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A Chromatographic Study of Phosphoglucomutase: Separation of Phospho- and Dephospho-Enzyme Forms*

J. A. YANKEELOV, JR.,† H. ROBERT HORTON,‡ AND D. E. KOSHLAND, JR.

From the Brookhaven National Laboratory, Upton, N. Y., and the Rockefeller Institute, New York, N. Y.

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A chromatographic method has been developed which permits the isolation of phosphorylated and dephosphorylated enzymatically active forms of phosphoglucomutase. The enzyme, obtained from rabbit muscle, is chromatographed at *p*H 7 on carboxymethyl-cellulose, eluting with a linear gradient of sodium phosphate buffer. Inactive protein, if present, passes through the column and is separated from the active enzyme forms which are subsequently eluted. Chromatographed enzyme can be stored at approximately 0° as a slurry in *p*H 5–buffered ammonium sulfate; or, alternatively, in liquid nitrogen as frozen pellets of neutral buffered solutions. That a homogeneous enzyme of high purity can be obtained by such chromatography was evidenced by the constant specific enzymatic activity exhibited by the fractions belonging to a single peak, by the enzyme's subsequent elution as a single peak upon repeated chromatography, and by its homogeneity in equilibrium ultracentrifugation. The phosphorylated and dephosphorylated nature of the separated phosphoglucomutase components was identified by ^{32}P-substrate labeling experiments. Amino acid analyses were performed, and the composition of the chromatographed phosphoenzyme was compared with that published for phosphoglucomutase in earlier reports.

Recent studies concerning the relationship of structural characteristics of phosphoglucomutase to its activity (Koshland *et al.*, 1962), in some cases involving rather small but measurable changes, emphasized the desirability of finding a reproducible method for obtaining enzyme samples of the highest purity. Moreover, according to the mechanism of action proposed by Najjar and Pullman (1954), this muscle enzyme must exist in two catalytically active forms, one a phosphoprotein and the other a nonphosphorylated species. A separation of these two forms would be important not only for the study of this enzyme, but also as an indication that other enzymes existing in two catalytically active forms might be separable chromatographically.

Earlier work had indicated that use of CM-cellulose[1] offered a suitable means of chromatographing the enzyme (Ray and Koshland, 1962). The present communication describes the development of a method to achieve this separation and some of the properties of rabbit muscle phosphoglucomutase.

EXPERIMENTAL

Enzyme Preparation.—Enzyme used in this study was prepared from rabbit skeletal muscle according to the method of Najjar (1955), with the following modifications. In many instances when storage was desirable, freshly ground muscle in individual polyethylene bags was frozen at −25°. The frozen muscle was allowed to defrost overnight at 4° and was then extracted for 40 minutes with 5 volumes of cold deionized water. The extract was adjusted to *p*H 5.0 with 1.0 M acetic acid, heated to 65°, and cooled to 4° by immersing the flask in an ethylene glycol–water bath at −10°. The mixture was adjusted to 25% saturation by addition of solid ammonium sulfate prior to centrifuging the heat-coagulated protein, a modification similar to that recently suggested by Najjar (1962). The supernatant was then adjusted to 65% saturation, and after standing overnight at 4° the precipitated enzyme was collected by centrifugation. The protein was then dissolved in an equal volume of sodium acetate, heated to 63° for 3 minutes, and centrifuged, and the supernatant was adjusted to a protein concentration of 5 mg/ml. The solution was brought to 50% saturation with ammonium sulfate and the sedimented protein was removed by centrifuging. After dropwise addition of saturated ammonium sulfate to 60% saturation over a period of 2–3 hours, the solution was allowed to settle overnight in the cold prior to centrifuging the crystallized protein.

The crystallized enzyme was stored at 0–4° as a suspended slurry in 65% saturated ammonium sulfate, 0.15 M in sodium acetate buffer, *p*H 5.0.

Enzyme Assay.—The assay method employed was essentially that described by Najjar (1955), except that the incubation mixture contained histidine (final concentration 0.04 M) and Tris (0.013 M) buffer rather than cysteine. No preactivation of the enzyme samples was employed. The unit of activity was that defined by Najjar, viz., that amount of phosphoglucomutase which catalyzes the formation of 1 mg of acid-labile P in 5 minutes' incubation at 30°.

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† Present address: Department of Biochemistry, University of Louisville, Louisville, Kentucky.

‡ Postdoctoral Fellow of the National Academy of Sciences—National Research Council, supported by the Air Force Office of Scientific Research.

[1] Abbreviations used in this work: CM-, carboxymethyl-; Tris, tris(hydroxymethyl)aminomethane.

