The Isoenzymes of Phosphoglucomutase*

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ABSTRACT: Phosphoglucomutase from a number of mammalian sources has been studied by electrophoresis. The phosphoglucomutase present in most tissues consists of a number of electrophoretically distinct enzymes. By column chromatography partial resolution of these isoenzymes can be achieved. They could not be distinguished catalytically. None appeared to be dephosphoenzyme. Addition of *p*-mercuribenzoate or partial iodination caused some interconversion of the isoenzymes. Dialysis against the substrates in order to produce the phosphorylated and dephosphorylated enzyme did not alter the electrophoretic variants, nor did prior activation of the enzyme. In human red blood cells a second allele for phosphoglucomutase was encountered, providing an additional mechanism for enzyme heterogeneity.

To account for the polymorphism of phosphoglucomutase, three mechanisms may be proposed. The first of these was advanced by Najjar and Pullman (1954), who postulated a mechanism of enzyme action such that phosphorylated and dephosphorylated enzyme species must exist. It was subsequently shown that these two enzymatic forms could be separated by column chromatography, and differences in their properties were reported (McCoy and Najjar, 1959; Yankeelov *et al.*, 1964; Yankeelov and Koshland, 1965). The second mechanism for phosphoglucomutase heterogeneity was advanced by Harris and his coworkers; they found in an electrophoretic study of human red blood cell phosphoglucomutase that a number of different phosphoglucomutases could be identified, and that three separate genetic loci were present (Spencer *et al.*, 1964; Hopkinson and Harris, 1968).

A third mechanism for enzyme heterogeneity has recently been proposed by Kaplan and coworkers in the case of malic dehydrogenase (Kitto *et al.*, 1966). They found a number of electrophoretically distinct malic dehydrogenases, which had the same amino acid composition, the same catalytic characteristics, and nearly the same reactions with specific antibody. These "conformers" differed significantly in optical rotatory dispersion, and were interconvertible with urea or by iodination.

In this paper we present evidence that some aspects of phosphoglucomutase heterogeneity are best explained by the presence of different conformations of the same enzyme molecule, similar to that postulated by Kaplan for malic dehydrogenase.

Material and Methods

The enzyme assay was similar to the spectrophotometric assay of Joshi *et al.* (1967), and contained in a final volume of 3.0 ml: imidazole-HCl (pH 7.5), 120 μmoles; $MgCl_2$, 10 μmoles; EDTA (pH 7.5), 5 μmoles; TPN, 0.5 μmole; glucose 6-phosphate dehydrogenase, 10 μg; glucose 1-phosphate, 5 μmoles; glucose 1,6-diphosphate, 10 mμmoles; and the enzyme source. One unit of enzyme catalyzed the reduction of 1 μmole of TPN/min at 25°. The enzyme was usually preactivated (Harshman *et al.*, 1965) by dilution into imidazole buffer containing 3 mM $MgCl_2$ and 1.5 mM EDTA, and incubation at room temperature for 10 min before the assay was begun.

The enzyme was obtained from a number of sources. Human phosphoglucomutase was obtained from autopsy material, rabbit embryos were obtained frozen, and the tissues of other species were fresh. The tissues were homogenized in cold water at a concentration of 100 mg/ml, and centrifuged at 100,000*g* for 1 hr. Some human muscle samples were obtained by biopsy. The use of fresh tissue did not lead to different results.

For purification of human phosphoglucomutase, 50–100 g of muscle obtained at autopsy was homogenized in a Waring Blendor in five times its volume of cold 1×10^{-4} M EDTA (pH 7.5). The homogenate was centrifuged and the precipitate was discarded. Such an extract contained enzyme at a specific activity of 10–25 units/mg. The heat step introduced by Najjar (1948) was not used. Solid $(NH_4)_2SO_4$ was added to a concentration of 45% at 4°, and the precipitate obtained was discarded. Further $(NH_4)_2SO_4$ was added to a concentration of 65%, and after standing overnight the precipitate was collected by centrifugation. It was resuspended in 1×10^{-4} M EDTA (pH 7.5) (1/20th the volume of the original extract) and dialyzed against 1×10^{-4} M EDTA. The resulting solution contained 50–65% of the starting amount of enzyme, at a specific activity of 20–38 units/mg. Such extracts were used for column chromatography, as described below. Enzyme that emerged from CM-cellulose or DEAE-cellulose columns with the void volume was not further purified, but subsequent enzyme peaks (*e.g.*, Figure 3) had specific activities of 55–160 units/mg. Purified rabbit muscle phosphogluco-

