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Physical and Chemical Studies on Crystalline Rabbit Muscle Phosphoglucomutase. Multiple Forms*

Sidney Harshman and Howard R. Six

ABSTRACT: Crystalline preparations of rabbit muscle phosphoglucomutase have been resolved into four fractions using the technique of isoelectric focusing. The fractions contained 82, 8, 6, and 4%, respectively, of the original enzyme and each was enzymatically active and recovered in the phosphorylated form.

Similar fractionation was demonstrated using acrylamide gel electrophoresis. Physical and chemical studies on the parent crystals indicated that the phosphoglucomutase "isozymes" were not due to molecular aggregation or subunit rearrangement. The molecular weight of crystalline rabbit muscle phosphoglucomutase by sedimentation equilibrium was found to be 64,900. Sedimentation equilibrium analyses of reduced and carboxymethylated phosphoglucomutase in the presence of 5 M guanidine hydrochloride gave a molecular weight of 64,000, indicating that there are no subunit structures in rabbit muscle phosphoglucomutase. The $s_{20,w}$ value, calculated from sedimentation velocity data, was 3.82 S. Calculation of the partial specific volume from amino acid composition data gives a value of 0.737. The isoelectric pH of phosphoglucomutase, using the techniques of isoelectric focusing, was found to be 6.80.

The enzyme PGM[1] from rabbit muscle has been reported to be a single homogeneous polypeptide chain with a molecular weight of 74,000 (Taylor *et al.*, 1956) and an isoelectric point at pH 8.6 (Boser, 1955). The available physical data have been summarized (Najjar, 1962). Recently a number of laboratories have reported data that are at variance with these results. The molecular weight of PGM has been reported to be 62,000 (Filmer and Koshland, 1963; Yankeelov *et al.*, 1964; Handler *et al.*, 1965; Hashimoto and Handler, 1966). The original value of 74,000 was obtained using sedimentation velocity methods, while the lower value of 62,000 is based on data obtained by the method of Yphantis (1964). Of greater significance are the more recent reports of multiple forms of PGM. Using the technique of starch gel electrophoresis, Spencer *et al.* (1964), Hopkinson and Harris (1965), and Luan Eng (1966) have reported multiple forms of PGM from human red blood cells, all of which appear to be in the phosphorylated form (Coifman and Epstein, 1969). Similar findings have been reported by Dawson and Mitchell (1969) in a study of muscle tissues from a variety of sources. In addition, two laboratories have reported that resolution of phosphoglucomutases into two components could be achieved by ion-exchange chromatography on CM-Sephadex (Joshi *et al.*, 1967) and on CM-cellulose (Yankeelov *et al.*, 1964).

We report here that the technique of isoelectric focusing resolves crystalline preparations of PGM into four enzymatically active phosphorylated forms. We also report on studies of the parent PGM crystals by Sephadex chromatography, sedimentation velocity, sedimentation equilibrium, and amino

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[1] Abbreviation used that is not listed in *Biochemistry* 5, 1445 (1966), is: PGM, phosphoglucomutase.

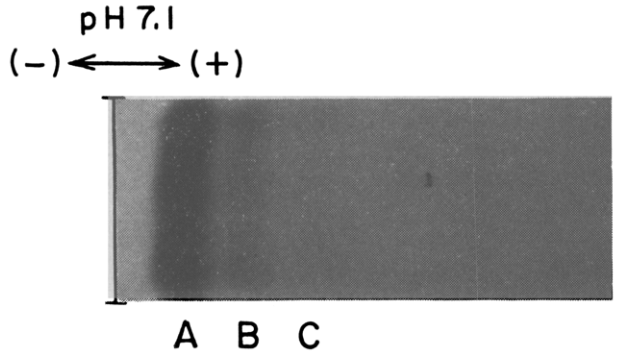

FIGURE 1: Electrophoresis of [^{32}P]PGM in acrylamide gel slabs. The conditions for electophoresis and for staining the proteins are as given in Methods. To determine the radioactivity in each protein band, slices of gel containing individual protein bands were soaked in 0.15 ml of 10 N NaOH at room temperature overnight and the solubilized ^{32}P was counted using an Ansitron scintillation counter (Wallingford, Conn.).

