP (500 μl/100 ml of 1 M) solution is cautiously added to the neutralized solution while it is stirred. After 30 min the pH is adjusted to 5.5 by adding 2 N HCl. The

[6] All the buffers used in this procedure are adjusted to the stated pH (to 0.01 unit) at 25°.

precipitate formed during these manipulations is finally removed by centrifugation.

The clear supernatant is then dialyzed against two precooled 16-l. baths of 5 mM succinate–1 mM EDTA solution at pH 5.5, for 15 and 5 hr, respectively. Completion of dialysis is verified by testing for sulfate with $BaCl_2$. The enzyme solution must be completely sulfate free before it can be used for the subsequent steps. The solution can be frozen at this stage and kept at $-20°$ until further processing. The same is true after each of the two chromatographic procedures. The apparent specific activity before dialysis is about 15 units/mg. It rises to a value of about 25 units/mg after dialysis, essentially due to removal of low molecular weight material absorbing strongly at 260 mμ.

Chromatography on DEAE-Cellulose. Dialysate (about 30 ml) containing 1250 mg of protein is allowed to enter the DEAE-column under gravity. The pH of the dialysate is 5.5, while the pH of the equilibrated column is 6.75.

STAGE 1. The column is now percolated with 400 ml of phosphate buffer obtained from a linear gradient device of the following composition. A reservoir, containing 300 ml of 50 mM sodium phosphate buffer, pH 6.75, is connected to a mixing chamber containing 300 ml of 5 mM sodium phosphate buffer at pH 6.75. The 400-ml effluent is collected into a single beaker and can be discarded, since it contains protein but no hexokinase. The flow rate should be 100 ml/hr, a value which is usually the maximum flow rate obtained with all columns operated with a hydrostatic pressure head of 150 cm, measured between the tip of the column and the gradient device.

STAGE 2. Chromatography is continued in this stage by a linear NaCl gradient in 50 mM sodium phosphate buffer at pH 6.75, generated with the same gradient device as used in stage 1. The reservoir contains 400 ml of 50 mM sodium phosphate–350 mM NaCl at pH 6.75; the mixing vessel 400 ml of 50 mM sodium phosphate at pH 6.75. The outflow is collected in fractions of 6 ml. Flow rate and the hydrostatic pressure head should be the same as in stage 1. The activity is eluted between tubes 20 and 50, with a peak of about 250 units/mg at tube 23. The fractions of 190 units/mg and higher activity are pooled, usually amounting to 30–50% of the total initial activity.

Chromatography on TEAE-Cellulose. To the enzyme solution obtained in the former step 1 M DFP solution 100 μl is added for each 100 ml of solution, prior to dialysis.[4] The solution is then dialyzed against 4 l. of 5 mM succinate–1 mM EDTA, pH 6.0, for 15 hr. About 60 mg of enzyme can be processed in this step. The enzyme is then adsorbed on the TEAE-column at a flow rate of about 15 ml/hr. The enzyme is eluted from the column with a pH gradient generated by a constant volume method. The constant-volume mixing chamber should contain 125 ml of 5 mM succinate–1 mM EDTA, pH 6.0, and the reservoir contains the same buffer at pH 4.6. This latter buffer is made by back-titrating the pH 6.0 buffer to pH 4.6 with HCl. The gradient device is placed above the column with a hydrostatic pressure head of 100–150 cm. By this means the required flow rate of 25 ml/hr can always be established. The fraction volume should be 5 ml/tube. All the tubes above 600 units/mg are pooled, yielding 80% of the total activity placed on the column.

Final Rechromatography. The TEAE-cellulose chromatographic procedure is repeated. The enzyme solution is adjusted to pH 6.0 with solid Tris prior to adsorption on the column.

Concentration Procedure. The enzyme is concentrated, when required, as follows. The enzyme obtained in the second or the rechromatography step is adjusted to pH 8.0 by cautiously adding solid Tris while gently stirring on a magnetic stirrer. The enzyme is then adsorbed (at 25 ml/hr) on a well-packed DEAE-cellulose column having a volume of 1 (for ~25 mg of protein) or 3 ml (for ~60 mg), equilibrated with 5 mM sodium phosphate–1 mM EDTA, at pH 7.8–8.0. It can be quantitatively eluted with 1.0 M NaCl in 0.1 M sodium phosphate of pH 5.5. The collection of the enzyme in the case of 1- and 3-ml columns can be started after a volume of 0.5 and 1.5 ml has been discarded from each column, respectively. It is possible to determine this point with an accuracy of 1 drop, by collecting the outflow drop by drop into a large volume of 10% TCA solution: collection begins after the drop that causes turbidity. The concentration rises very sharply with the next drop, and the enzyme is eluted to an extent of about 80% with 1.0 and 1.5 ml (for these two columns) of the NaCl–phosphate solution. Elution is complete when about 2 ml and 3.0 ml, respectively, are collected. The columns can be used repeatedly without repacking after reequilibration.

Crystallization. The method of Darrow and Colowick (1962) is used. However, ammonium sulfate recrystallized from a slightly ammoniacal solution of EDTA (2×10^{-2} M) should preferably be used for the pure enzyme. The material, after the second chromatography, crystallizes with great ease. Multiple recrystallizations are, however, neither needed nor recommended, as they lead to some activity loss.

Results

Preparation of Crude Extract. Disintegration of the yeast cells has been accomplished by three methods. The first method consisted of homogenizing small pieces of Dry Ice in an electric homogenizer and then crumbling about 200 g of yeast cake into the Dry Ice. The mixture was homogenized for 5 min (van Hofsten and Tjeder, 1961). In the other procedures ether or toluene was cooled to $-30°$ with Dry Ice prior to crumbling the yeast cake into the cooled mixture. While the final specific activities obtained by all three methods are comparable, the homogenization procedure was not used further. It could deal only with small quantities, and gave the lowest yields because of the inefficiency of rupturing the cells. Only about 40% of the cells were seen under microscopic examination to be disintegrated. Ether and toluene are equally effective both in the amount of yeast cells disintegrated