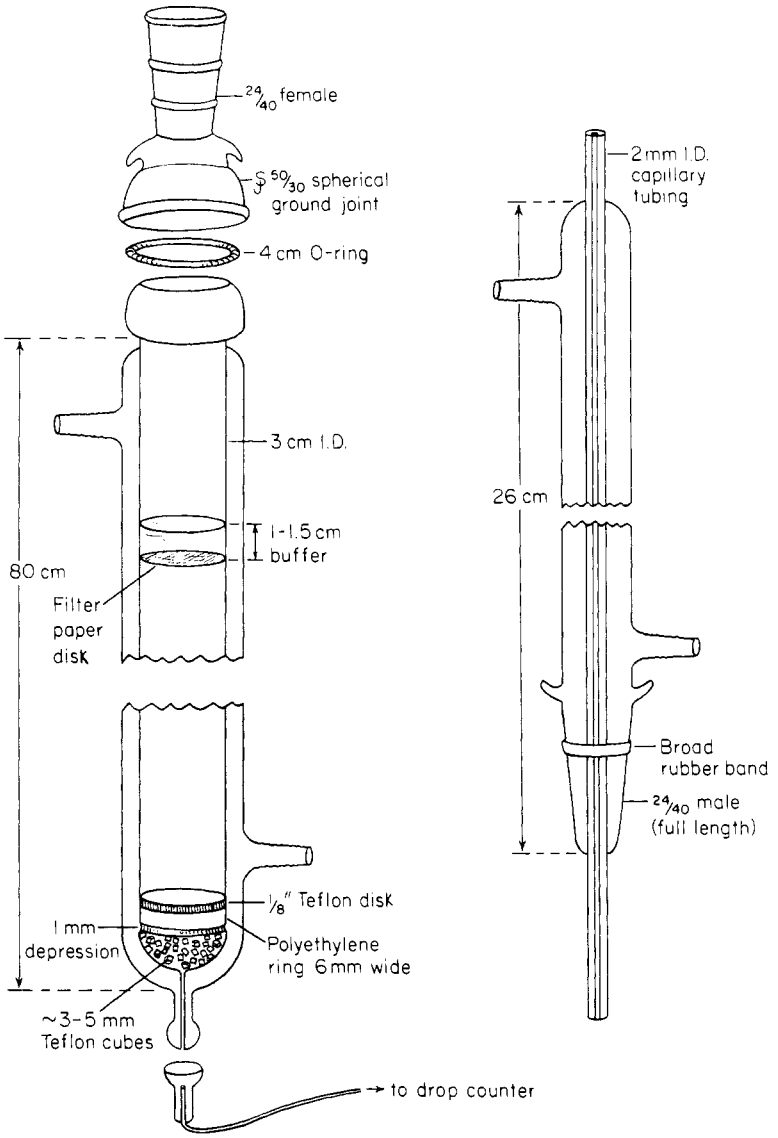

FIG. 1.—Apparatus used with refrigerated fraction collector for chromatographing phosphoglucomutase. *Left,* jacketed column assembly; *right,* prechilling device for delivery of eluent to column.

Protein concentrations were estimated by absorbancy at 278 mμ, employing 7.70 as the absorbancy index of an enzyme solution of 1 g/100 ml in a quartz cell of 1 cm optical path.

Resin Preparation.—CM-cellulose suitable for phosphoglucomutase chromatography was prepared according to the directions of Peterson and Sober (1956). Since unsatisfactory results were obtained with CM-cellulose of variable particle size, the resin used here was settled in dilute buffer from approximately 20 cm of supernatant within 20–25 minutes.

In general, regeneration of CM-cellulose with sodium hydroxide–sodium chloride was not satisfactory with the resin packed in large columns. The following regeneration scheme was found to provide material with properties well suited for column chromatography. Previously untreated CM-cellulose powder, or that removed from a used column, was suspended in about 4 volumes of deionized water, and fines were removed after 20 minutes' undisturbed standing. This settling procedure was repeated until the supernatant was essentially clear of fine particles. The resin was then suspended in a similar volume of 0.5 M NaCl adjusted to *p*H 11 with NaOH, and the suspension was titrated to *p*H 11 with a solution 0.5 M in NaCl and 0.5 M in NaOH. This mixture was stirred for 30 minutes, then allowed to settle for 25 minutes before the supernatant and unsettled fines were removed. The resin was then suspended and settled twice in *p*H 11, 0.5 M NaCl, each time with removal of the supernatant and fines after 25 minutes' settling. Thus regenerated, the resin was washed free of alkali by repeatedly resuspending and settling in deionized water until the supernatant was essentially neutral. The cellulose was then resuspended and settled numerous times using *p*H 7 sodium phosphate buffer, 0.1 M in Na^+ and 0.001 M in Na^+. CM-cellulose prepared in this manner could be stored at 0–4° in dilute buffer, or as a frozen suspension. Each batch of resin was settled free of fines in 0.001 M buffer immediately prior to pouring a column.

Column Design and Operation.—Chromatography of the enzyme was performed at 2–4° using a refrigerated fraction collector. A simple prechilling device for delivering cold buffer to a jacketed column is shown in Figure 1. By employing a series of adapters constructed to receive the prechiller it was possible to assemble the unit with columns of various diameters, thus increasing the applicability of the system to the chromatography of a range of sample sizes.