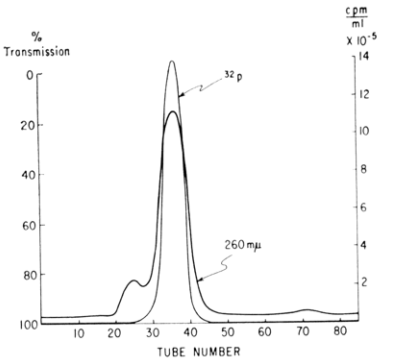

FIGURE 2: Chromatograph of crystalline rabbit muscle PGM on Sephadex G-100 columns; 203.6 mg of [^{32}P]PGM was chromatographed on a column 3.5 × 24 cm of Sephadex G-100 superfine. The eluting buffer was 0.1 M sodium acetate (pH 5.0) and 50-drop (3.0-ml) fractions were collected. Per cent transmission was recorded automatically using a Uvicord (LKB Products, Stockholm, Sweden) and the radioactivity was determined on aliquots from each sample tube using a gas-flow autoscaler (Nuclear-Chicago, Des Plaines, Ill.).

acid analysis, which indicate that the multiple forms of PGM do not arise by molecular aggregation or by subunit rearrangement.

Materials and Methods

Enzymes. PGM and [^{32}P]PGM were prepared as previously described (Najjar, 1962; Harshman and Najjar, 1965).

Amino Acid Analysis. Amino acid compositions were determined by the method of Piez and Morris (1960) using a Technicon amino acid analyzer (Tarrytown, N. Y.) equipped with both C_2- and B-type resins and range expanders. Samples were hydrolyzed in 6 N HCl in sealed, evacuated tubes at 110° for 20, 41, and 64 hr. To assure maximum recovery of tyrosine, additional samples were hydrolyzed for 20 and 41 hr in the presence of 0.9% phenol. Such treatment did not alter the yield of tyrosine from PGM. Cysteine and methionine residues were determined after oxidation to cysteic acid and methionine sulfone as described by Moore (1963) and analyzed on a Beckman-Spinco Model 120C amino acid analyzer (Palo Alto, Calif.), equipped with both sample injectors,[2] and an Infotronics Model CRS-12AB digital readout system.

Ultracentrifugation. Sedimentation analyses were performed using a Beckman Model E ultracentrifuge equipped with both the Rayleigh interference and the schlieren optical systems.

Sedimentation velocity analysis was done at a rotor speed of 59,780 rpm using Kel-F double-sector synthetic boundary cells and rotor Model No. An-D. Sedimentation coefficients were corrected to standard conditions of water at 20° and extrapolated to zero protein concentration. Molecular weights were calculated as described by Svedberg and Pedersen (1940).

Sedimentation equilibrium studies were done using the meniscus depletion method of Yphantis (1964). The rotor speed was 23,150 rpm. Kel-F double-sector cells with 12-mm optical path and quartz windows offset 11, 6, 0, −6, and −11°, respectively, were used in a Model An-G rotor. Aliquots of each sample sufficient to make 3-mm columns were overlaid on Beckman-Spinco FC43 (perfluorotributylamine). Equilibrium was achieved in 24 hr.

Acrylamide Gel Electrophoresis. The gels were prepared using a 5% solution of cyanogum 41 with "bis" added (Fisher Scientific Corp., Atlanta, Ga.). To this solution 100 mg/ml of ammonium persulfate and 0.1 ml/100 ml of 3-dimethylaminopropionitrile were added and the mixture was quickly poured into molds to give, after setting, gel slabs 6.6 × 30.5 × 0.3 cm (Raymond and Wang, 1960). The gels were washed repeatedly in 0.01 M phosphate buffer (pH 7.1) and the electrophoresis was run in the same buffer. The protein bands were visualized using buffalo black dye in methanol–water–acetic acid (5:5:1, v/v; Raymond and Wang, 1960). The electrophoresis was run horizontally using the RSCo Model E800-2B instrument (Research Specialties Co., Richmond, Calif.) cooled to 0° at a constant voltage of 750 V for 4 hr.