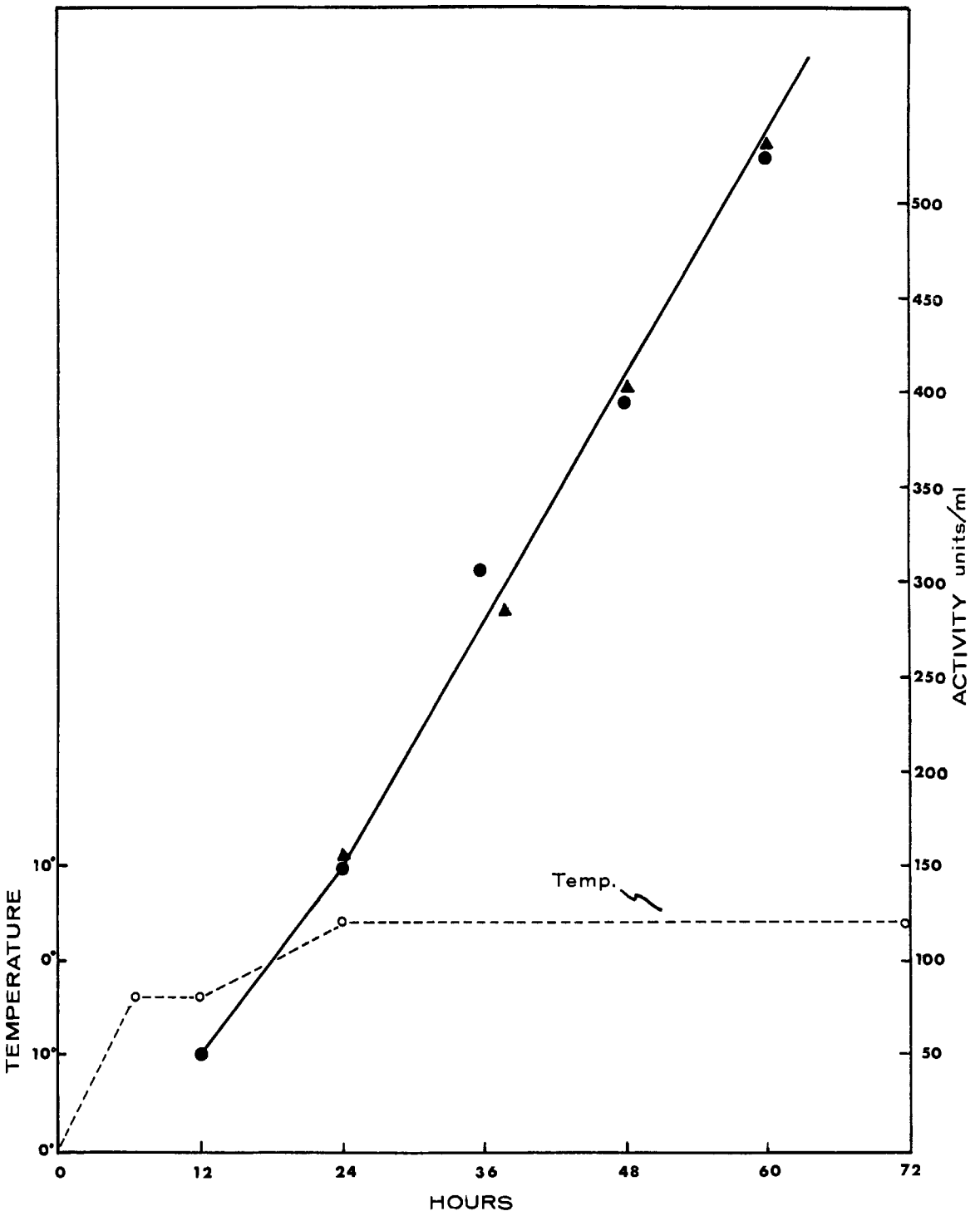

FIGURE 1: Time and temperature dependence of the appearance of yeast hexokinase activity in the crude extract. Activity (right-hand ordinate) is shown for melting in the absence (●) of DFP or in presence (▲) of DFP (5×10^{-2} M). Temperature (left-hand ordinate) (○– – –○) was read at one point within the yeast cells. Hexokinase activity begins to appear at −4° when melting (seen from the eutectic line) occurs. Samples removed before this melting at 0–6 hr show a low activity similar to the first melted sample at 12 hr, but this activity is due to the thawing introduced in order to assay these.

and the final yield obtained. Ether, because it requires extremely cautious handling methods, is not recommended for general use. In addition, it required complete removal from the material under a strong fan draught at −20° for 36 hr, since residual traces of ether led to some inactivation of hexokinase. Toluene

