

PHOSPHOGLUCOMUTASE, V. MULTIPLE FORMS OF PHOSPHOGLUCOMUTASE

BY J. G. JOSHI, J. HOOPER, T. KUWAKI, T. SAKURADA,
J. R. SWANSON, AND P. HANDLER

DEPARTMENT OF BIOCHEMISTRY, DUKE UNIVERSITY MEDICAL CENTER,
DURHAM, NORTH CAROLINA

Communicated March 30, 1967

Previous publications from this laboratory which described the purification and properties of phosphoglucomutase (PGM) from diverse biological sources indicated that it is probably the expression of a homologous gene.¹⁻⁷ In a continuation of these studies it was observed that extracts of human muscle contained two PGM's separable by *O*-(carboxymethyl) (CM)-Sephadex column chromatography. This finding prompted a search for similar pairs of PGM's in other organisms. Although the relative proportions vary considerably, two forms of PGM are present in a variety of species including rabbit muscle, the usual source of this enzyme for studies of its mechanism of action.

Experimental.—Materials: Glucose 1-phosphate, glucose 1,6-diphosphate, and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Company. CM-Sephadex, *O*-(diethylaminoethyl) (DEAE) Sephadex, and Sephadex G-100 were obtained from the Pharmacia Chemical Company. Human muscle was procured from the surgical pathology laboratory at Duke Hospital and stored frozen until use. Frozen muscle from rabbits of desired strain was obtained from Pel-Freezer. Albino rats were from a local strain; sweet potato and white potato were locally procured.

Methods: PGM activity was estimated by a coupled assay method. The assay medium contained 40 μ moles imidazole HCl-buffer, pH 7.5, 2 μ moles of glucose 1-phosphate, 3.5 μ moles of glucose 1,6-diphosphate, 0.5 μ mole of triphosphopyridine nucleotide (TPN⁺), 1 enzyme unit of glucose 6-phosphate dehydrogenase, and the source of PGM in a total volume of 1 ml. Activity was determined at room temperature by measuring the rate of increase in optical density at 340 μ . One unit of enzyme activity catalyzed the formation of 1 μ mole of glucose 6-phosphate in 1 min.

Tissues were homogenized with 10 vol of ice-cold water in a Waring Blendor for 45 sec; the homogenates were filtered through glass wool and filtrates centrifuged at 18,000 $\times g$ for 20 min at 4°. The supernatants were dialyzed against 1 mM Na⁺ (Na₂HPO₄-HCl), pH 7, containing 0.1 mM ethylenediaminetetraacetate (EDTA), centrifuged, and the supernatants used for column chromatography. CM-Sephadex column chromatography was employed for a semiquantitative detection of PGM's in tissue extracts. The prepared extract was applied to a 2.5- \times 40-cm column of CM-Sephadex, previously equilibrated with the dialyzing buffer. The column was then eluted with the Na⁺ gradient generated by a mixing chamber and reservoir which contained 1 liter each of 1.0 mM Na⁺ and 33 mM Na⁺, respectively, and 0.1 mM EDTA.

DEAE-Sephadex chromatography was performed similarly. The gradient bottles contained 1 liter each of 1 mM Na⁺ and 70 mM Na⁺, respectively, and 0.1 mM EDTA. The buffers used for CM-Sephadex and DEAE-Sephadex chromatography were prepared by diluting 0.1 *M* Na⁺ (Na₂HPO₄-HCl) stock solution, pH 7.0, without further correction for the change in pH caused by dilution. Sephadex G-100 column chromatography was used as a final step of purification. A 2.8- \times 100-cm column of Sephadex G-100, with a bed volume of 600 ml, was equilibrated with 0.1 *M* phosphate, pH 7, containing 1 mM Mg⁺⁺ and 0.1 mM EDTA, and eluted with the same buffer at a rate of 0.5 ml per min: 3.0-ml fractions were collected. All preparative procedures were conducted at 0 to -4° unless stated otherwise.