Electrofocusing. The electrofocusing column used had a volume capacity of 440 ml and was equipped with double-cooling jackets (LKB-Produktor, Stockholm, Sweden). The pH 3–10 gradient was achieved using ampholyte LKB 8141 and the pH 6–8 gradient with LKB 8154. Both of these compounds are mixtures of low molecular weight polyaminopolycarboxylic acids. The procedure used followed that of Vesterberg *et al.* (1967), except that the initial sucrose gradients were established by a continuous-pumping technique (Ayad *et al.*, 1968). The linear nature of the gradient was confirmed by refractive index analysis using the refractomotor Model Abbe-3L (Bausch & Lomb Optical, Rochester, N. Y.). The "dense" solution contained 200 ml of sucrose, 7.5 ml of ampholyte (40% w/v), and 7.5 ml of water, while the "light" solution contained 212.5 ml of water and 2.5 ml of ampholyte (40% w/v). The sample, PGM (10–30 mg), in a volume of 10 ml, was introduced after 200 ml of the gradient was pumped. With the pH 3–10 gradient the run was started at constant milliamperage (5.0 mA) which required approximately 300 V. After 14 hr the current was adjusted to a constant voltage of 900 V and the milliamperage fell eventually to 2.0. Total running time was 60 hr. With the pH 6–8 columns the initial conditions were the same, *i.e.*, 5 mA constant at about 300 V, which was

[2] The injectors were custom built to Dr. S. Moore's specifications by the Rockefeller University machine shop.

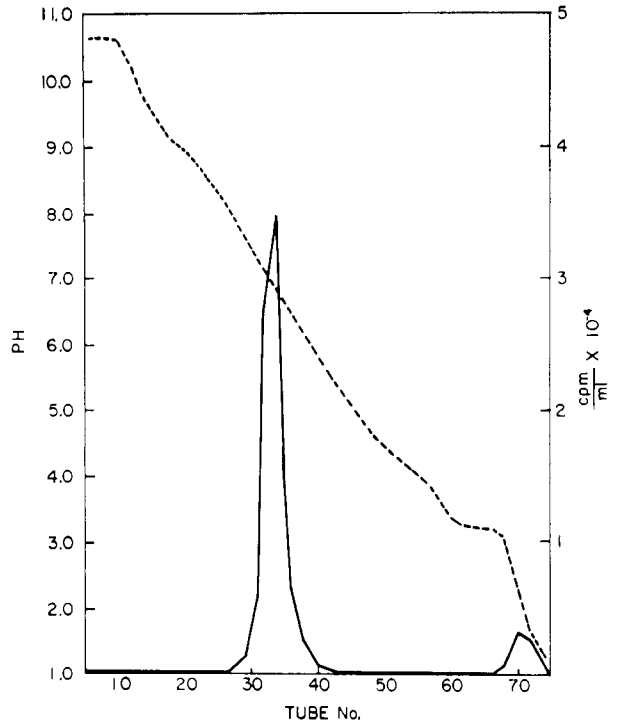

FIGURE 3: Electrophoresis focusing of [^{32}P]PGM on a pH 3–10 gradient. The data were obtained with 16 mg of [^{32}P]PGM. After attaining equilibrium, 6-ml fractions were collected and aliquots were analyzed for ^{32}P content using an Ansitron liquid scintillation counter (Wallingford, Conn.). For further details, see text.

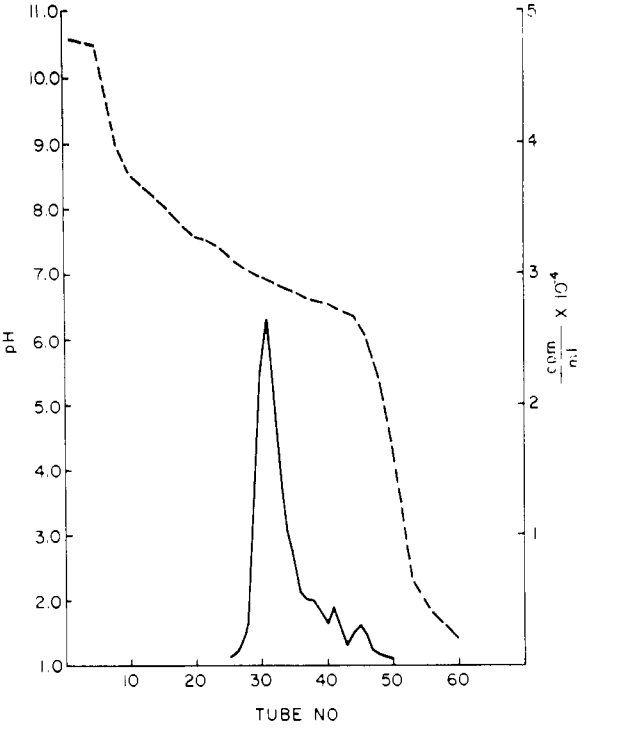

FIGURE 4: Electrophoresis focusing of [^{32}P]PGM on a pH 6–8 gradient. For details, see Figure 5 except that the [^{32}P]PGM concentration was 12 mg and the fraction volumes were 7.0 ml.

changed after 10 hr to 800 V constant with the milliamperage falling to 1.6. Total running time was 50 hr. The columns were maintained at 4°.