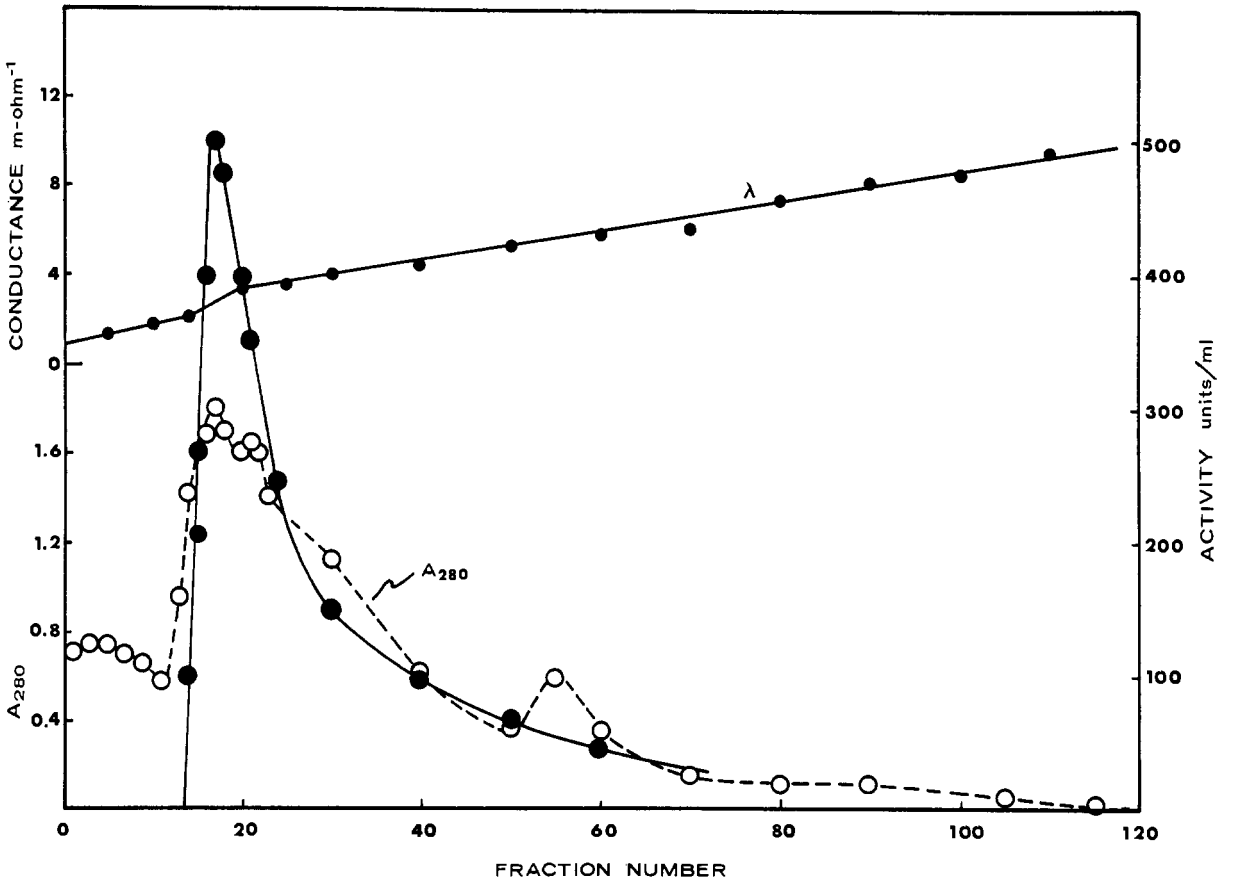

FIGURE 2: Chromatography on DEAE-cellulose (phase II) of the dialyzed ammonium sulfate fraction. ●——●, hexokinase activity in units per milliliter at 25°. O- - -O, protein concentration measured at 280 mμ. Elution by linear NaCl gradient in sodium phosphate (5×10^{-2} M, pH 6.75). The gradient indicated is the observed conductance in the fractions and is shown in the upper graph.

was used for all routine preparations. During the melting incubation of the yeast, hexokinase activity begins to be released when the temperature is above −4°, which appears to be the eutectic temperature of the mixture (Figure 1). The liberation of hexokinase is not influenced by the addition of DFP to the slurry. The amount of hexokinase liberated rises to a maximum over 72 hr and then levels off. The melting incubation was halted after 36 hr, so as to minimize exposure to the proteolytic enzymes present. At the stage of the dialysate (after the acid and the ammonium sulfate fractionations) the following enzymes were tested for by standard methods and were found to be absent: β-aspartokinase, creatine kinase, glycerate kinase, glycerokinase, and aldolase.

DEAE-Cellulose Chromatography. FIRST PHASE. The column was washed with 400 ml of phosphate buffer removing 400 mg of the 1250 mg applied. The maximum amount of protein that can be eluted, even with larger volumes, is 75% of that applied to the column. Washing the column to completion is, however, not recommended because the subsequent sodium chloride gradient will not yield hexokinase of specific activity greater than 150 units/mg.

SECOND PHASE. The sodium chloride gradient elutes 80% of the total activity placed on the column. Of this only 30–50% is of sufficiently high specific activity to be collected. The rest is generally discarded as it has been found to give unsatisfactory chromatography if it is dialyzed and rechromatographed. This portion is, however, retained in those cases in which a total analysis of the activity profile is desired (Figure 2).

TEAE-Cellulose Chromatography. As stated previously, some batches of TEAE-cellulose gave unsatisfactory behavior. All samples which give good results display a smooth titration curve in the region of interest (*ca.* pH 6) with practically no indication of a titrating group in this region[7] (and were more fibrous, and brownish instead of white). The celluloses causing

[7] Titrations of cellulose were performed as described in the catalog "Materials of Ion Exchange Absorption and Gel Filtration" supplied by the Bio-Rad Laboratories.

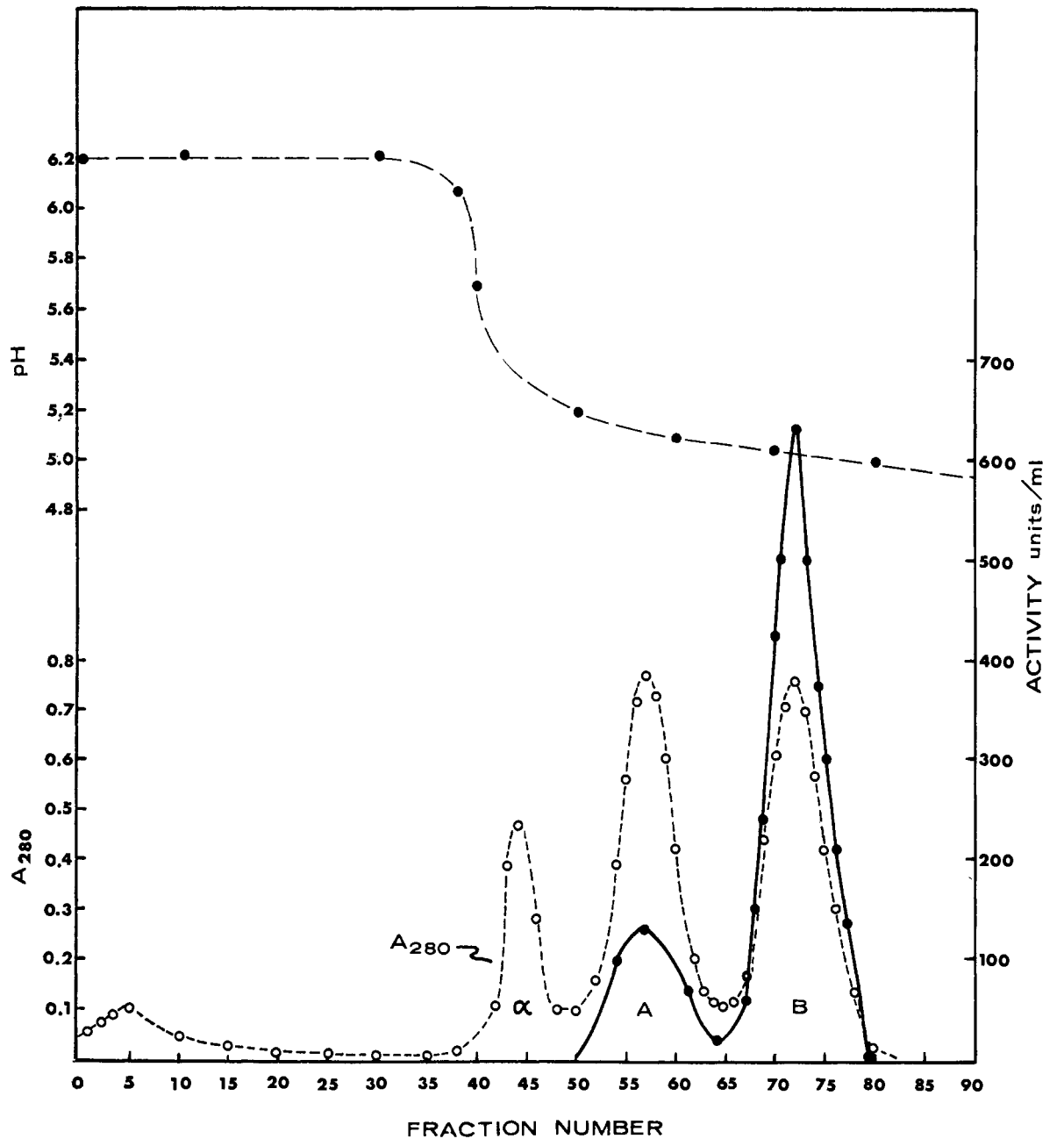

FIGURE 3: Chromatography on TEAE-cellulose of the eluate from the first column. ●——●, hexokinase activity in units per milliliter at 25°. O- - -O, protein concentration, measured at 280 mμ. Elution is by pH gradient, pH 6.00 to 4.60; observed pH values (on upper scale).