Purification of phosphoglucomutases from rabbit muscle: The procedure used was generally similar to that described by Najjar,⁸ modified to separate both enzymes and improve yields. About 1 kg of muscle from New Zealand white rabbits was extracted at 0° with 3 vol of water

for 30 min. The extract was filtered through two layers of cheesecloth and the pH of the filtrate adjusted to 5.0 with 1 *M* acetic acid. The solution was then brought to 60° in boiling-water bath and cooled immediately. Solid ammonium sulfate was added to 0.25 saturation and the inactive precipitate was centrifuged and discarded. The supernatant was brought to 0.75 saturation with solid ammonium sulfate. After 1 hr the precipitate was centrifuged, dissolved in 0.15 *M* acetate buffer, pH 5, heated to 60° as before, cooled, centrifuged, and the supernatant brought to 0.7 ammonium sulfate saturation. The precipitate was centrifuged and dissolved in 1 mM Na⁺ buffer, pH 7, containing 0.1 mM EDTA, and dialyzed against 100 vol of the same buffer for 12 hr with one change after every 4 hr. Any precipitate formed was removed by centrifugation and the supernatant applied to a CM-Sephadex column. The column was washed with 500 ml of the same buffer, and then eluted. Peak II enzyme appeared at about 0.015 *M* Na⁺ concentration.

The breakthrough volume contained peak I enzyme which was concentrated by ammonium sulfate precipitation (0.70 saturation). After dialysis against 1 mM Na⁺, 0.1 mM EDTA, pH 7.0, this fraction was further purified by DEAE-Sephadex column chromatography; the enzyme appeared at about 0.04 *M* Na⁺ concentration.

Heavier impurities which remained in both preparations (peak I and peak II), were readily removed by chromatography on Sephadex G-100. Under the conditions described, peak I and peak II enzymes were both eluted between tubes 71 and 91, and gave superimposable symmetrical protein and activity peaks. About 200 mg of peak II and 10 mg peak I enzyme were obtained, respectively. The total units recovered represented 70–80% of the initial activity. When commercial crystalline enzyme from the Sigma Chemical Co., prepared by the conventional method, was similarly fractionated chromatographically, a similar distribution was obtained.

Comparison of peak I and peak II phosphoglucomutases: About 6–10 mg of enzyme in 1 ml was denatured by dialysis overnight against 50 ml of 10 *M* urea; when the urea was removed by dialysis against water, the enzyme precipitated. Twice-crystallized trypsin (Worthington) was then added (protein to trypsin, 100:1) and the pH was adjusted to 8.0 with ammonium carbonate. After 12 hr at 40°, the solutions were lyophilized, the residue dissolved in dilute ammonia, and spotted on Whatman 3MM filter paper. Electrophoresis was conducted at pH 6.5 (pyridine, 200; acetic acid, 8; and water to make 2000 ml) for 2 hr at 800 volts. The papers were dried and chromatography was conducted in *n*-butanol-pyridine-acetic-water (30:6:20:24) for 10 hr. The dried papers were sprayed with ninhydrin, the spots marked with pencil, and then resprayed with Pauley's reagent. Total amino acid analysis of each form of PGM was performed using a standard technique,⁹ with the Beckman Spinco automatic amino acid analyzer.

Effects of insulin and epinephrine on enzyme activity in vivo: Two separate experiments were conducted. In the first experiment, 36 male rats, weighing between 180 and 200 gm were divided into two equal groups. Group A was fed a stock diet *ad libitum*, and group B was fasted for 36 hr prior to hormone injection. Six rats from each group were given 0.2 units of insulin subcutaneously, six received 0.2 mg of epinephrine, and six served as controls. In the second experiment, the animals were fasted for 72 hr, after which appropriate groups were given two doses of 0.6 mg of adrenaline or 0.6 units of insulin at 4-hr intervals on the first day and a third injection the following day.

All the animals were sacrificed 3 hr after the last injection; liver and leg muscle were removed and homogenized in ice-cold 0.9% NaCl. After centrifugation at 18,000 × *g* for 10 min, 0.4-ml aliquots of each supernatant were transferred to test tubes containing, in a total volume of 0.1 ml, 200 μmoles of imidazole-HCl buffer, pH 7.5, and 0.5 μmole of Mg⁺⁺. PGM activity was determined using 10- or 20-μl aliquots immediately and after 1 hr of preincubation in this buffer at room temperature. Protein was estimated from absorbancy at 280 mμ.