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mutase has a specific activity in our assay of 150–170 units/mg. Rabbit muscle phosphoglucomutase was also purified by the method of Najjar (1948), including the heating step at low pH.

Starch gel electrophoresis was carried out according to the method of Fine and Costello (1963), in 11% hydrolyzed starch, in phosphate–citrate buffer at pH 7.0. The staining mixture was that of Spencer *et al.* (1964), except that nitro blue tetrazolium was used. Glucose 1,6-diphosphate was routinely added (final concentration 3.0 mM), and the staining mixture was poured over the sliced gel in warm 1% agarose.

Glucose 1-phosphate and glucose 1,6-diphosphate were from Sigma Chemical Co. Glucose 6-phosphate dehydrogenase, TPN, and rabbit muscle phosphoglucomutase were from Boehringer-Mannheim Co. Imidazole was from Eastman. Urea and guanidine hydrochloride were recrystallized from methanol. Rabbit embryos were from Pel-Freez Co., Rogers, Ark.

Results

A typical electrophoretic separation of the multiple forms of phosphoglucomutase activity from human tissues is shown in Figure 1. All the active enzyme forms moved toward the positive pole at pH 7.0, and were equally spaced. In the figure, a maximum of four spots are shown, but with heavier staining, as many as six or seven spots could be demonstrated, with progressively less activity in those migrating farthest. There was some variation in the intensity of the different spots in extracts from different tissues, but almost always the slowest migrating enzyme was the largest in amount. For convenience, we have called these isoenzymes A, B, C, D, etc.

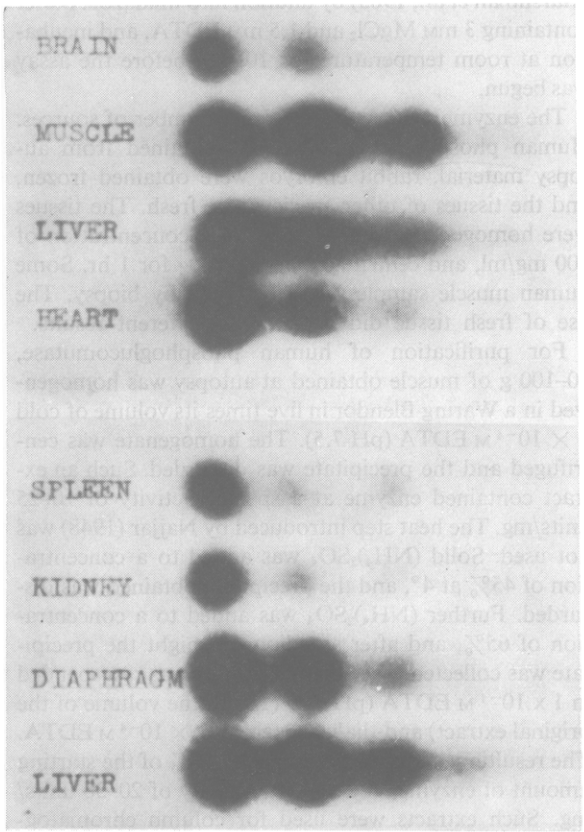

FIGURE 1: Electrophoresis in starch gel of phosphoglucomutase from human autopsy tissues. All the tissues were from the same individual, except for the last sample of liver. The anode is to the right, and the origin was near the left-hand margin.