complete failure of chromatographic resolution show a conspicuous inflection around pH 6.0, indicating the presence of a group of pK 6.0. The most reliable criterion for anion-exchange cellulose of the required properties is the shape of the gradient curve obtained from a column without protein applied. All the samples which lead to a complete failure are characterized by a near-vertical decline of the pH from 6.0 to 5.0 within 3–5 tubes starting around tube no. 70, when run under the conditions described above. The successful celluloses display this drop in pH around tube 40. The sharp drop in pH ends around pH 5.6 and a subsequent gradual decline of the pH is observed. This decline coincides with the theoretical gradient curve obtained under the same conditions without cellulose. Since the two hexokinases in question chromatograph at pH ~5.3 and ~5.1, respectively, no resolution can be expected with celluloses of the former type, where the gradually declining titration region is missing.

On analysis of the protein profile it will be seen that

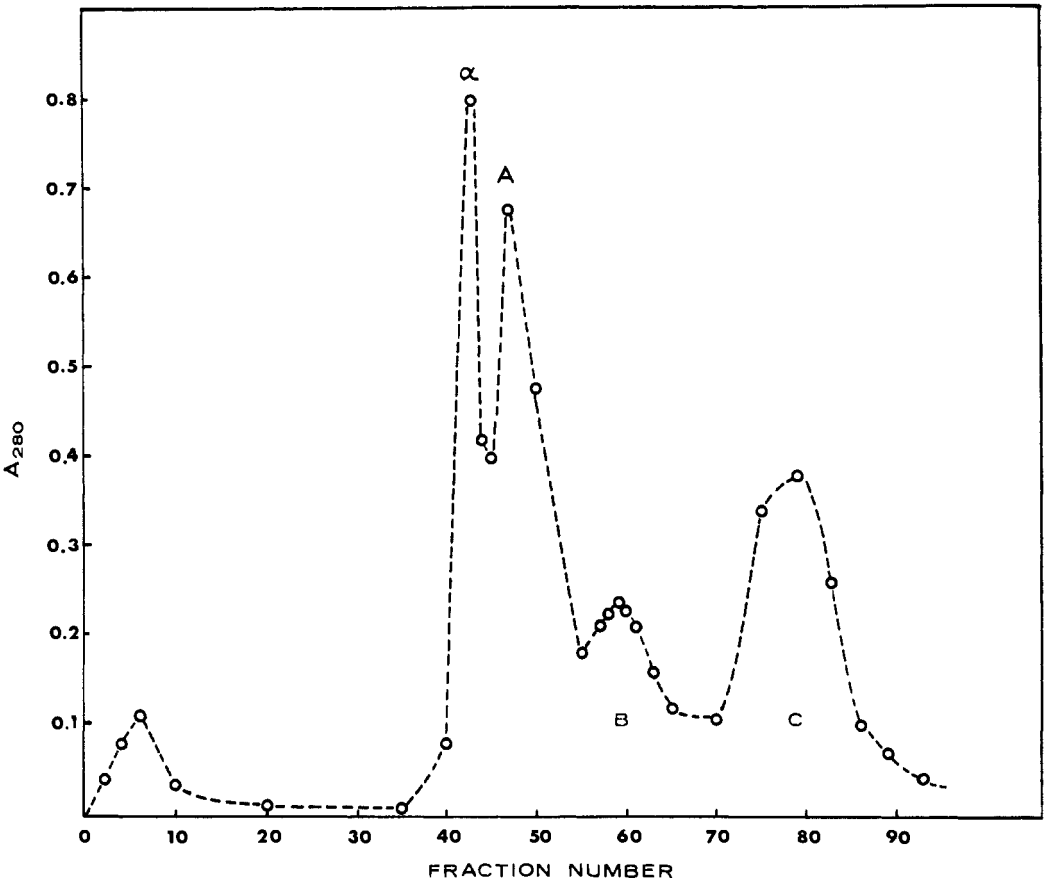

FIGURE 4: Chromatography on TEAE-cellulose of the first column eluate when unprotected by DFP. Protein concentration was measured at 280 mμ. Comparison with Figure 3 shows the appearance here of a fourth peak, designated C. The amount of material under peak C corresponds to the loss of material noted in peak B. The specific enzymic activities of the peak fractions were 170 (A peak), 300 (B peak), and 370 (C peak) units per milligram (at 25°). The first peak was inactive, indicating its equivalence to the α peak of Figure 3.

three clearly separated peaks are eluted (Figure 3). The material under the first peak (α) is inactive. The material of the second peak (around tube 55), designated peak A, is active hexokinase; the specific activity of the peak tube in A was found never to surmount values of 250 units/mg. The third peak (located around tube 70), designated peak B, comprises highly active hexokinase. The peak tubes have a specific activity of 650–750 units/mg. Peak B represents about 80–90% of the activity placed on the column. It has been found that the proportion of peak A to peak B (in the chromatograms of the fraction applied routinely to the second column) varies from one preparation to another, with the ratio of the protein amount in A and B lying between 0.5 and 1. If the material from the DEAE-cellulose column was not treated with DFP prior to the 15-hr dialysis at 4° at pH 6.0, the pattern of active species obtained in the TEAE-cellulose chromatography was then altered from the normal. The material in peak B was reduced by about 60% of its expected amount while a fourth peak eluting between tubes 75 and 100 appeared. This peak accounted for nearly all of the protein missing from the B peak. On rechromatography this new peak can attain a specific activity of 675 units/mg. The A peak was unaltered in amount. This interesting observation suggests that proteolytic attack is directed mainly at the B peak to produce a new active form, but that A does not arise by such proteolysis (Figure 4).

When chromatography of the discarded fraction from the first column was carried out on TEAE-cellulose, it was found to contain further amounts of peaks A and B. Peak A was present in approximately three times the amount of B but the specific activities of both species never approached those of the original cut. Only the latter was, therefore, used for the preparation of the pure enzyme. On occasion the third peak found in the unprotected preparation could also be detected in this portion of the DEAE-cellulose-chromatographed material. The amounts in this peak accounted for only a small portion of the total activity eluted from the DEAE-cellulose column.

Rechromatography. When peak A and peak B were separately rechromatographed on TEAE-cellulose under

TABLE I: Summary of Purification Procedure.