Results and Discussion.—The results of the chromatographic fractionation of PGM activities of various tissue extracts are summarized in Table 1. A typical elution pattern from CM-Sephadex is shown in Figure 1. The presence of two PGM's seems to be a general phenomenon. The major PGM activity in muscle from man, flounder, rat, and a non-New Zealand white rabbit, and in rat and rabbit livers appeared as peak I enzyme, i.e., in the breakthrough volume. The remainder, peak II, was eluted at about 0.015 *M* Na⁺ concentration. Independent rechromatography

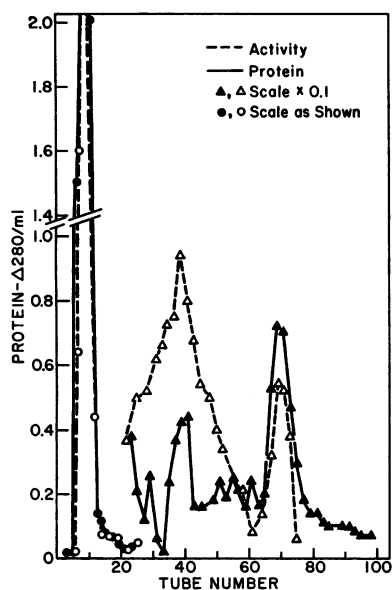


FIG. 1.—Chromatography of human muscle extracts on CM-Sephadex column.

mato-graphy of peaks I and II from human and rabbit muscle, as well as yeast, did not alter their elution patterns.

Occasionally a small third peak, peak III, appeared at 0.02 M Na⁺. A small amount of peak III enzyme from human muscle was labeled with glucose 1-phosphate-P³², mixed with peaks I and II and the mixture rechromatographed as before. The eluted fractions were assayed for radioactivity and enzyme activity. All radioactivity then appeared in the peak II fraction, indicating that peak III enzyme is a dephosphorylated form of peak II enzyme. Similar conversion of peak III to peak II enzyme was observed with rabbit and yeast enzymes. Indeed, rechromatography of an aged preparation of pure peak II from a yeast autolysate yielded a small additional activity peak at the peak III position with a corresponding decrease in peak II. A similar observation has been reported for rabbit muscle enzyme fractionated by CM-cellulose column chromatography.^{10, 11}

Peak II PGM exceeded the peak I form only in New Zealand rabbit muscle, yeast, and the sweet potato. It is noteworthy that the very small amount of peak II PGM in flounder muscle had escaped detection in earlier DEAE-cellulose chromatography.⁴

A fourth peak, the dephospho form of peak I enzyme, was detected only in the sweet potato and in fresh rabbit liver, although it might well appear in other tissues when other extraction procedures are employed. The actual relative amounts of

TABLE 1
SEPARATION OF PHOSPHOGLUCOMUTASES BY COLUMN CHROMATOGRAPHY

Species	Tissue	Chromatography on	PGM Eluted in Peak (%)			
			I	II	III	IV
Human	Muscle	CM-Sephadex	80	14	6	—
Rabbit (New Zealand white)	Liver	CM-Sephadex	6	54	11	29
	Muscle	CM-Sephadex	20	70	10	—
		CM-cellulose ¹¹	20	70	10	—
	Liver*	CM-Sephadex	92	8	—	—
	Muscle*	CM-Sephadex	80	15	5	—
Rat (Adult)	Muscle	CM-Sephadex	64	24	12	—
	Liver	CM-Sephadex	92	8	1	—
(Newborn)	Liver	CM-Sephadex	96-97	3-4	—	—
Flounder	Muscle	DEAE-cellulose ⁴	>99	—	—	—
		CM-Sephadex	99.5	0.5	—	—
Sweet potato		DEAE-Sephadex	1-2	60	20	18
White potato		DEAE-Sephadex		30-40		60-70
Yeast		CM-cellulose	10	85	5	—
		CM-cellulose ¹²	5	95	0.0	—
<i>E. coli</i>		CM-Sephadex	>99.9	—	—	—

Literature values taken from data in references cited.