A comparison of phosphoglucomutases from rabbit, human, rat, and chicken sources is shown in Figure 2. In each case the separation of the isoenzymes from each other is the same, although the over-all rate of migration from different species varied considerably.

A number of experiments were tried under different conditions in an effort to purify the individual isoenzymes. Using DEAE-cellulose, enzyme A was the first to appear on gradient elution, while the faster migrating enzymes, B–D, were retained. Although peaks of enzyme activity were often obtained, particularly with the human enzyme, which was better retained, these peaks did not correspond to the distinct electrophoretic entities (Figure 3). Although it seemed possible that there was conversion of one isoenzyme into another after separation on the column, a more likely explanation was that the initial separation itself was incomplete. In support of this, we found that the electrophoretic pattern of any fraction was the same after many weeks of storage at 4°. Secondly, the elution pattern from DEAE-cellulose was variable; sometimes the second peak consisted almost entirely of isoenzyme B, while more often it was a mixture of A and B.

Rabbit muscle phosphoglucomutase, either freshly prepared from tissue, or obtained commercially as crystalline enzyme, gave similar patterns on DEAE-cellulose. Uniformly the rabbit enzyme was less well retained on the column and large but variable amounts appeared with the breakthrough protein peak.

When partially or fully purified rabbit muscle phosphosglucomutase was chromatographed on CM-cellulose, a number of peaks were again obtained. Otherwise, the behavior of the phosphoglucomutases tested was the reverse of that seen with DEAE, that is, the slowest moving enzyme on electrophoresis (enzyme A) was the best retained, and enzyme peaks appearing after

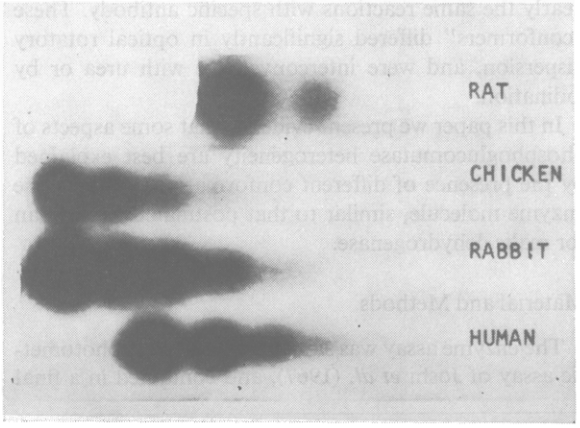

FIGURE 2: Electrophoresis in starch gel of phosphoglucomutase from muscle of the species listed. Origin was at the left-hand margin.