Step	Vol. (ml)	Total Protein (mg)	Total Act. (units)	Sp Act. (units/mg at 25°)	Yield (%)
Crude extract	850	200,000	360,000	1.8	100
Supernatant after acid fractionation	800	60,000	300,000	5.0	83
Supernatant after $(NH_4)_2SO_4$	95	11,000	180,000	16.3	60
Dialyzed enzyme	145	6,800	180,000	26.5	60
DEAE-cellulose eluate	200	370	81,000	220	22.5
TEAE-cellulose eluate	—	100	65,000	650	18.1
Rechromatography	—	70	52,000	750	14.4

TABLE II: Proteolytic Contamination as Assayed by the Casein Digestion Method at Various Stages of the Purification Process.

Sample	DFP[a]	Conditions	ΔA_{280}/mg of Protein	Ratio[b]	Trypsin Equiv (μg/mg of protein)
Crude extract	−	Standard[c]	0.250		3.58
After first dialysis	−	Standard	0.125	23	2.42
	+	5 mM succinate, pH 5.5, 4°, 24 hr	0.0076[d]		
First column eluate	−	Standard	0.040	260	0.69
	+	5 mM succinate, pH 6, 4°, 24 hr	0.000154[d]		

[a] 5×10^{-3} M final concentration. [b] Ratio of proteolytic activities in DFP-untreated and treated cases. [c] 0.057 M Tris, pH 7.7, ionic strength 0.11, 0.0025 M $CaCl_2$, 35°, 10 min. [d] Interpolated to 10 min assuming linearity with time.

the conditions described, both peaks eluted at their characteristic positions, *i.e.*, they behave as two distinct species. The specific activity of peak A even after rechromatography does not rise above 250 units/mg. Under peak B the specific activity is found to be uniform over the whole peak and in the region of 800 units/mg. Further chromatography does not increase the specific activity.[8] The rechromatographed hexokinase B material, when crystallized by the method of Darrow and Colowick (1962), gives crystals instantaneously, but this step was not routinely applied. A summary of the purification procedure is given in Table I.

Proteolytic Contamination. From the start it was observed that some yeast proteases remain with hexokinase after ammonium sulfate fractionation and these give degraded forms of the enzyme even though some hexokinase activity may remain. Precautions described under Methods were, therefore, rigorously applied to prevent proteolysis, at all stages. Examinations were made for the level of proteolytic activity present at three main stages, namely after acidification, during the dialysis of the ammonium sulfate fraction and during dialysis of the first column eluate. The actual proteolytic activity, under the precise conditions of isolation, was measured on both protein and synthetic substrate and its effect on hexokinase activity followed. Parallel experiments were carried out to determine the total extent of proteolytic activity present in the unprotected state in arbitrary standard conditions (pH 7.5, 35°).

Thus, in the case of the casein digestion assay under standard conditions, to determine the total potential protease activity present, the protein samples were adjusted to these conditions by dialysis against the assay buffer without any addition of DFP. To measure caseinolytic activity under isolation conditions, the casein solution itself was adjusted by dialysis to the

[8] It must be emphasized that the yields and activities that we report would be about 50% higher on the scale used by Darrow and Colowick (1962) and in most previous reports. There the units are determined at 30°, whereas we use the more usual standard of 25°. Since the Q_{10} has been found to be 2.2, the activity values we report must be increased by 50% for the comparison.

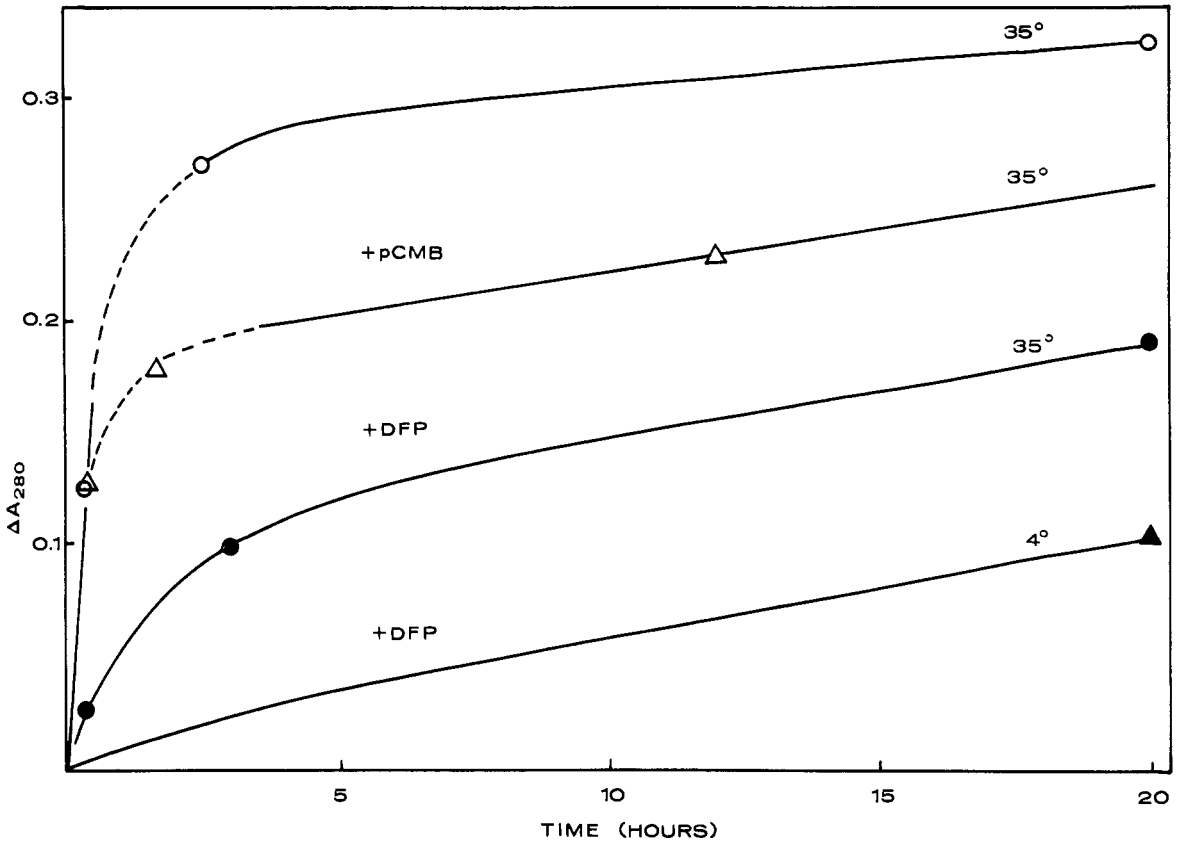

FIGURE 5: Influence of DFP and PMB on the proteolysis of the dialyzed ammonium sulfate fraction. A_{280} is a measure of the TCA-soluble material released. ○, control, pH 7.5, 35°; ●, DFP (5×10^{-3} M), pH 7.5, 35°; ▲, under isolation conditions: DFP (5×10^{-3} M), succinate (5×10^{-3} M), pH 5.5, 4°; △, PMB (2×10^{-4} M), pH 7.5, 35°. The reagents were present before (0.5 hr) and during the proteolysis measurements.