* Rabbit strain unknown but other than New Zealand white.

the four peaks from sweet potato have varied greatly with the age of the preparation, the condition of the tissue, length of time in storage, and the time of harvest. The figures shown in Table 1 are from one analysis only. Thus all the species tested, from man to yeast, exhibit two chromatographically separable forms of PGM. In contrast, all the activity in *Escherichia coli* appeared in the breakthrough. However, it should be noted that *E. coli* does contain yet another functional PGM, a "glucose phosphate transferase," which is specific for glucose rather than glucose 1,6-diphosphate as a cofactor for catalysis of the same over-all reaction.¹³

Comparison of two forms of phosphoglucumutase: In the recent past, several enzymes have been shown to exist in more than one form,¹⁴⁻¹⁷ and more such instances are being encountered. Where these enzymes have been shown to be comprised of more than one nonidentical polypeptide chain, the multiple forms have resulted from different combinations thereof. However, PGM appears to be a single polypeptide chain. In view of the highly unequal amounts of the two forms in most sources, it is unlikely that PGM occurs as a dimer which is separated into two enzymically active monomers during the isolation procedure. Conceivably, mild proteolysis or deamidation of the native enzyme *in vivo* or *in vitro*^{18, 19} might cause the emergence of two discrete activity peaks on CM-Sephadex column chromatography. These possibilities were tested as follows:

(1) Crude extracts of rabbit and human muscle in water or 0.9 per cent NaCl and yeast autolysate were centrifuged in a sucrose density gradient and the fractions assayed for PGM activity (Fig. 2a). Since all purification procedures for PGM have included a heat step, yeast autolysate which had been heated to 65° in 0.15

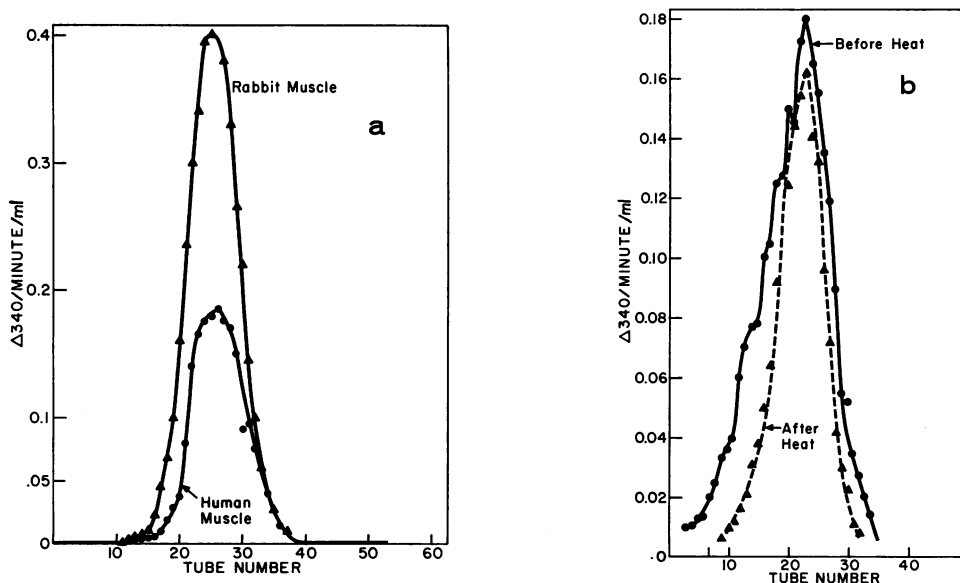


FIG. 2.—Distribution of PGM in sucrose density gradient in (a) crude extracts of human and rabbit muscle and in (b) yeast autolysates before and after heat treatment. Tissue homogenates were prepared as described in the text, dialyzed against 0.05 M phosphate, pH 7.0, containing 0.1 mM EDTA and 1.0 mM Mg^{++} . Sucrose solutions were prepared in the same buffer. All samples were dialyzed against the buffer, centrifuged, and 0.2 ml of the supernatant was used per gradient tube.

TABLE 2
AMINO ACID COMPOSITION OF PHOSPHOGLUCOMUTASES FROM RABBIT MUSCLE

Amino acid	24 Hr Hydrolysis		72 Hr Hydrolysis	
	I	II	I	II
Lysine	39.1	38.2	40.0	39.3
Histidine	8.3	10.0	8.6	10.2
Ammonia		39.9	43.4	42.4
Arginine	24.9	24.4	26.0	25.5
Tryptophan*	2.8	2.2	2.6	1.9
Aspartic acid	62.9	65.8	59.5	62.8
Threonine	30.0	31.0	30.0	30.4
Serine	36.0	37.3	30.2	32.5
Glutamic acid	52.2	54.5	52.1	51.3
Proline	30.9	30.4	30.8	26.5
Glycine	53.4	58.2	52.1	54.1
Alanine	46.7	52.9	41.9	51.1
Valine	24.0	26.5	29.9	34.6
Methionine	14.2	11.8	12.9	11.2
Isoleucine	24.6	27.2	34.6	37.5
Leucine	50.6	42.2	49.4	42.4
Tyrosine	21.0	17.2	19.5	15.4
Phenylalanine	31.6	31.5	30.6	31.0

Residues calculated for 60,000 molecular weight.