Results

Fractionation of PGM Using Acrylamide Gel Electrophoresis. Crystalline PGM preparations routinely show a single, broad band, corresponding to the serum γ-globulin region when tested using the usual paper strip electrophoresis technique at pH 8.6 in Veronal buffer. Such analyses are regularly done in our laboratory as a monitoring method to check for the chance contamination of the PGM crystals with albumin. As a more rigorous test of homogeneity, we elected to submit samples of PGM to the more stringent technique of gel electrophoresis. Unexpectedly both PGM and its [^{32}P]PGM derivative showed three components in acrylamide gel, all of which stained with buffalo blue dye and all of which were radioactive (Figure 1). Analysis of the radioactivity in each band showed the concentrations of the components to be distributed 85, 11, 4%, from slowest to fastest moving bands, respectively. Addition to the buffer of either 0.01 M mercaptoethanol or 0.005 M dithiothreitol did not change the distribution pattern, suggesting that the multiple phosphorylated forms of PGM observed in gel electrophoresis did not arise by multimer formation related to intramolecular disulfide-bond formation.

Purification of Crystalline PGM by Sephadex G-100 Chromatography. In order to further exclude the possibility that the multiple forms of PGM were aggregates of the parent molecule, samples of enzyme were submitted to chromatographic sieving on Sephadex. Chromatography of crystalline preparations of rabbit muscle PGM on G-100 Sephadex revealed a small amount of material with an apparent molecular size greater than that of PGM. Investigation showed that the contaminant was present in all PGM preparations tested, that it had no detectable enzymatic activity, and that it was not radiolabeled when the mixture was preincubated with G-6-^{32}P. A typical chromatogram is shown in Figure 2. Since the multiple forms of PGM were each shown to be labeled with ^{32}P, it is clear that the small amount of nonlabeled protein contaminant, 10% or less of the total protein, is unrelated to the observed multiple forms of the enzyme. Moreover, the symmetry of the peak indicates a homogeneous population of enzyme molecules and the position of elution is consistent with a molecular weight of approximately 60,000–70,000.

Fractionation of PGM Using Isoelectric Focusing. To confirm the results obtained with acrylamide gel and to exclude the possibility that the multiple forms of PGM were due to interactions of the protein with the gel, PGM, and [^{32}P]PGM, both before and after chromatography on Sephadex G-100, were examined by the electrophoresis focusing technique. With the pH 3–10 ampholyte gradient (Figure 3) a distinct skewing of the peak was observed, and with the more shallow pH 6–8 gradient (Figure 4), four distinct peaks are clearly evident. They contain 82, 8, 6, and 4%, respectively, of the original enzyme. Enzymatic assay (Najjar, 1962) for PGM showed that all of the fractions were active and that the specific activities, based on radioactivity, were approximately the same. All of the fractions were activatable from 1.5–3-fold by preincubation with magnesium ion and imidazole (Robinson *et al.*, 1965; Harshman *et al.*, 1965). Moreover, refocusing of a given fraction did not regenerate any of the other fractions, indicating

TABLE I: A Comparison of the Amino Acid Composition of "Purified" Rabbit Muscle PGM with Composition Data Reported by Others.[a]

	"Purified"[a] PGM	Yankeelov *et al.* (1964)	Sloan and Mercer (1964)
Asp	71	67	66
Thr	31	32	33
Ser	33	36	38
Glu	54	53	58
Pro	26	27	30
Gly	54	54	55
Ala	54	53	53
Half-Cys	6	6	6
Val	39	41	38
Met	12	12	10
Ile	48	45	37
Leu	48	45	44
Tyr	16	17	17
Phe	30	33	31
Lys	42	41	41
His	12	11	12
Arg	26	26	26
Trp	ND	4	8–9
Molecular weight[b]	66,900	66,500	66,900
Partial specific[b] volume	0.737	0.736	0.731