TABLE III: Rate of Self-Digestion at Various Stages of the Purification Procedure.

Sample	DFP[a]	Conditions	ΔA_{280}/mg of Protein	Extent of Proteolysis (%)	Act. Loss (%)
Supernatant after acidification	−	pH 4.4, 4°, 48 hr	0.1	10	<5[b]
After first dialysis	+	5 mM succinate, pH 5.5, 4°, 24 hr	0.166	16.6	<5[b]
First (DEAE-cellulose) column eluate	+	5 mM succinate, pH 6.0, 4°, 24 hr	0.0134	1.3	<5[b]

[a] 2.5×10^{-3} M final concentration. [b] Not significantly different from 0% in the hexokinase assay used.

required conditions. In this latter case the incubation time was extended to 24 hr, which is the time that the samples are exposed in the isolation procedure. The absorbance increment was in all cases a linear function of the amount of specimen protein added. It is seen (Table II) that, although the potential proteolytic activity, at all levels is initially very high and indeed remains potentially high during the isolation, it is effectively reduced by the addition of DFP and by the reductions in temperature and pH. In the case of the first dialysate, a reduction factor of 23 could be achieved, while in the first column eluate there was a 260-fold decrease in proteolytic activity. It was shown, however, by the self-digestion experiments (Table III) that even this dramatically decreased proteolytic activity tends to form a substantial amount of low molecular weight

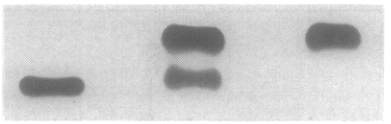

FIGURE 6: Cellulose acetate electrophoresis (for conditions, see text) of yeast hexokinase. Left to right, the samples are hexokinase B, a mixture of hexokinase A and B, hexokinase A. The positive pole is at the top and the negative pole is below.

material because of the prolonged exposure times of the samples during dialysis (24 hr). This low molecular weight material amounts to 16% for the first dialysate and 1.3% for the first column eluate. It is striking to note that despite the high proteolytic digestion taking place under the above conditions no enzyme activity losses were concurrently observed during the exposure. It would seem then that some other protein present, and not hexokinase, is being attacked under these particular conditions. That interfering proteases were finally totally removed was shown by the stability measurements on rechromatographed hexokinase that are reported below.

The influence of DFP and PMB was studied on self-digestion of the dialyzed ammonium sulfate fraction (Figure 5). In each case a 29-mg (10 ml) sample of protein in 0.05 M potassium phosphate buffer, pH 7.5, was incubated at 35°. Aliquots (2 ml) were withdrawn at the times indicated, 5% TCA (6 ml) was added to arrest reaction, and the material-solubilized in the digestion was measured.

It is seen that both reagents are inhibitory but neither completely inactivates. Of the two only DFP is initially

TABLE IV: Activities of Hexokinases A and B on Some Hexoses.

Substrate	Relative Activities[a]	
	Hexokinase A	Hexokinase B
D-Glucose	1.00	1.00
D-Mannose	0.60	0.28
D-Fructose	2.60	1.29
D-Galactose	~0[b]	~0[b]
3-Deoxy-D-glucose	0	0
3-Deoxy-D-mannose	0	0

[a] The activity on each substrate (at 0.027 M, with ATP and Mg^{2+} in saturation, at 25°) is expressed relative to that on glucose for each enzyme. The actual activities here are obtainable from the specific activities on glucose (in the same conditions) of the two enzyme preparations used, namely 180 units/mg for A and 780 units/mg for B. [b] Activity on galactose was just detectable, but too small to be accurately determined.

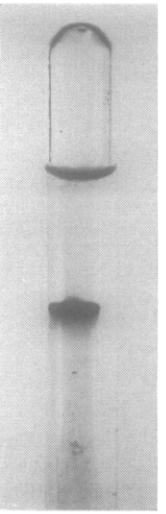

FIGURE 7: Polyacrylamide disc electrophoresis of hexokinase B (specific activity at 25°, 800 units/mg). Running pH was 8.5, 4° at a current of 1 ma/gel tube for 2.5 hr. The method was that of Davis (1964) applying the protein in a layer (100 μl) of sucrose (40%) directly above the spacer gel.

effective (78% inhibition) while PMB inhibition appears to be time dependent. After 2 hr this inhibition amounts to only 25%. The combination of both reagents does not increase the inhibition, in fact there is a decrease in the amount of inhibition. The crude extract was further tested for the presence of esterase activity against BAEE and BTEE. While there was no measurable activity on the arginine substrate, BTEE was rapidly hydrolyzed and the amount of activity expressed in chymotrypsin equivalents was approximately 6.5 μg/mg of specimen protein. This activity while not affected by DFP or TPCK exhibited an absolute requirement for the presence of calcium ions. The esteratic activity can be abolished by the addition of EDTA or PMB. The PMB inhibition is, however, time dependent. No Ca^{2+}-dependent proteolytic activity was detected on casein substrate, thus negating the possibility that the enzyme is a protease as well as an esterase.

Properties of the Purified Enzyme. SPECIFICITY. The phosphorylating ability of hexokinase A and B on glucose, galactose, mannose, and fructose has been examined; 3-deoxyglucose and 3-deoxymannose have been tested as substrates on yeast hexokinase for the first time (Table IV). Fructose and mannose are phosphorylated at a lower relative rate by hexokinase B as compared to hexokinase A. Hexokinase B, however, phosphorylates both substrates at much higher absolute rates than hexokinase A.