* Values not accurate: no separate method was used for their detection.

saturated ammonium sulfate was compared with an unheated control (Fig. 2b). In all cases, the activity peaks appeared at the positions expected for a pure protein with a molecular weight of about 62,000.^{1, 2, 6, 10, 11} Although human and rabbit muscle extracts gave symmetrical activity peaks, yeast autolysates exhibited a nonsymmetrical peak with a maximum at a position similar to that of the other PGM's tested. Heat treatment sharpened the peak. However, when heated²¹ and crude autolysates were independently analyzed by chromatography over a G-200 column (3-4 × 100 cm), only one symmetrical activity peak emerged in both instances.

(2) Twenty grams of rat muscle were dissected from a single animal. Ten grams were extracted with water immediately, and the remainder after three hours at room temperature. The two extracts were independently subjected to CM-Sephadex column chromatography. Although the second sample had lost about 30 per cent of the total activity, three activity peaks emerged in both cases, and in the same relative proportions, indicating that the presence of two major forms of PGM is not an artifact introduced by proteolysis. Further, whereas autolysates of a wild-type yeast exhibited two PGM's separable on CM-cellulose and by electrophoresis, similar autolysates of a galactose mutant¹² exhibited only ten per cent of the normal total activity, all of which was in peak I.

(3) The amino acid compositions of pure PGM I and PGM II from rabbit muscle show significant differences (Table 2). Both enzymes have essentially equal numbers of arginine, lysine, aspartic acid, and glutamic acid residues. Although the values for ammonia are comparable, this is not a reliable assay and the two enzymes may differ in their amide/dicarboxylic acid distributions. Peak I enzyme definitely has more leucine, tyrosine, and methionine, while peak II enzyme has more histidine, alanine, and valine. The total amino acid composition shown for peak II enzyme compares well with that described by Ray and Koshland who isolated this enzyme by CM-cellulose chromatography.¹⁰ As shown in Figure 3, there are also highly significant differences in the fingerprint maps of the two enzyme forms. About 44

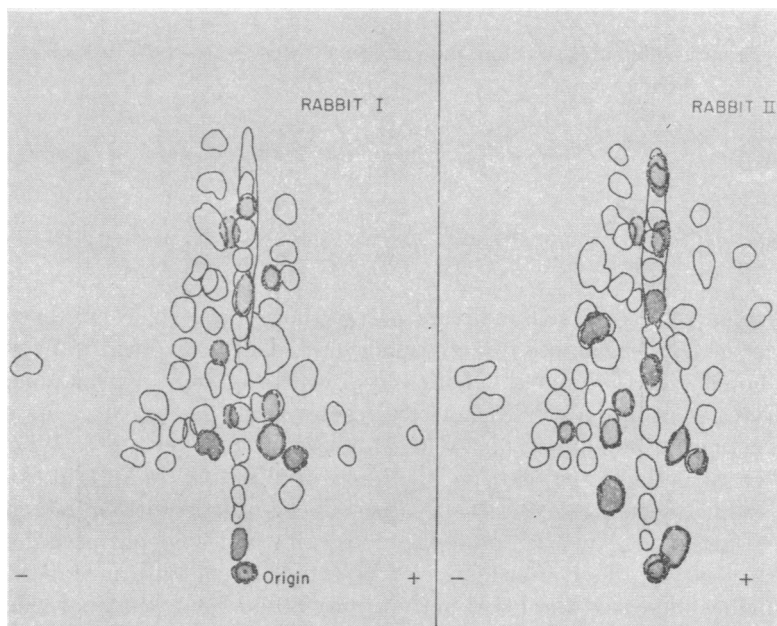


FIG. 3.—Fingerprint maps of the tryptic digest of PGM I and PGM II from rabbit muscle indicating peptides showing positive reaction to ninhydrin ○, and to Pauley's reagent ●. For details, see text.