[a] Residues of amino acid are calculated from the average values obtained after 20, 41, and 64 hr of hydrolysis and are calculated relative to cysteine which was taken as six residues per mole (Bocchini *et al.*, 1967). Serine and threonine values remained stable for 41 hr of hydrolysis and were used as such. Isoleucine and leucine were calculated from the maximum yields obtained after 64 hr of hydrolysis. For further details, see Methods. [b] The calculations for molecular weights and for partial specific volumes are based on the amino acid compositions given. In the case of "purified" PGM an amide content of 57 residues is assumed and the tryptophan content is taken to be four residues per mole (Yankeelov *et al.*, 1964).

that the four forms observed do not represent a simple equilibrium mixture. These data further show that the major component in crystalline rabbit muscle PGM is isoelectric at pH 6.80.

In view of the demonstrations that our preparations of crystalline rabbit muscle PGM were (a) contaminated with small amounts of inert protein and (b) contained multiple phosphorylated forms of enzyme, we elected to reinvestigate certain parameters of the parent crystals.

Amino Acid Composition and Partial Specific Volume of PGM. Since a number of laboratories have reported amino acid compositions for rabbit muscle PGM (Najjar, 1962; Sloan and Mercer, 1964; Yankeelov *et al.*, 1964), it was of interest to reexamine this question using "purified" PGM. Accordingly, after chromatography on Sephadex G-100, aliquots of PGM were hydrolyzed and the number of amino acid residues, corrected for hydrolytic losses, was determined (Table I). It is apparent that the amino acid composition of "purified" PGM does not significantly differ from that reported for CM-cellulose chromatographed rabbit muscle PGM (Yankeelov *et al.*, 1964). These results indicate also that the amino acid distribution in the contaminant protein is "normal" and, therefore, at a 10% level, it does not significantly modulate the amino acid composition data for PGM. An additional piece of information can be obtained from the amino acid composition data. By summation of the individual amino acid specific volumes (Cohn and Edsall, 1943; McMeekin and Marshall, 1952), the specific volume for PGM can be calculated to be 0.737.

Determination of $s^0_{20,w}$ for "Purified" PGM. To determine the $s^0_{20,w}$ value for rabbit muscle PGM after Sephadex G-100 purification, various concentrations of "purified" PGM were analyzed in a Spinco Model E centrifuge at a rotor speed of 59,780 rpm in 0.10 M sodium phosphate buffer (pH 6.8) (Figure 5). The s values, corrected to $s_{20,w}$, for 1.82, 1.35, 0.91, and 0.45 g per 100 ml of PGM were 3.41, 3.84, 3.67, and 3.77 S, respectively. The $s_{20,w}$ value obtained with the 1.35-g/100 ml sample is clearly displaced and was not used in constructing the extrapolation curve for zero concentration. The extrapolated $s_{20,w}$ value obtained for zero concentration of "purified" PGM was 3.82 S which agrees well with the reported value of 3.69 S (Taylor *et al.*, 1956). Further, the observed symmetry of the peaks during centrifugation is an additional indication of the molecular homogeneity of the "purified" PGM preparation.

Determination of the Molecular Weight of PGM by Sedimentation Equilibrium. To determine the molecular weight of PGM, analysis of "purified" PGM was undertaken using the sedimentation–equilibrium method (Yphantis, 1964). Accordingly, dilutions of PGM were made in 0.05 M potassium phosphate buffer (pH 6.8) to give solutions containing 0.75, 0.60, 0.40, and 0.20 mg per ml of protein. The values for d in C/dx^2 obtained at each concentration were 1.793, 1.855, 1.910, and 1.970, respectively. From the extrapolation of the calculated molecular weights to zero protein concentration (Figure 6) a molecular weight for PGM of 64,900 was obtained. It is of particular interest that similar values for the molecular weight of PGM were obtained when samples of PGM, which were reduced and carboxymethylated (Crestfield *et al.*, 1963) were subjected to sedimentation equilibrium analysis in the presence of 5 M guanidine hydrochloride. Since this concentration of guanidine hydrochloride has been shown to be sufficient to cause dissociation of most multimer proteins (Reithel, 1963), the fact that such treatment did not change the molecular weight values for PGM may be taken as strong support for the view that PGM has no subunit structure (Najjar, 1962; Harshman and Najjar, 1965).