HOMOGENEITY. Cellulose acetate electrophoresis (Beckman microzone electrophoresis system) at pH 8.5, 0.1 M Tris, 250 v at room temperature, of the rechromatographed hexokinases A and B yielded a single discrete band for each enzyme, hexokinase A having a

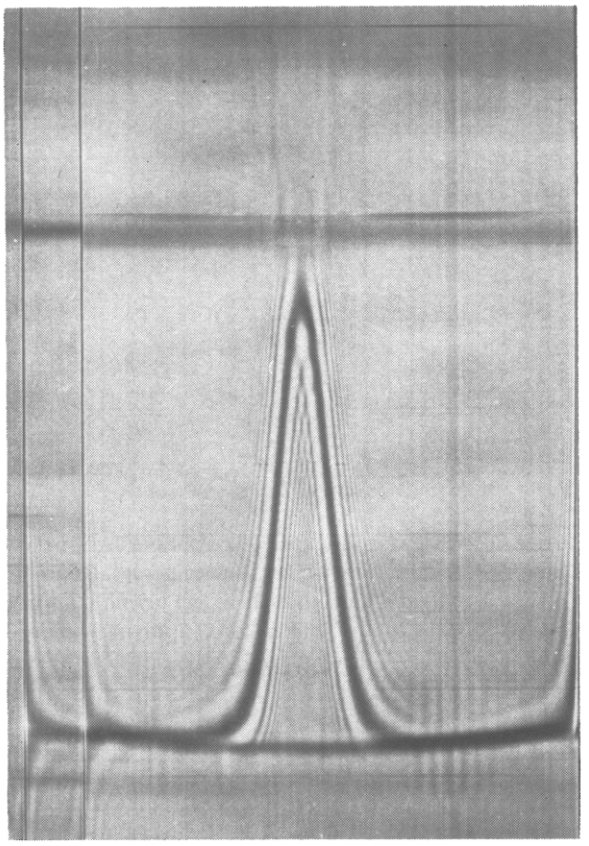

FIGURE 8: Sedimentation velocity pattern of hexokinase B (specific activity about 740 units/mg). Conditions: concentration, 7.4 mg/ml; pH 7.03; sodium phosphate, $\mu = 0.10$; 5°. Photographed at 128 min after attaining 50,740 rpm with a bar angle of 50° (obtained by Dr. M. Derechin).

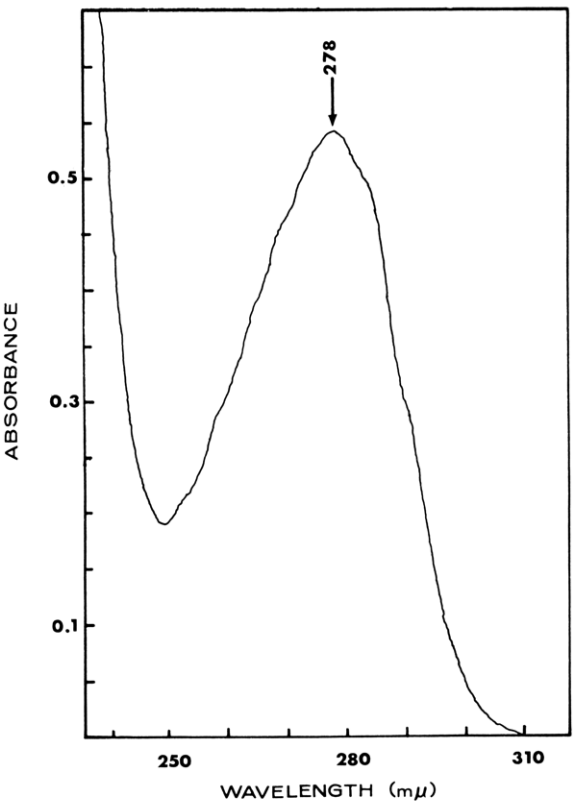

FIGURE 9: Absorption spectrum of hexokinase B in 5 mM sodium succinate of pH 5.0. The ionic strength was adjusted to 0.05 with sodium chloride. The protein concentration was 0.59 mg/ml.

greater mobility than B (Figure 6). In the same system at pH 5.5 each showed virtually no mobility (in a 20-min run). Electrophoresis on polyacrylamide gel was performed as described by Davis (1964). The system was run at 4° at pH 8.5. A rechromatographed sample of hexokinase B gave a single sharp band (Figure 7) which moved more slowly than a band obtained similarly with hexokinase A. Sedimentation velocity experiments in the Model E ultracentrifuge yielded a single sedimenting component at pH 7.03 (Figure 8). Further sedimentation behavior is described in the succeeding paper (Derechin *et al.*, 1966).

OTHER PROPERTIES. Hexokinase B was found to be stable as judged by absence of activity loss over 24 hr at 26° in 5 mM succinate buffer adjusted to pH 7.5 by the addition of Tris. The Q_{10}, for the activity of hexokinase B on glucose, was determined and found (at 25–30°) to be 2.2, identical with that reported by Darrow and Colowick (1962) for a yeast hexokinase preparation. The absorption spectrum of hexokinase B shows a maximum at 278 mμ (Figure 9). Visible and ultraviolet-absorbing cofactors are seen to be absent.[9]

Discussion

For preparation of yeast enzymes it is first necessary to break the cells and, in general, subsequently to release by some treatment the soluble enzyme from particulate or membrane material. Thus, for hexokinase, simple rupture of the yeast cells by mechanical methods (Sols *et al.*, 1958) or by prolonged drying (Darrow and Colowick, 1962) does not release soluble enzyme. The method resorted to for this subsequent step has in the past been autolysis at 37°. That such an enzymic attack has been found essential for the liberation, suggests that hexokinase, and other yeast enzymes behaving similarly, are bound to some structural component in the cell. In the present method, freezing and thawing (by any of several procedures) were shown to be a very satisfactory way of rupturing the cells. The hexokinase activity is present, but is not appreciably released in a soluble form (Figure 1). A long melting process at 4° is necessary for full release of soluble enzyme.

[9] In recent analysis by the anthrone method (A. H. Ramel, unpublished results), hexokinase B has also been shown to be free of carbohydrate material.

In the present preparation, it has been shown that substantial proteolytic activity is present when the hexokinase is liberated; this is to be expected, since previous studies by Sylven *et al.* (1959) and Cordonnier (1961) have shown that simple cell rupture of *Saccharomyces cerevisiae* releases several proteases and peptidases. The role that these proteolytic enzymes play in the liberation of yeast hexokinase is an intriguing question. There is no doubt that the presence of a proteolytic enzyme is of prime importance in the liberation of yeast hexokinase in the Darrow and Colowick procedure, because addition of DFP during autolysis in those conditions, *i.e.*, at 37° in 0.2 M dibasic sodium phosphate, completely prevents the liberation of hexokinase (Ramel, 1964). In contrast, DFP has no effect on the hexokinase liberation during the 36-hr melting at 4°, as reported above. The sharp difference in DFP effect in the two types of isolation procedure presages the possibility of distinct differences in the two enzymes. The processes that occur during the thawing stage at 4° here are otherwise not characterized. Acidification of the slurry to pH 4.5 completely abolishes the release of hexokinase. There is, therefore, still the possibility that some enzymic mechanism is involved in the release process.