to 46 of the theoretical 65 spots could be detected on the fingerprint maps. In both cases, a strong ninhydrin-positive reaction was seen at the origin, as well as along the center line of the chromatograms, indicating the presence of a mixture of neutral peptides not resolved by the procedure employed. However, the fact that more than 60 per cent of the theoretical peptides could be clearly distinguished again indicates that the native enzyme is a single chain of about 62,000 molecular weight, in accord with ultracentrifugal studies of this enzyme in urea and guanidine solution.²²

The evidence presented here demonstrates the ubiquity of two PGM's among higher organisms. The three enzymatically active components in human muscle extracts reported here are consistent with the appearance of three²⁴ rather than eight PGM activity bands reported to be detected by starch gel electrophoresis of human erythrocyte lysates.²³ Similar multiple bands have also been observed in muscle extracts of rainbow trout.²⁵

Harris has shown²⁴ that the electrophoretic pattern for PGM in lysates of human erythrocytes is remarkably constant for a single individual but varies considerably among individuals. They have therefore suggested that the enzyme, like alkaline phosphatase, is controlled by two separate genes, a suggestion first made by Tsoi and Douglas.¹² In view of the facts that considerable variation in the relative amounts of the two forms are observed both among individuals and between species and that the two forms differ in amino acid composition, it is clear that the two forms are phenotypic expressions of related but independent genes.

Effect of fasting and hormones on PGM activity of rat tissues: The significance of the presence of two distinctly different PGM's in the same tissue is not clear. Since

TABLE 3
EFFECT OF INSULIN AND EPINEPHRINE ON PGM ACTIVITY IN RAT TISSUE

Expt.		Preincubation with imidazole and Mg ⁺⁺	PGM		Activity (units/mg protein)		Epinephrine	
			Liver	Muscle	Liver	Muscle	Liver	Muscle
I	Fed	—	2.6	10.0	2.6	9.1	2.4	8.4
		+	3.6	16.8	3.3	14.0	3.1	15.1
	Fasted	—	1.5	7.0	1.5	5.6	1.3	6.5
		+	2.2	12.5	2.2	14.5	2.0	14.6
II	Fed	+	2.8	12.0	2.2	14.1	2.0	12.4
	Fasted	+	1.5	8.9	2.0	14.5	1.8	13.8

both enzymes were detected in livers of new-born rats, their existence, unlike glucokinase or UDP-galactose pyrophosphorylase, does not appear to be related to the development of the animal. Differential centrifugation of liver homogenates showed that essentially all PGM activity resides in the cytoplasm. As shown in Table 3, fasting for 36 hours reduced PGM activity by 50 per cent. Whether this represents a reduction in net enzyme synthesis, or alteration in V_{max} of existing enzyme by other means, remains to be ascertained. Little further reduction was seen when fasting was further prolonged. Insulin and epinephrine did not alter the PGM activity of the liver or muscle of fed rats. In contrast, in starved animals both hormones increased the PGM activities by 50 per cent, the final values being comparable to those of the fed control groups. In all cases, preincubation of the extracts with imidazole and Mg⁺⁺ stimulated the enzyme by 50–100 per cent, indicating that the activation process is not related to hormonal effects or the condition of the animal. In order to ascertain which of the two PGM's is affected by starvation, chromatographic fractionation of homogenates of livers from fasted (36 hr) and fed rats was conducted. The results clearly indicated that the major fraction, peak I, was markedly reduced, whereas peak II was essentially unaffected.

It is pertinent to note that it has been reported that total PGM activity in slow-growing hepatoma is considerably reduced, and in a fast-growing hepatoma, the K_m of PGM is significantly elevated.²⁶ In view of the results reported here, it will be of interest to ascertain which of the two enzymes is so affected. It is noteworthy, however, that in all these instances,^{26, 27} as reported here, PGM activity is increased to the same extent by preincubation of the enzyme with imidazole and magnesium, in accord with the demonstration that this phenomenon reflects the removal of inhibitory metal ions.^{5, 28}

Clearly, these studies are incomplete and pose several interesting questions. Experiments now in progress are designed to examine more thoroughly the amino acid sequence at the active site of the two enzymes in several different organisms, and to re-examine the catalytic properties of the two forms of PGM. The data here reported, however, further strengthen the case for the manner in which novel proteins appear in evolution. Gene duplication, independent of cell division, afforded the opportunity for subsequent independent mutational remodeling of the respective gene products. In this instance, both products are still functional phosphoglucomutase, neither of which appears to confer any unique advantage, or to have acquired new functional properties, unlike the various polypeptide chains of the hemoglobins or the homologous digestive enzymes of the pancreas. Hence, these phosphoglucomutases may be regarded as analogues of the early history of hemoglobins or the pancreatic enzymes.