Discussion

Crystalline preparations of rabbit muscle PGM can be resolved into four phosphorylated forms of the enzyme by the technique of isoelectric focusing (Figure 4). Reelectrophoresis of an isolated fraction does not regenerate the total pattern suggesting that the various phosphorylated forms of PGM are not a simple equilibrium mixture. Each of the forms is enzymatically active and can be recovered quantitatively. Thus the technique of isoelectric focusing for fractionating the phos-

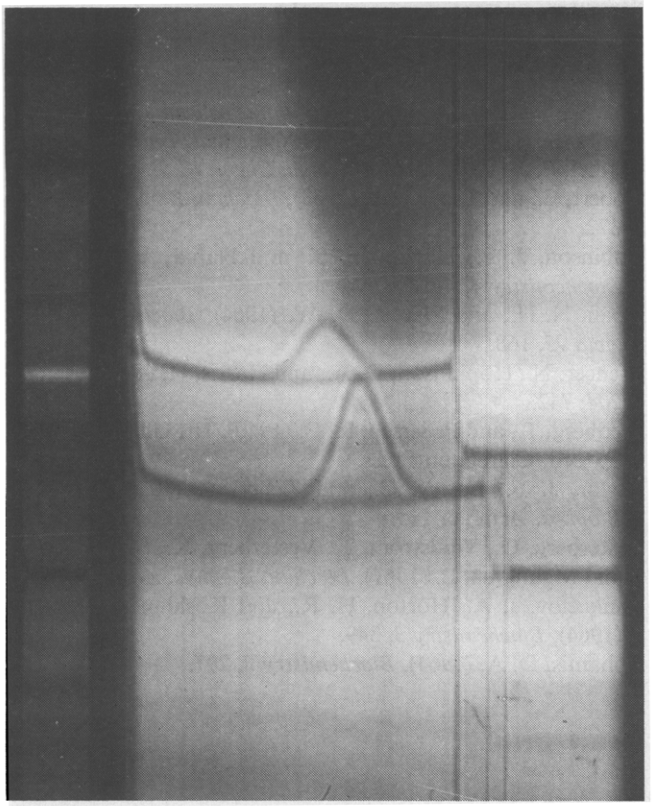

FIGURE 5: Sedimentation velocity analysis of PGM. The direction of sedimentation was from right to left. The protein concentration in the 2.5° angle cell (upper curve) was 4.5 mg/ml and the concentration in the standard cell (lower curve) was 9.0 mg/ml. The photo was taken 56 min after reaching speed at a bar angle of 70°. For further details, see text.

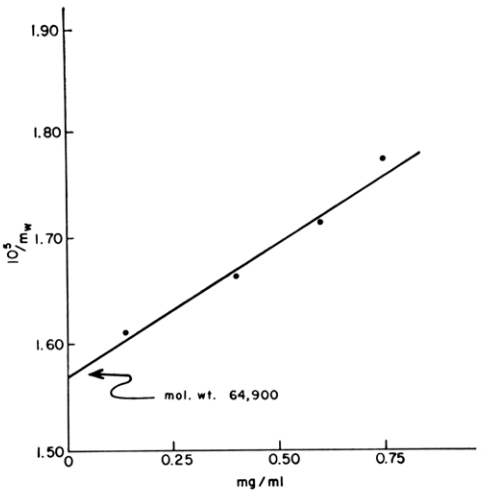

FIGURE 6: A plot of the protein concentration against the reciprocal of the molecular weight obtained from sedimentation equilibrium data. For details, see text.

phorylated forms of rabbit muscle PGM offers some distinct advantages over other fractionation procedures. Fractionations by acrylamide gel (Figure 1) or starch gel electrophoresis (Robert and Tsuyuk, 1963; Hopkinson and Harris, 1965; Luan Eng, 1966; Dawson and Mitchell, 1969; Coifman and Epstein, 1969) do not lend themselves easily to large-scale fractionation or to quantitative enzyme recovery. Ion-exchange methods also have been applied to PGM fractionation. However, the DEAE method does not work with rabbit muscle PGM (Dawson and Mitchell, 1969) and the CM-Sephadex method, which yields two forms of phosphorylated PGM (Joshi *et al.*, 1967), does not resolve the electrophoretically separable forms (Dawson and Mitchell, 1969).