Columns 60 × 3 cm were routinely employed for preparative-scale enzyme chromatography. The column was poured from CM-cellulose suspended in 3 volumes

of 0.001 M buffer, using a Teflon disk support (porous Teflon, grade 50–55, Fluoro-Plastics, Inc., Philadelphia 46, Pa.) as shown in Figure 1. The use of a Teflon support was preferred to that of sintered glass because of the affinity of proteins for glass at low ionic strengths (Shapira, 1959) and because of the greater ease of cleaning the apparatus. The first few centimeters of resin were allowed to settle with the column exit valve (greaseless Teflon and glass) closed; the remainder of the column was packed by gravity with the exit valve adjusted to permit a flow of *ca.* 60 ml/hour until a height of about 63 cm was reached (allowing 3 cm to compensate for subsequent shrinkage). A properly fitted disk of filter paper (1 mm smaller than the column diameter), allowed to settle into place on top of the column, was found to afford complete protection of the surface against disturbance, and yet follow the shrinkage and expansion of the CM-cellulose accompanying changes in ionic strength. The column could then be washed with 0.1 M buffer at a rate of about 60 ml/hour until a sharp increase in phosphate occurred in the effluent, as determined by the Fiske-Subbarow colorimetric assay (Fiske and Subbarow, 1925). The column was then equilibrated with 0.001 M buffer by washing at a flow rate of 30 ml/hour in the cold for approximately 16 hours. Column equilibration was checked by comparing phosphate concentration of the effluent with that of the 0.001 M buffer before applying the enzyme. The hold-up volume of the columns was 0.4–0.5 of the gross resin volume. Based on a single determination, the water bound to the cellulose appeared to be about 0.3 of the gross column volume.

In gradient elution with columns of large diameter the possibility exists that nonuniform percolation of the gradient through the packing could result in decreased resolution and reproducibility. A test with bromophenol blue showed that dropwise delivery to the surface of a buffer pool in a 3.0-cm column resulted in discrete rings which descended with virtually no dispersal. However, allowing the drops to fall 15–20 cm before striking the surface of the buffer resulted in sufficient turbulence to promote good dispersal of the dye. Consequently, for gradient elution the apparatus was arranged so that cold eluent dropped 15–20 cm into a pool of buffer 1–1.5 cm above the filter paper–protected surface of the column (Fig. 1).

Application of Enzyme to the Column.—The enzyme was dissolved in deionized water to a concentration of *ca.* 10 mg/ml., and dialyzed for 24 hours against large volumes of cold 0.001 M *p*H 7 phosphate buffer. Following dialysis the enzyme solution was centrifuged to remove any insoluble material which might have accumulated, and then diluted with 0.001 M buffer to a protein concentration of 2.0–2.5 mg/ml. The enzyme solution was applied to the 0.001 M buffer–equilibrated column at a flow rate of 30 ml/hour. For columns operated with the refrigerated fraction collector it was found convenient to add the cold enzyme solution from a jacketed separatory funnel (Teflon stopcock) connected to the prechiller and column assembly with a short length of 0.095-in. o.d. polyethylene tubing (PE-240, Clay-Adams, Inc.). The column was then washed with 270 ml (slightly in excess of the hold-up volume) of 0.001 M buffer to remove unabsorbed protein.

Evidence indicated that the maximum load of sample compatible with resolution of active enzyme peaks was dependent on the quality of the enzyme preparation being chromatographed. Thus with crystallized phosphoglucomutase of high specific activity (16–20 units/mg) approximately 500 mg could be satisfactorily chromatographed on a 60 × 3.0–cm column, whereas with preparations of lower specific activity (10 units/mg) satisfactory separation of active components could be obtained with application of as much as 750 mg to the column.

Elution.—A linear gradient of *p*H 7 phosphate buffer, 0.001 M–0.033 M in Na^+ (total volume = 2000 ml for 60 × 3.0–cm column), was applied to the column at a rate of 30 ml/hour from bottles of matched cross-sectional area (Bock and Ling, 1954), using a micropump.

Storage of Chromatographed Enzyme at Liquid Nitrogen Temperature.—Since occasionally an unexplained loss in enzymatic activity was observed on storage in ammonium sulfate suspensions, a method of storing chromatographed enzyme in liquid nitrogen was developed. Small volumes of enzyme in a suitable buffer could be frozen by dropwise expression from a syringe into a pool of liquid nitrogen, while larger volumes could be conveniently frozen by dropwise addition from a separatory funnel. In either case the rate of addition of enzyme to liquid nitrogen was slow enough to permit formation of individual pellets rather than inconvenient clumps. After freezing was completed the intact pellets were transferred in polyethylene cartridges to a stainless steel canister for storage in a liquid-nitrogen refrigerator.

Samples of the enzyme could be removed as needed and thawed in a 30° bath. Activity losses throughout the entire freezing and thawing procedure were marginal (less than 5%) in samples containing enzyme concentrations below 10 mg/ml in *p*H 7 or 8 buffers of sodium phosphate or Tris-chloride. It seems probable that any buffer which maintains the native enzyme through a dialysis procedure would be satisfactory for cryogenic storage.