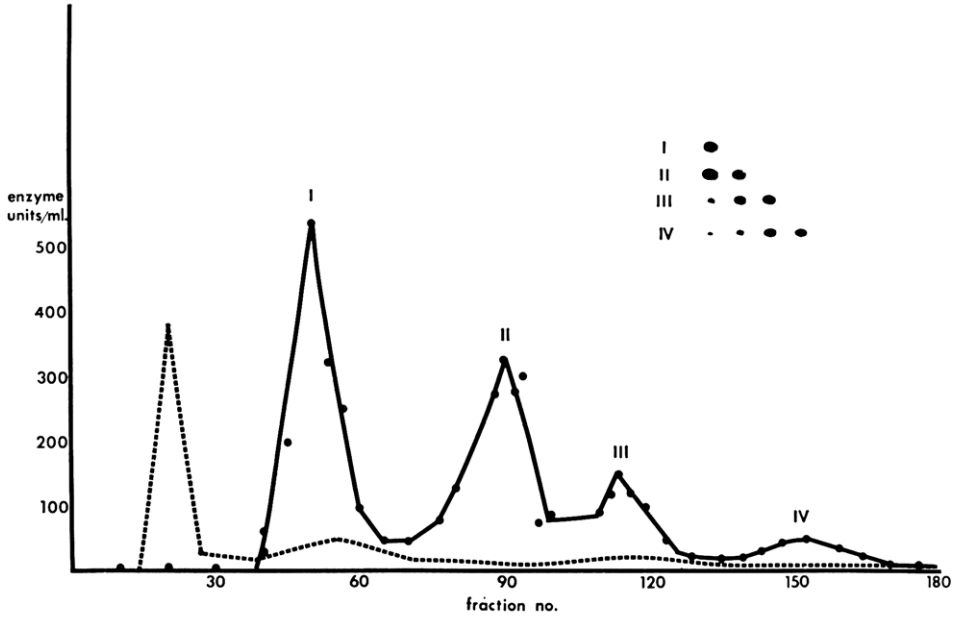

FIGURE 3: Chromatography on DEAE-cellulose (40 × 5 cm) of an $(NH_4)_2SO_4$ fractionated human muscle extract, containing 1500 units of enzyme and 40 mg of protein. The column was equilibrated with 0.001 M potassium phosphate buffer (pH 7.5) with 1×10^{-4} M EDTA. To 1000 ml of this buffer was added 1000 ml of the buffer containing 0.1 M KCl, to produce a linear gradient. Fraction size was 8.0 ml. The vertical axis refers to enzyme units (× 2000) and to absorbancy at 280 mμ (× 4000). The electrophoretic pattern of the four phosphoglucomutase peaks is shown at the upper right.

the breakthrough peak were enriched to varying degrees with A relative to the starting material (Figure 4).

If these various forms of phosphoglucomutase, distinct on electrophoresis and partially separable on column chromatography, are conformational isomers of each other, it should be possible to show their interconversion. This was demonstrated by the addition to the enzyme of *p*-mercuribenzoate or by partial iodination (Figure 5). The enzyme used was a human muscle phosphoglucomutase, consisting of peak III from a DEAE column, and consisted largely of isoenzymes B and C. Addition of iodine to the enzyme solution resulted in no detectable change in enzyme activity, and produced a marked change in mobility, with the appearance of isoenzymes A, D, and E. Addition of *p*-mercuribenzoate (10^{-6} M) also altered the mobility, as shown in Figure 5. The effects of *p*-mercuribenzoate on the purified rabbit muscle enzyme are seen in Figure 6. There was a marked change in mobility, accompanied by slight reduction in activity. We did not carry out the addition of *p*-mercuribenzoate further than 3.4 moles/mole of enzyme, although it has been shown (Bocchini *et al.*, 1967) that a total of six sulfhydryl groups can be titrated with prolonged incubation. The altered mobility of the enzyme with *p*-mercuribenzoate addition, but not with iodina-

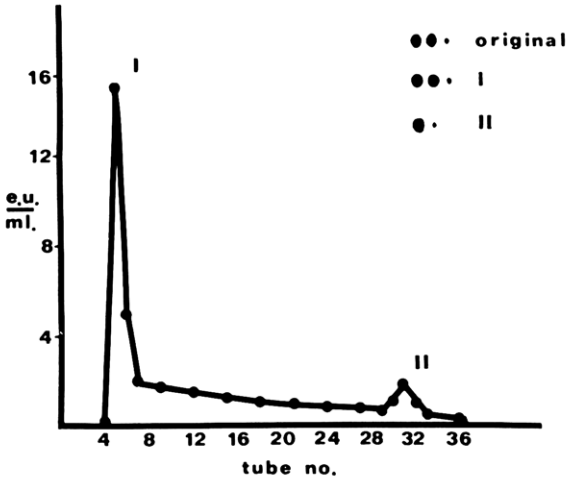

FIGURE 4: Chromatography on CM-cellulose (10 × 1 cm) of rabbit muscle phosphoglucomutase prepared by fractionation according to Najjar (1948), 180 enzyme units. Starting buffer was 0.001 M potassium phosphate (pH 7.0) containing 1×10^{-4} M EDTA. At tube 24 the buffer was changed to 0.1 M. Fraction size was 1.3 ml. The electrophoretic pattern of the two peaks is shown at the right.