Chromatography of crystalline PGM on Sephadex G-100 (Figure 2) reveals the presence of small amounts (10%) of a contaminant, nonfunctional protein. However, such contaminants are not thought to play a role in the fractionation reported here. This conclusion derives from the fact that all of the forms of PGM detected during isoelectric focusing react with G-6-^{32}P whereas the contaminant protein does not.

A number of studies on the parent PGM preparations indicate that the fractionation achieved by electrophoresis focusing is not due to aggregation of monomer units. The symmetry of the elution pattern of the PGM from Sephadex G-100 suggests a homogeneity of molecular size (Figure 2). The failure of added mercaptoethanol or dithiothreitol to alter the pattern observed in acrylamide gel (Figure 1) argues against the formation of intramolecular disulfide bonds. The linear fit of the data from sedimentation equilibrium studies (Figure 6) also argues against the formation of aggregates as does the symmetry of the peaks obtained during sedimentation velocity studies (Figure 5). It is of particular interest that 5 M guanidine hydrochloride does not alter the molecular weight as determined by sedimentation equilibrium. Such a result indicates the absence of subunit structure in PGM, which precludes the formation of multimers of the type found in lactic acid dehydrogenase isozymes (Markert, 1968).

Since the same preparations of PGM were used in both the sedimentation equilibrium and the sedimentation velocity studies, a direct comparison can be made of the molecular weight estimates of PGM obtained by these two methods. Sedimentation equilibrium data gave a molecular weight value of 64,900. An $s_{20,w}$ value of 3.82×10^{-13} S was obtained from the sedimentation velocity studies. Using the $s_{20,w}$ value and the values 4.83×10^{-7} cm^2 sec^{-1} (Taylor *et al.*, 1956) for $D_{20,w}$, and 0.737 for $\bar{V}$ (Table I), a molecular weight of 73,200 was calculated. The basis for the discrepancy in the molecular weights of PGM as calculated by each method is unknown. The possibility exists that the reported value for $D_{20,w}$ of 4.83×10^{-7} (Taylor *et al.*, 1956) is low. Calculation of $D_{20,w}$, assuming a molecular weight of 65,000 (Figure 3), gives a value of 5.45×10^{-7}, which agrees closely with typical values observed for other proteins in this molecular weight class (Edsall, 1953).

Acknowledgment

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The Role of G Factor in Protein Synthesis. Studies on a Temperature-Sensitive *Escherichia coli* Mutant with an Altered G Factor*

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ABSTRACT: A temperature-sensitive *Escherichia coli* mutant with a specific defect in a supernatant factor required for protein synthesis has been studied. The altered factor has been identified as G by DEAE-Sephadex column chromatography: in a polyuridylic acid-directed cell-free system the addition of wild-type G restores the ability of the mutant supernatant to synthesize polyphenylalanine. When NH_4Cl-washed ribosomes and purified T factor from the mutant are incubated in the presence of polyuridylic acid, guanosine triphosphate, and [^{14}C]phenylalanine transfer ribonucleic acid, the binding of aminoacyl transfer ribonucleic acid to ribosomes proceeds normally. Paper chromatography of the products of the binding reaction shows the presence of a dipeptide peak with trace amount of tripeptides. The addition of wild-type G to the binding mixture results in a decrease of the dipeptide peak with parallel increase of tripeptides and longer polypeptide chains. Experiments with puromycin indicate that ribosomes from the mutant are not impaired in their ability to form a peptide bond with the antibiotic. In conclusion washed ribosomes from the mutant are able to synthesize a single peptide bond. Chain elongation depends upon the addition of G factor prepared from wild type.

In bacterial extracts distinct factors for initiation (Stanley *et al.*, 1966; Eisenstadt and Brawerman, 1966; Revel and Gros, 1966; Clark and Marcker, 1965; Brown and Doty, 1968), elongation (Nakamoto *et al.*, 1963; Lucas-Lenard and Lipmann, 1966), and termination (Capecchi, 1967; Ganoza and Nakamoto, 1966; Takanami and Yan, 1965; Bretscher *et al.*, 1965) have been described. Our knowledge of the number of protein factors required for polypeptide synthesis has been growing very fast but, so far, the genetic approach to the problem has been neglected.

Several laboratories have isolated temperature-sensitive mutants of *Escherichia coli* (Kohiyama *et al.*, 1966; S. Brenner, personal communication) and strains with altered aminoacyl-

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