Results

Initial Distribution Patterns.—During the early stages of this investigation chromatography of phosphoglucomutase on 60 × 3.0–cm columns of CM-cellulose was performed using a linear gradient of *p*H 7 phosphate buffer, 0.001 M–0.10 M in Na^+ as described in Figure 2. The protein (initial specific activity 17 units/mg) was separated into three well-resolved peaks, as indicated by the distribution of 278 mμ absorbancy in the figure. The breakthrough peak, for the most part, exhibited little enzymatic activity, while the second and third peaks had specific activities of approximately 30 and 21 units/mg, respectively. When the enzyme was labeled with ^{32}P via labeled substrates (Ray and Koshland, 1963), the enzymatically active protein in peak II contained most of the radioactivity, while that in peak III was devoid of radioactivity. The appearance of a partial bifurcation in the peak II material suggested the possibility that two forms of phosphorylated protein of equal enzymatic activity were being eluted together in this chromatographic system. A small portion of the enzyme had apparently passed through the column without being bound to the CM-cellulose, and appeared in the breakthrough peak together with denatured or otherwise inactive protein.

When a preparation of phosphoglucomutase with lower enzymatic activity (9.6 units/mg) was chromatographed using this system, the distribution pattern shown in Figure 3 was obtained. Partial resolution of weakly absorbed protein occurred; peaks II and III were again well separated, peak II exhibiting higher phosphoglucomutase activity. An indication of the degree of homogeneity achieved by the chromatographic separation of this preparation, with low initial activity, was provided by the patterns obtained when peak II and peak III proteins were subjected to starch-

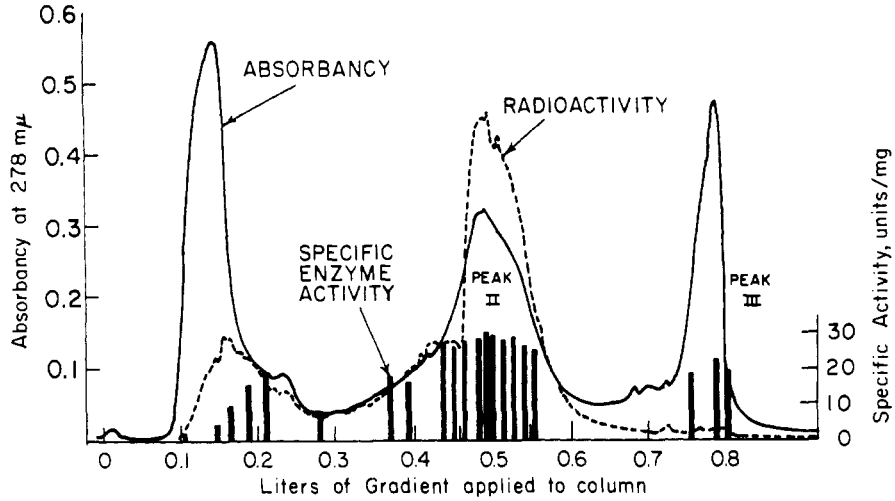

FIG. 2.—Initial distribution pattern, ^{32}P-labeled phosphoglucomutase with specific enzymatic activity 17 units/mg, applied to 60 × 3.0-cm column of CM-cellulose, washed with 70 ml of *p*H 7 phosphate buffer, 0.001 M in Na^+, and eluted with linear gradient of *p*H 7 phosphate buffer, 0.001–0.10 M in Na^+, 3000 ml total volume. Continuous line represents 278 mμ absorbancy; broken line, radioactivity; vertical bars, specific enzymatic activity of individual fractions.

gel electrophoresis at *p*H values above and below the isoelectric points, as shown in Figure 4.

The development of the elution method described under Experimental resulted from attempts to determine if peak II enzyme could be separated into more than one component by employing half the gradient. The distribution obtained when crystallized phosphoglucomutase was chromatographed at *p*H 7 on 60 × 3.0-cm columns, eluting with half the gradient, is shown in Figure 5. Phosphorylated enzyme with specific enzymatic activity of 28–32 units/mg was eluted in two peaks, I and II, while dephosphoenzyme with specific activity of 22–27 units/mg appeared in peak III. Specific enzymatic activity and specific radioactivity remained relatively constant throughout the individual fractions associated with a single peak.

Chromatography of Previously Chromatographed Enzyme.—Further evidence concerning the nature and homogeneity of chromatographically separated phosphoglucomutase components was obtained by subsequent chromatographic studies. When peak II enzyme was collected, crystallized with ammonium sulfate, subjected to the dialysis procedure, and rechromatographed on CM-cellulose, a single peak emerged in the peak II position, as shown in Figure 6. Specific enzymatic activity was constant across the peak, 31–32 units/mg. No breakthrough peak of inactive protein was eluted.