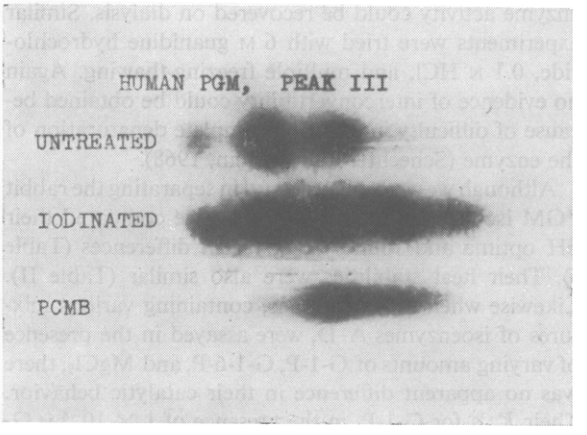

FIGURE 5: Electrophoresis of human muscle phosphoglucomutase after partial iodination and after addition of *p*-mercuribenzoate. The iodination was carried out by the method of Kitto *et al.* (1966), using nonradioactive iodine.

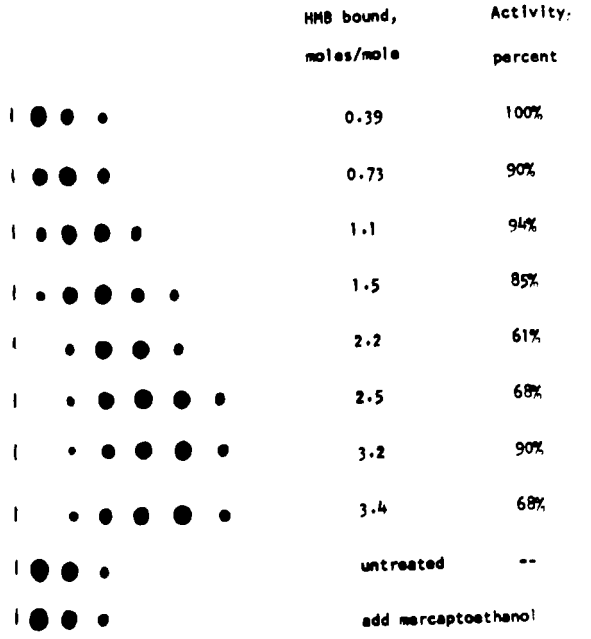

FIGURE 6: Effect of the addition of *p*-mercuribenzoate to purified rabbit muscle *p*-mercuribenzoate (Boehringer-Mannheim). The reaction with the mercurial was carried out in Tris buffer (pH 7.5) at room temperature, at an enzyme concentration of 9.1×10^{-6} M; each electrophoretic sample was a separate aliquot. The activity measurement after the addition of 3.2 moles of *p*-mercuribenzoate was probably erroneous.

tion, was reversible with the addition of 2-mercaptoethanol. Mercaptoethanol alone had no effect.

Interconvertibility was also tested by denaturing the enzyme in 8 M urea with 0.1 M 2-mercaptoethanol. We were unable, however, to fully denature and renature the enzyme. When purified rabbit muscle phosphoglucomutase was used at enzyme concentrations of 0.1 mg/ml or higher, the enzyme was not fully denatured, and on dilution directly into an assay mixture showed residual activity, or on dialysis and electrophoresis showed reduced activity with the same pattern as the starting material. More dilute solutions of the enzyme were totally denatured in 8 M urea as tested by assay, and no enzyme activity could be recovered on dialysis. Similar experiments were tried with 6 M guanidine hydrochloride, 0.1 N HCl, and multiple freezing-thawing. Again no evidence of interconvertibility could be obtained because of difficulty in ensuring complete denaturation of the enzyme (Schechter and Epstein, 1968).

Although we were unsuccessful in separating the rabbit PGM isoenzymes from each other, we compared their pH optima and found no apparent differences (Table I). Their heat stabilities were also similar (Table II). Likewise when these fractions, containing various mixtures of isoenzymes A–D, were assayed in the presence of varying amounts of G-1-P, G-1-6-P, and $MgCl_2$, there was no apparent difference in their catalytic behavior. Their K_m's for G-1-P, in the presence of 1×10^{-6} M G-2-P, were 1×10^{-5} M, in agreement with Handler's value for rabbit muscle phosphoglucomutase (Handler *et al.*, 1965).