The presence of lipid solvents is not essential for the liberation of hexokinase. Initial cell rupture can be obtained in satisfactory conditions either by freezing and thawing combined with mechanical stress, as in the Dry Ice procedure that we used, or by dispersal in a liquid vehicle at low temperature followed by thawing, as in our toluene treatment. We have taken hexokinase, liberated by the Dry Ice method alone, through purification and chromatographic procedures, to give a high specific activity product. Hence, there is no support here for the concept that the enzyme is initially bound *via* a lipid–protein complex. The toluene method is preferred for the routine preparation, however, because of its complete efficiency in cell rupture in larger scale operation.

At all stages following the point of liberation of the hexokinase by thawing, proteolytic activity is held at negligible levels in this procedure. The DFP effect here is striking, giving 80% removal of potential proteolytic activity. The other factors that prevent proteolytic action are the pH and temperature values used until the proteases are finally totally removed by the fractionation and chromatographic steps. The results of the self-digestion tests cannot exclude that minor structural changes occur in the protein without activity loss. However, when those results are taken together with the actual assays of proteolytic activity under the isolation conditions, and the constancy of the chromatographic behavior of the two active species in a large number of preparations, the conclusion that proteolytic attack is absent is very strongly supported.

The relationship between hexokinase A and hexokinase B poses an interesting problem. Both are distinct, homogeneous species. The results of the self-digestion experiments, performed under our isolation conditions, eliminate the possibility that peak A is derived from peak B during the later stages of the isolation procedure; if this were the case, a drop in enzyme activity would be detected. Further, in the succeeding paper it will be shown that hexokinase A does not accumulate on incubation of hexokinase B with the crude yeast protein mixture. We have noted here chromatographic evidence that also shows that the yeast proteases produce other species but not A from B. Since hexokinases A and B have almost the same sedimentation coefficient (Derechin *et al.*, 1966) and A moves faster than B in electrophoresis (in two systems), there must be a charge difference between them, but it is not clear why these mobilities are reversed in chromatography on the anionic exchanger. This may reflect a critical ionization difference in the two molecules between the pH of chromatography (about pH 5.2) and the pH in electrophoresis (pH 8.5). In electrophoresis at pH 5.5, mobilities of both were very small, so this point could not be tested directly. An alternative interpretation would be that the two proteins differ considerably in structure and their adsorption affinities. It has not been possible to prevent or to promote the formation of either hexokinase A or B by the addition of 1% glucose to all stages of the purification procedure. The distinctive properties of hexokinases A and B, their different substrate specificities, and our inability to form one from the other by exposure to yeast proteases are facts that are most easily explained if they are two different hexokinases coexisting in the yeast cell (perhaps in different sites). Further evidence to test this suggestion is being examined.

The pitfalls of enzyme isolation procedures, depending on the presence of intrinsic yeast proteases to liberate the enzyme, have been previously documented by Ramel (1964) and Kenkare *et al.* (1964). The isolation of a single species in such a system is no guarantee of having an undegraded enzyme. This can be shown by the striking differences reported in the following paper (Derechin *et al.*, 1966) between our hexokinase B and yeast hexokinases prepared by others. Until purification procedures of yeast enzymes can be shown to be either lacking in, or protected from, those proteases which attack the enzyme under isolation, reports of isoenzymes must be viewed with caution. Indeed, any isolation system for an enzyme in which there are proteases present must adhere to the same rigid requirement before claims of a nondegraded species can be entertained. It is probable that some of the wide variation in properties of an enzyme isolated by various workers under slightly different conditions can be accounted for by the degree of proteolytic degradation that the enzyme has been subjected to during the purification procedure, where, as in hexokinase, partial activity is retained after some proteolysis.

The present method appears to be a suitable one for preparation of other yeast enzymes at sites similar to those of hexokinase. Certainly, other preparations of yeast enzymes should be scrutinized for the effects of proteolytic attack, which our evidence shows must normally occur unless precautions of the type described here are applied. In general, isolation by the present

procedure should be efficient and conservative.

In summary, a method is described for the preparation of yeast hexokinase without apparent proteolytic degradation, to yield a pure, homogeneous, stable enzyme, hexokinase B (with concurrent disclosure of another species, hexokinase A, present in large amount). Taking only hexokinase B, the yield is about three times better than that obtained in the best previous preparations of pure yeast hexokinase, while the time occupied in preparation is considerably reduced. If we were to use the activity units of Darrow and Colowick (1962) (see footnote 8), hexokinase B here would attain a maximum activity of about 1300 units/mg. This final specific enzymic activity is more than twice that obtained by other methods.

References

Ågren, G., Engström, L., and Eklund, S. (1963), *Biochem. Biophys. Acta 67*, 143.

Bailey, K., and Webb, E. C. (1948), *Biochem. J. 42*, 61.

Cordonnier, R. (1961), *Compt. Rend. 253*, 910.

Darrow, R. A., and Colowick, S. P. (1962), *Methods Enzymol. 5*, 226.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Derechin, M., Ramel, A. H., Lazarus, N. R., and Barnard, E. A. (1966), *Biochemistry 5*, 4017 (this issue; following paper).

Hummel, B. C. W. (1959), *Can. J. Biochem. Physiol. 37*, 1393.

Kenkare, U., Schulze, I. T., Gazith, J., and Colowick, S. P. (1964), Abstracts VI, Vol. 6, International Congress of Biochemistry, New York, N. Y., p 477.

Kunitz, M. (1947), *J. Gen. Physiol. 30*, 291.

Kunitz, M., and McDonald, M. R. (1946), *J. Gen. Physiol. 29*, 393.

Laskowsky, M. (1956), *Methods Enzymol. 2*, 33.

Layne, E. (1957), *Methods Enzymol. 3*, 448.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Meyerhof, O. (1927), *Biochem. Z. 183*, 176.

Murray, D. H., and Prokop, J. (1965), *J. Pharm. Sci. 54*, 1637.

Ramel, A. H. (1964), Habilitation Thesis, University of Basel, Basel, Switzerland.

Ramel, A. H., Stellwagen, E., and Schachman, H. K. (1961), *Federation Proc. 20*, 387.

Schachman, H. K. (1960), *Brookhaven Symp. Biol. 13*, 49.

Schwert, G. W., and Takenaka, Y. (1955), *Biochim. Biophys. Acta 16*, 570.

Sols, A., de la Fuente, G., Villar-Palasi, C., and Asensio, C. (1958), *Biochim. Biophys. Acta 30*, 92.

Sylven, B., Tobias, C. A., Malmgren, H., Ottoson, R., and Thorell, B. (1959), *Exptl. Cell Res. 16*, 75.

Trayser, K. A., and Colowick, S. P. (1961), *Arch. Biochem. Biophys. 97*, 177.

van Hofsten, B., and Tjeder, A. (1961), *J. Biochem. Microbiol. Tech. Eng. 3*, 175.