When twice-chromatographed phosphoenzyme, which had appeared as a single peak, was incubated with ^{32}P-labeled substrate at 30° and again chromatographed, the distribution pattern shown in Figure 7 was observed. Approximately one-half the enzyme had become dephosphorylated, and was eluted as nonradioactive peak III, in the same position as the peak III component of untreated enzyme crystallized from rabbit muscle. A small portion of material exhibiting 278 mμ absorbancy was eluted in the region corresponding to the trailing edge of the breakthrough peak encountered in initial chromatographic separations of phosphoglucomutase. This material, however, differed in several respects from protein appearing in the break-through peak of untreated phosphoglucomutase chromatograms. It exhibited no detectable enzymatic activity, it contained approximately six times the specific radioactivity of peak II, and it was not readily precipitated in ammonium sulfate or trichloroacetic acid solutions.

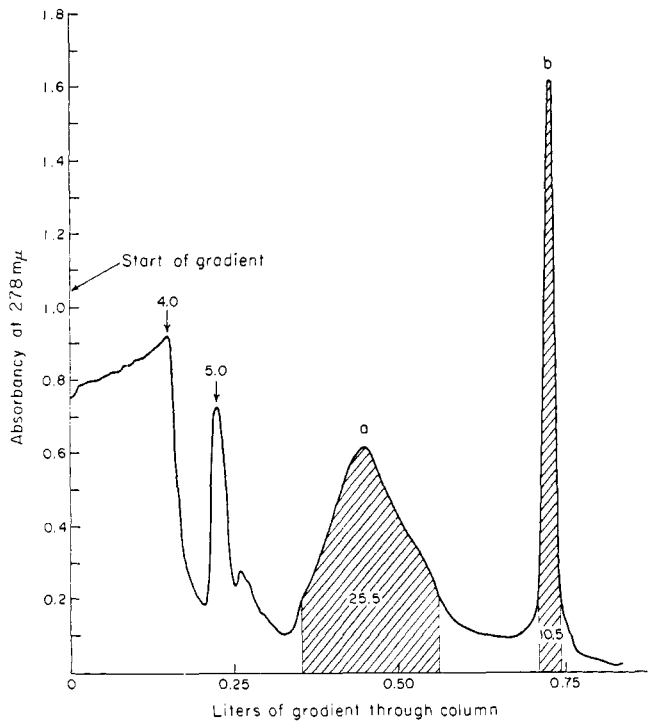

FIG. 3.—Initial distribution pattern of phosphoglucomutase of lower specific activity, 9.6 units/mg, upon CM-cellulose chromatography using conditions described in Fig. 2 legend. Continuous line represents 278 mμ absorbancy; numbers refer to specific enzymatic activities, units/mg; cross-hatched areas *a* and *b* indicate proteins collected for starch-gel electrophoresis, Fig. 4.

Molecular Weight of Phospho- and Dephospho-Enzyme.—Peak II and Peak III phosphoglucomutase from the chromatogram illustrated in Figure 7 were subjected to equilibrium ultracentrifugation, using the method of Yphantis (1962). The molecular weights of enzyme preparations from both peaks II and III were found to be 62,000 and the linearity of the plots indicated the material was homogeneous (Filmer and Koshland, 1963).

Based on its specific radioactivity, peak II phosphoenzyme prepared in this manner contained 1.03 g-atoms of labeled P per 62,000 g of protein.