TABLE I: Activity[a] of Mixtures of Phosphoglucomutase Isoenzymes at Differing pH Values.

Isoenzyme Composition:	100% A	50% A and 50% B	50% C and 50% D
pH	Peak I[b] (%)	Peak II (%)	Peak III (%)
8.5	35	37	41
8.0	70	63	68
7.5	97	85	90
7.0	100	100	100
6.5	74	76	60
6.0	23	21	21
5.5	4	5	5

[a] The assays were performed in imidazole-HCl buffers of varying pH values, and the results are expressed as the activity relative to the maximum at pH 7.0. [b] The enzyme peaks were obtained from a DEAE column similar to that described in Figure 3, with the starting material a rabbit muscle enzyme prepared by the $(NH_4)_2SO_4$ and heating fractionation of Najjar (1948). The isoenzyme composition of the indicated peaks was estimated by electrophoresis.

Harris and his coworkers have presented evidence based on genetic frequencies and electrophoretic studies, that there are three genetic loci for phosphoglucomutase in man (Spencer *et al.*, 1964; Hopkinson and Harris, 1966, 1968). On comparison of our results with his published reports, it is clear that the phosphoglucomutase variants described in the above paragraphs were all of locus phosphoglucomutase$_1$, as designated by Harris. Our experience with phosphoglucomutase$_2$ and phosphoglucomutase$_3$ has been limited but we have encountered phosphoglucomutase$_2$ in human tissue extracts. In the human red cell phosphoglucomutase$_2$ is well represented, and this is shown in Figure 7. Under the electrophoretic conditions we used, the enzymes resulting from expression of phosphoglucomutase$_2$ locus are overlapped by isoenzymes C, D, and E of locus phosphoglucomutase$_1$, and we did not observe the phosphoglucomutase$_2$ enzymes in tissue extracts containing large amounts of total enzyme. It may be that phosphoglucomutase$_2$ is less stable in autopsy material, although it did not appear to be unstable in stored red cell extracts. We did not observe phosphoglucomutase$_3$ at any time, but Harris found the largest amounts of phosphoglucomutase$_3$ in placentas and cultured skin fibroblasts, which we did not employ.

We have briefly examined the embryonic expression of the different phosphoglucomutase loci in rabbit embryo tissues, using embryos of 30-, 50-, and 75-mm crown-rump length. In these embryos, there was a marked increase in the total amount of enzyme in muscle and a slight increase in total enzyme in brain and liver (Table III). In all of them the pattern was the same as in the adult, *i.e.*, a preponderance of phosphoglucomutase$_1$ enzymes and small amounts of faster moving enzymes which may have been phosphoglucomutase$_2$

TABLE II: Heat Stability[a] of Mixtures of Phosphoglucomutase Isoenzymes.

	Isoenzyme Composition:	100% A	50% A and 50% B	10% A, 50% B, and 40% C	10% B, 70% C, and 20% D
Temp (°C)	min	Fraction I[b] (%)	Fraction II (%)	Fraction III (%)	Fraction IV (%)
58	0	100	100	100	100
58	2	76	85	80	88
58	5	76	85	80	89
58	10	51	60	45	67
63	5	41	27	14	43
64	7	32	23	15	31

[a] Enzyme fractions were incubated for the stated time in 0.1 M sodium acetate (pH 5.0) and diluted approximately 20-fold with cold imidazole buffer for immediate activation and assay. The results are expressed as the activity relative to that of the unheated enzyme fraction. [b] The enzyme fractions were eluted from starch grain (Fine and Costello, 1967) after electrophoresis of rabbit muscle phosphoglucomutase purified by the method of Najjar (1948). The isoenzyme composition of each fraction was estimated from starch gel electrophoresis.