- ¹ Joshi, J. G., and P. Handler, *J. Biol. Chem.*, **239**, 2741 (1964).
- ² Joshi, J. G., T. Hashimoto, K. Hanabusa, H. G. Dougherty, and P. Handler, in *Evolving Genes and Proteins*, ed. V. Bryson and H. J. Vogel (New York: Academic Press, Inc., 1965), p. 207.
- ³ Hanabusa, K., H. G. Dougherty, C. del Rio, T. Hashimoto, and P. Handler, *J. Biol. Chem.*, **241**, 3930 (1966).
- ⁴ Hashimoto, T., and P. Handler, *J. Biol. Chem.*, **241**, 3940 (1966).
- ⁵ Hashimoto, T., J. G. Joshi, C. del Rio, and P. Handler, *J. Biol. Chem.*, in press.
- ⁶ Handler, P., T. Hashimoto, J. G. Joshi, H. G. Dougherty, K. Hanabusa, and C. del Rio, *Israel J. Med. Sci.*, **1**, 1173 (1965).
- ⁷ Joshi, J. G., J. Hooper, J. R. Swanson, T. Sakurada, T. Kuwaki, and P. Handler, *Federation Proc.*, in press.
- ⁸ Najjar, V. A., in *Methods in Enzymology*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press, Inc., 1955), vol. 1, p. 294.
- ⁹ Speckman, D. H., W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
- ¹⁰ Ray, W. J., and D. E. Koshland, Jr., *J. Biol. Chem.*, **237**, 2493 (1962).
- ¹¹ Yankeelov, J. A., H. R. Horton, and D. E. Koshland, *Biochemistry*, **3**, 349 (1964).
- ¹² Tsoi, A., and H. C. Douglas, *Biochim. Biophys. Acta*, **92**, 513 (1964).
- ¹³ Fujimoto, A., P. Ingram, R. A. Smith, *Biochim. Biophys. Acta*, **96**, 91 (1965).
- ¹⁴ Kaplan, N. O., in *Evolving Genes and Proteins*, ed. V. Bryson and H. J. Vogel (New York: Academic Press, Inc., 1965), p. 243.
- ¹⁵ Anstall, H. B., C. Capp, J. M. Trujillo, *Science*, **154**, 657 (1966).
- ¹⁶ Dawson, D. W., H. S. Eppenberger, N. O. Kaplan, *J. Biol. Chem.*, **242**, 204 (1967).
- ¹⁷ Yoshida, A., *J. Biol. Chem.*, **241**, 4966 (1966).
- ¹⁸ Carey, F. G., I. Fridovich, and P. Handler, *Biochim. Biophys. Acta*, **53**, 440 (1961).
- ¹⁹ Joshi, J. G., and P. Handler, *J. Biol. Chem.*, **237**, 929 (1962).
- ²⁰ Martin, R. G., and B. N. Ames, *J. Biol. Chem.*, **236**, 1373 (1961).
- ²¹ McCoy, E. E., and V. A. Najjar, *J. Biol. Chem.*, **234**, 3017 (1959).
- ²² Nelson, C., J. G. Joshi, J. Hooper, and P. Handler, in preparation.
- ²³ Spencer, N., D. A. Hopkin, and H. Harris, *Nature*, **204**, 742 (1964).
- ²⁴ Harris, H., *Proc. Royal Soc. (London), Ser. B*, **164**, 298 (1966).
- ²⁵ Roberts, E., and H. Tsuyuki, *Biochim. Biophys. Acta*, **73**, 673 (1963).
- ²⁶ Weber, G., G. Banerjee, and H. Morris, *Cancer Res.*, **21**, 933 (1966).
- ²⁷ Weber, G., M. C. Henry, S. R. Wagle, and D. C. Wagle, in *Advances in Enzyme Regulation* (New York: Pergamon Press, 1963), vol. 2, p. 335.
- ²⁸ Milstein, C., *Biochem. J.*, **79**, 574 (1961).