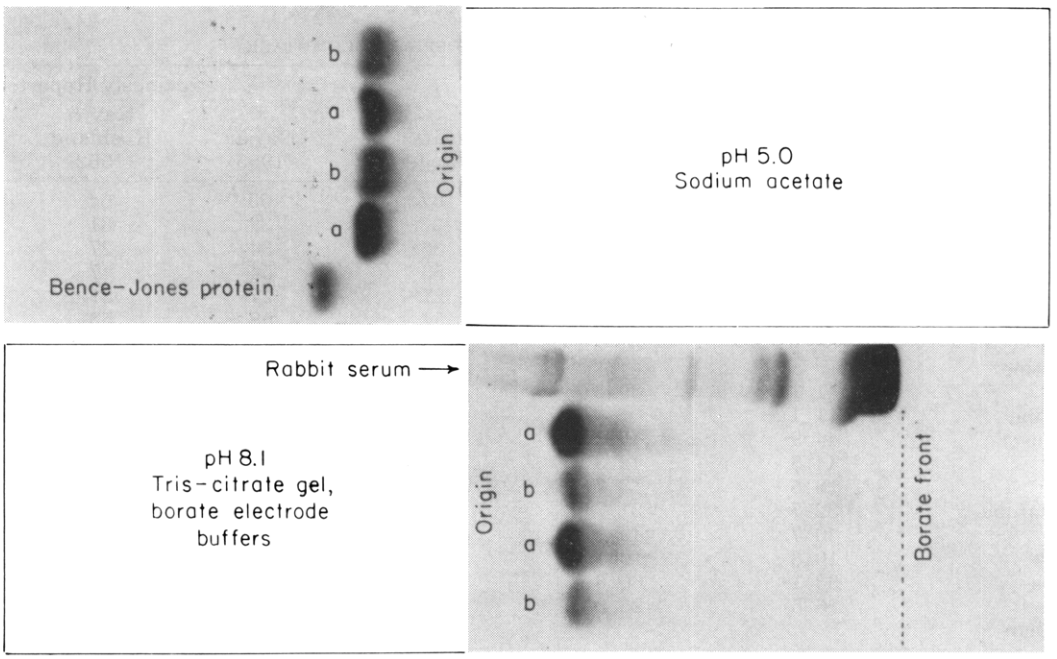

FIG. 4.—Distribution of chromatographed phosphoglucomutase in starch-gel electrophoresis. Designations *a* and *b* refer to components of the chromatographic separation shown in Fig. 3. Conditions: acetate, $\mu = 0.03$, 100 v, 20 ma, 19 hours; Tris-citrate, $\mu = 0.03$, and borate buffer, 0.3 N, *p*H 8.1, 100 v, 25 ma, 23 hours.

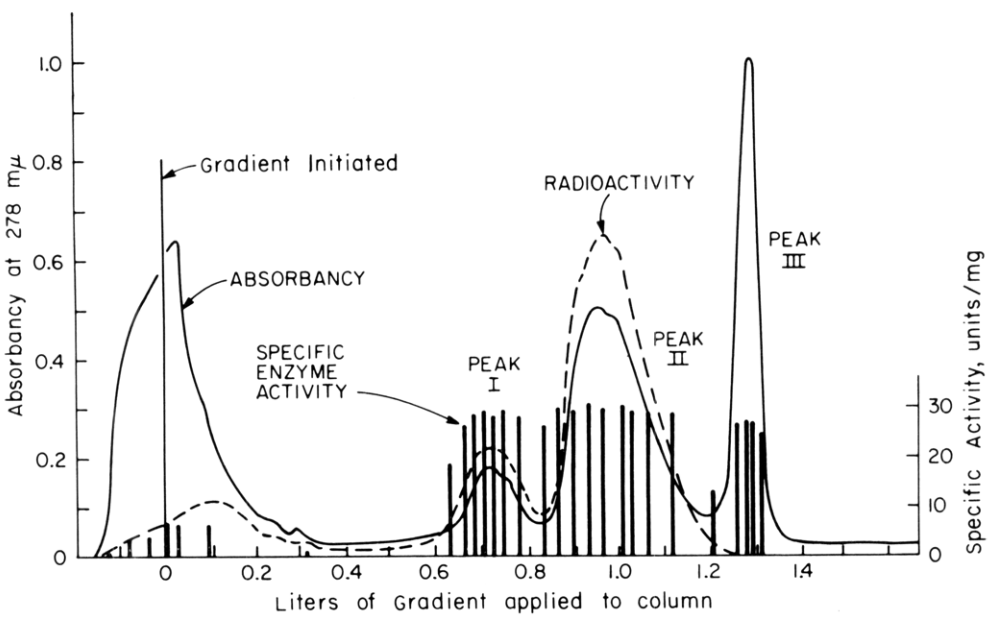

FIG. 5.—Initial distribution pattern of phosphoglucomutase applied to 60 × 3.0-cm column of CM-cellulose, washed with 270 ml of 0.001 M buffer, and eluted with linear gradient of *p*H 7 phosphate buffer, 0.001–0.033 M in Na^+, 2000 ml total volume (one-half the gradient described in Fig. 2).

Amino Acid Composition.—The amino acid content of chromatographed phosphoenzyme was determined using the automatic apparatus of Spackman *et al.* (1958). Average values for the chromatographed enzyme are presented in Table I, together with previously reported values for comparison. Analysis of 24-hour acid hydrolysates from six separately chromatographed enzyme preparations, including a twice-chromatographed sample, gave results consistent to within ±3%. Tryptophan was determined by the spectrophotometric method of Goodwin and Morton (1946), and methionine and half-cystine were analyzed by a performic acid oxidation procedure described by Moore (1963). The amino acid analyses of 24-hour acid hydrolysates accounted for 99% of the nitrogen content of chromatographed phosphoglucomutase samples, but only 91% of the ash-free dry weight of the dialyzed and lyophilized protein. The lower recovery on the weight basis, in view of the quantitative nitrogen recovery, may have resulted from bound salt not detected in the ashing procedure.