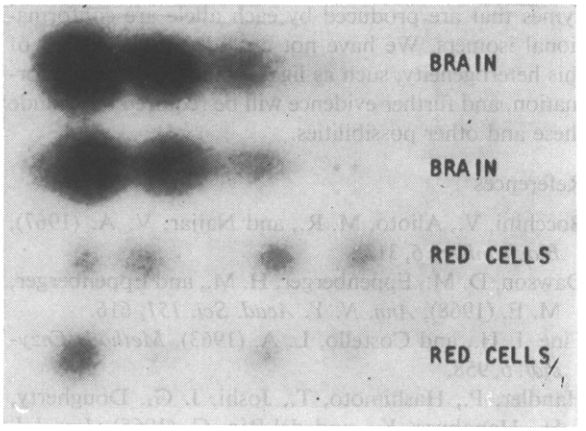

FIGURE 7: Electrophoresis of two samples of human brain and two red blood cell extracts from different individuals. In the red cell extracts the two isoenzymes migrating further toward the right (anode) would correspond to Harris' phosphoglucomutase$_2$ locus and should be contrasted with the trace amounts of isoenzymes C and D in the brain extracts.

and phosphoglucomutase$_3$ (thus far not well identified in the rabbit).

Harris has calculated the frequency of mutation at locus phosphoglucomutase$_1$, and has demonstrated the expression of both alleles in the heterozygote. An example of this can be seen in Figure 8, using phosphoglucomutase from human autopsy muscle. All four visible isoenzymes are shifted in their mobility to an equal degree in the mutant phosphoglucomutase, emphasizing that these four isoenzymes are all espressions of phosphoglucomutase$_1$ locus. In comparing the expression of the two alleles of phosphoglucomutase$_1$ in liver, muscle, and other tissues, we occasionally found a minor degree of preferential expression in one tissue *vs.* another.

Handler and coworkers (Joshi *et al.*, 1967; Hooper *et al.*, 1968) also presented evidence for several genetic loci of phosphoglucomutase from a variety of sources. It has been shown that the separated enzymes differ somewhat in their amino acid composition, differ more widely in their fingerprint patterns after tryptic digestion, but have similar amino acid sequences around the phosphorylated serine residue. It is difficult to compare our results with his, since the techniques differ, but it seems likely that his work refers to the separated phosphoglucomutase$_1$ and phosphoglucomutase$_2$ groups of enzymes, according to the terminology we are using. It should be noted, however, that Handler's peak 1 enzyme consists of the breakthrough peak, and our results with CM-cellulose would tend to indicate that the breakthrough enzyme is essentially identical with the starting material,

TABLE III

	Embryos[a]			
	30 mm	50 mm	75 mm	Adult
Brain	2.0[b]	3.0	3.5	1.7
Liver	26	27	29	14
Muscle	3.0	6.7	16.8	96

[a] Crown-rump length. [b] The values given are enzyme units per gram of tissue.

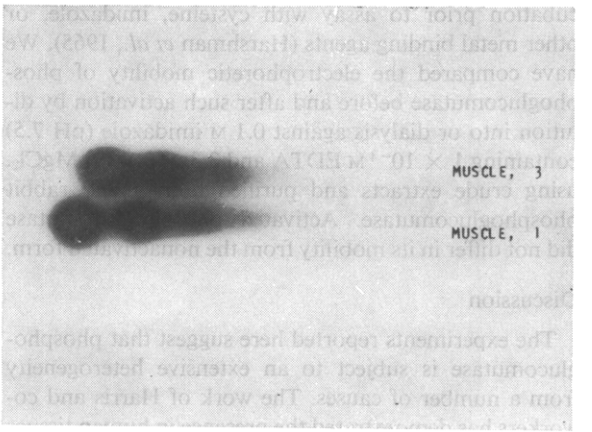

FIGURE 8: Electrophoresis of two human muscle extracts, the lower one showing the commoner phosphoglucomutase$_1$ form, while the upper one shows an altered mobility of all isoenzymes and would correspond to Harris' phosphoglucomutase$_1^2$ mutation at phosphoglucomutase$_1$ locus.

and that only subsequent peaks could be distinct electrophoretic entities.