DISCUSSION

Chromatography of phosphoglucomutase preparations on CM-cellulose columns, employing a linear gradient of *p*H 7 phosphate buffer of low ionic strength, affords a means of separating enzymatically active components from one another and from inert protein. If an enzyme sample suffers partial denaturation before it is

TABLE I
AMINO ACID COMPOSITION OF PHOSPHOGLUCOMUTASE

Amino Acid	24-Hour Hydrolysis	48-Hour Hydrolysis[a]	Nearest Integer[b]	Previously Reported[c]		
				Najjar (1955)	Ray & Koshland (1962)	Boser (1955)
Aspartic acid	66.9	65.2	67	63	64	84
Threonine	30.1	27.5	32[d]	32	31	17
Serine	32.0	27.3	36[d]	34	27	39
Glutamic acid	52.9	51.2	53	54	47	57
Proline	26.8	26.1	27	27	31	37
Glycine	54.2	52.2	54	58	53	35
Alanine	52.9	51.2	53	55	53	68
Half-cystine			6[e]	6	12	23
Valine	41.1	39.3	41	37	43	38
Methionine	(11.1)		12[e]	18	11	
Isoleucine	44.9	45.9	45[f]	35	52	39
Leucine	44.5	42.3	45	46	46	
Tyrosine	16.5	13.7	17[d]	17	16	30[g]
Phenylalanine	32.7	31.7	33	28	33	61
Lysine	40.7	40.2	41	38	50	40
Histidine	10.8	10.1	11	11	13	3[g]
Ammonia	63.1	66.6	63[h]			
Arginine	26.5	25.3	26	23	29	42
Tryptophan			4[i]	0	4	2

[a] 48-Hour hydrolysis results are generally in agreement with those of 24-hour hydrolysis, except for low recoveries of serine and tyrosine, suggesting that hydrolysis of phosphoglucomutase is essentially complete within 24 hours at 110° in constant-boiling hydrochloric acid. [b] Summation of the integral residue values from this study gives a molecular weight of 65,900. [c] Comparisons based on number of residues per 62,000 g of protein. [d] Results for labile amino acids corrected approximately for decomposition during 24 hours of acid hydrolysis of deaerated samples by applying the factors: threonine, 0.95; serine, 0.90; tyrosine, 0.95 (Liu *et al.*, 1963). [e] Methionine and half-cystine determined by performic acid oxidation (Moore, 1963), employing 48-hour acid hydrolysis at 115° rather than 18 hours at 110°. Shorter periods of hydrolysis of the oxidized samples resulted in incomplete release of constituent residues. Values obtained: half-cystine = 5.98; methionine = 12.03. [f] Isoleucine = the sum of isoleucine and alloisoleucine. [g] Tyrosine and histidine values in agreement with those reported by Anderson and Jollès (1957). [h] Value for ammonia represents only an approximation for asparagine plus glutamine, not corrected for citrate buffer. [i] Tryptophan determined spectrophotometrically (Goodwin and Morton, 1946). Value obtained: 3.92.

chromatographed, inactivated material passes through the column prior to the elution of the major active species. When prepared from rabbit muscle extracts by the Najjar (1955) procedure without modification, three-times-crystallized phosphoglucomutase showed no significant difference in chromatographic distribution or in amino acid composition of peak II from that obtained by the modified procedure described in this paper. Thus it can be concluded that preparations of the muscle enzyme contain several chromatographically distinguishable components (cf. Najjar, 1962).

When CM-cellulose chromatography of crystallized enzyme was performed at *p*H 6, under otherwise similar conditions, a single peak containing all the enzyme was obtained. This is in sharp contrast to the separation of phosphorylated and nonphosphorylated enzyme molecules when chromatographed at *p*H 7. Protein recoveries averaged 94% for the *p*H 7 procedure while phosphoglucomutase activity recovered in the major peaks averaged 70% of the total activity applied to the column.

Evidence indicative of the homogeneity of the separated components included the constant specific activity across each peak, the linearity of graphs of fringe height plotted logarithmically against distance from the center of rotation in the sedimentation equilibrium studies (Filmer and Koshland, 1963), and, in the case of peak II, the failure to achieve further separation upon repeated chromatography. The observed reproducibility and absence of an inert breakthrough peak upon repeated chromatography excludes the possibility that the separations were merely artifacts of the chromatographic process. Thus the separated components are not simply partially denatured forms generated during the dialysis and chromatographic operations.

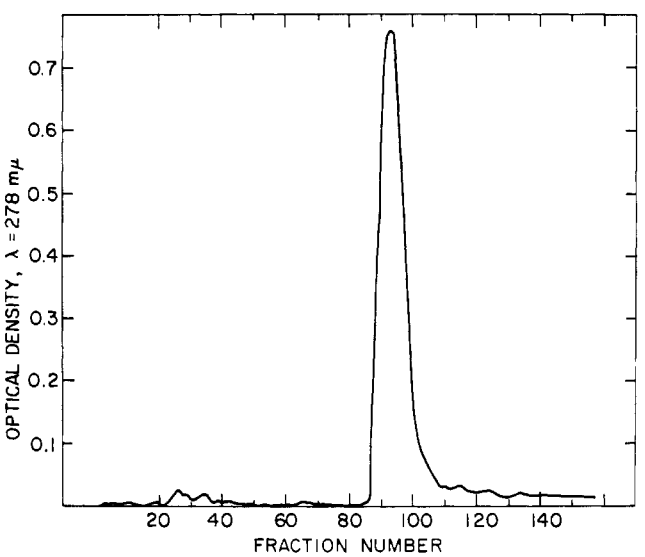

FIG. 6.—Appearance of chromatographically separated peak II phosphoglucomutase upon repeated chromatography under identical conditions.

While the present chromatographic system offers a method for obtaining samples of phosphoglucomutase of uniform purity, the use of solutions of low ionic strength necessitates considerable care in order to achieve reproducible separations. Among the other systems examined with respect to the chromatographic behavior of phosphoglucomutase were columns of phosphocellulose. It was reasoned that the similarity of the resin to the substrate might offer unique absorption selectivity for this enzyme. Attempts to chromatograph phosphoglucomutase on phosphocellulose, however, resulted in rather dif-

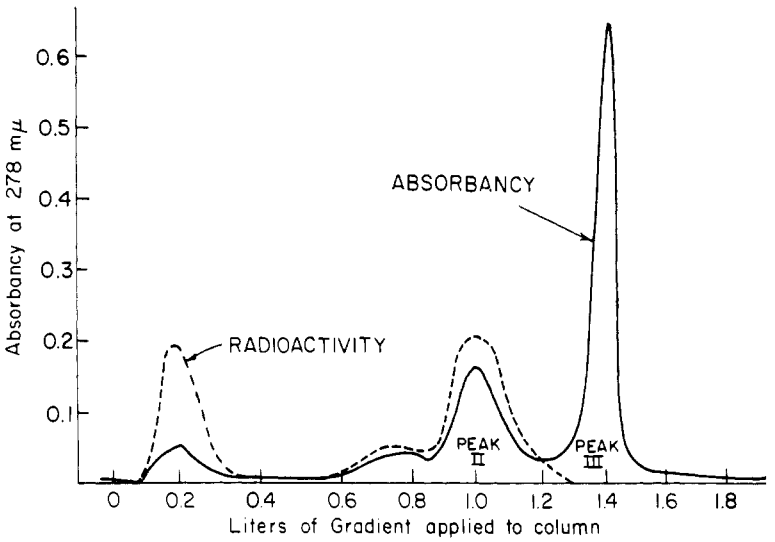

FIG. 7.—Distribution of twice-chromatographed peak II phosphoglucomutase (appearing as a single peak, Fig. 6) after incubation with ^{32}P-labeled substrate, upon repeated CM-cellulose chromatography. Chromatographic conditions as in Fig. 5.

fuse spreading of enzymatic activity and poorer resolution than that achieved with CM-cellulose. Similar experiments with sulfoethyl-Sephadex resulted in severe tailing of the eluted protein.

The major active components of muscle phosphoglucomutase separated by *p*H 7 chromatography on CM-cellulose (peaks II and III) may be identified as phospho- and dephospho-enzyme by their positions in the elution pattern and by their respective contents of exchangeable phosphate. With respect to both eluate volume and specific conductance measurements, these constituents chromatographed in analogous positions to those of phospho- and dephospho-enzyme prepared by incubation of isolated phospho-enzyme with substrate. It should be noted, however, that while peak II enzyme possessed reproducibly high specific enzymatic activity, on rare occasions peak III protein was obtained which exhibited low enzymatic activity (e.g., Fig. 3). This finding suggests that the present system may, under certain circumstances, be ineffective in separating dephospho-enzyme of uniform purity. Consequently, the most reliable means of preparing dephospho-enzyme appears to be by dephosphorylating and rechromatographing isolated phosphoenzyme.

The exchangeable phosphorus content of the enzyme, 1.03 mole/62,000 g, agrees with that observed by Anderson and Jollès (1957), and by Sidbury and Najjar (1957). The amino acid composition of chromatographically isolated phosphoglucomutase differs markedly from values reported by early workers using paper chromatography (Anderson and Jollès, 1957; Boser, 1955), and is more nearly in agreement with values given by Ray and Koshland (1962) and Najjar (1962). The chromatographed enzyme was found to contain 6 half-cystine residues and 36 serines, in agreement with the latter, but contained 4 tryptophan residues and only 12 methionines, in agreement with the former authors.

That the two active forms of phosphoglucomutase can be chromatographically separated is, of course, of significant value for kinetic and mechanistic studies concerning this enzyme. In the case of ribonuclease, enzyme which has been modified by the dinitrophenylation of a single lysyl residue can be separated from native enzyme and other dinitrophenylation products by chromatography on IRC-50 (Hirs, 1962). In that case, excellent separation of two similar proteins of minimal lability can be achieved using a resin of high resolving power and 0.2 M sodium phosphate buffer. The fact that phosphoglucomutase, an enzyme of 62,000 mw and much greater lability, can be separated into phosphorylated and nonphosphorylated components by conventional methods of protein chromatography using columns of CM-cellulose under mild conditions is encouraging for other similar cases, for example, the transaminases which also appear to exist in two stable chemically distinct forms.

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