The third major kind of heterogeneity of phosphoglucomutase is that related to the reversible interconversion of the phospho and dephospho forms of the enzyme. We attempted a number of ways to demonstrate the presence of dephosphoenzyme, but did not succeed in doing so. The dephosphoenzyme is far more heat labile (McCoy and Najjar, 1959). Heating extracts of tissues or enzyme at various stages of purification to 58° for varying periods of time, to produce a decline in total enzyme activity (Table II), did not alter the electrophoretic pattern, except for the reduced total activity. Dialysis against glucose monophosphate should convert phosphoenzyme to dephosphoenzyme, while dialysis against glucose diphosphate should produce phosphoenzyme from any dephosphoenzyme present (McCoy and Najjar, 1958; Yankeelov and Koshland, 1965). Multiple trials, using a number of different concentrations of the substrates, $MgCl_2$, EDTA, and different pH values, also produced no alteration of the electrophoretic pattern.

It was rather surprising that no evidence for the dephosphoenzyme could be detected at any time in these electrophoretic experiments, when others (Yankeelov *et al.*, 1964; Joshi *et al.*, 1967) had little difficulty in separating it from phosphoenzyme by chromatographic methods. It is possible that the dephosphoenzyme is less stable during electrophoresis, or that the staining reaction does not detect it. Unfortunately, the experiments were negative ones and no explanation is at hand.

It has been known for some time that the enzyme activity of phosphoglucomutase can be increased by incubation prior to assay with cysteine, imidazole, or other metal binding agents (Harshman *et al.*, 1965). We have compared the electrophoretic mobility of phosphoglucomutase before and after such activation by dilution into or dialysis against 0.1 M imidazole (pH 7.5) containing 1×10^{-4} M EDTA and 3.3×10^{-3} M $MgCl_2$, using crude extracts and purified human and rabbit phosphoglucomutase. Activated phosphoglucomutase did not differ in its mobility from the nonactivated form.

Discussion

The experiments reported here suggest that phosphoglucomutase is subject to an extensive heterogeneity from a number of causes. The work of Harris and coworkers has demonstrated the presence in human tissues of three separate and distinct loci for phosphoglucomutase, which are variably expressed in different tissues, and of which phosphoglucomutase$_1$ predominates by far in liver, muscle, brain, heart, and other solid organs. Mutation of phosphoglucomutase$_1$, as well as the other loci, produces commonly observed genetic variants.

Phosphoglucomutase can exist in separate phospho and dephospho forms, and can, in addition, be activated by a variety of metal binding compounds. There is evidence to suggest that the phospho- and dephosphoenzyme differ in their conformation (Yankeelov and Koshland, 1965) and increase in specific activity of the enzyme with activation may also involve a local change in conformation (Bocchini *et al.*, 1967). We saw no evidence that either of these alterations in phosphoglucomutase activity and/or structure was reflected in an altered electrophoretic mobility.

Several enzymes are now thought to exist in different conformations of the same polypeptide chain. Mitochondrial malic dehydrogenase (Kitto *et al.*, 1966) and avian brain creatine kinase (Dawson *et al.*, 1968) have been reported to exist in two or more stable conformations, which are distinguishable but catalytically identical. This mechanism for enzyme heterogeneity is apparently distinct from allosteric transition by the binding or release of modifiers, and is clearly distinct from the formation of isoenzymes by the condensation of differing subunits. There is evidence from tryptic digestion fingerprints that phosphoglucomutase is a single-chain enzyme (Joshi *et al.*, 1967). which is further evidence against the presence of subunit permutations. We propose that the multiple forms of phosphoglucomutase may also be partly explicable on this basis of multiple conformations, and that the two or more isoenzymes that are produced by each allele are conformational isomers. We have not excluded other causes of this heterogeneity, such as ligand binding or amide formation, and further evidence will be required to exclude these and other possibilities.

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