

# The Purification and Properties of Phosphoglycerate Mutase from Chicken Breast Muscle\*

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## SUMMARY

A very rapid procedure for the preparation of phosphoglycerate mutase from chicken breast muscle is presented. Commercially available frozen chicken breasts may be used. The enzyme is obtained in high yields, and the specific activity of the crystalline homogenous preparations is equal to that of the crystalline enzymes from yeast and rabbit muscle.

The chicken breast enzyme requires 2,3-diphosphoglycerate for activity, and it is very similar in kinetic and molecular properties to the rabbit muscle phosphoglycerate mutase. Similarly, it also possesses 4 sulfhydryl groups per mole of enzyme. The molecular weight of the enzyme is 65,690.

With the use of a gel filtration technique, it has been demonstrated that 2 moles of 2,3-diphosphoglycerate may be bound per mole of the enzyme. It appears that the binding of this phosphoglycerate may entail phosphoenzyme formation. Again, as with other pure phosphoglycerate mutases, the chicken breast muscle mutase shows some 2,3-phosphoglycerate phosphatase activity (about 1/40,000 the mutase activity).

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Phosphoglycerate mutases are known to be of two types, 2,3-phosphoglycerate-dependent and 2,3-phosphoglycerate-independent (1). Of the former, the yeast and rabbit muscle enzymes have been extensively purified and crystallized (2, 3). However, in spite of fairly extensive molecular and kinetic studies from several laboratories (3-5), the intimate mechanism of action remains to be clarified (6, 7). The yeast and rabbit muscle enzymes show many similarities and differences. Although it is known that the yeast and rabbit muscle enzymes possess 2,3-P-glycerate phosphatase activity, and much evidence indicates that this phosphatase might be due to a dual lesser expression of the main mutase activity rather than to phosphatase contamination, clear evidence for phosphoenzyme formation has been obtained only with the rabbit muscle enzyme (6, 8). Further evidence

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for the identity of the phosphatase with the mutase activities was sought recently by immunological techniques (9). These experiments indicated that the yeast mutase and phosphatase activities are indeed activities of a single enzyme. Similar studies were not possible with the rabbit muscle enzyme since rabbits were used as recipients. Therefore, it was of interest to purify a 2,3-P-glycerate-dependent enzyme from another source both to conduct immunological studies and to find whether or not phosphoenzyme formation would occur with mutases other than the rabbit muscle enzyme.

As previously shown in a survey of some 50 sources (1), chicken breast appears to be a very rich source of 2,3-P-glycerate-dependent mutase. This fact and the finding that 2,3-P-glycerate offers remarkable protection against heat inactivation (8) were combined and utilized for the purification of the enzyme by a very rapid and reproducible procedure.

This paper presents the procedure for the purification of the phosphoglycerate mutase from chicken breast muscle and some of its properties.

## EXPERIMENTAL PROCEDURE

*Material and Methods*—Frozen chicken breasts from Ocoma Foods Company were purchased at the hospital kitchens. P-glycerate was obtained as the barium salt from Schwarz, ammonium sulfate (enzyme grade) from Mann, and Sephadex G-25 from Pharmacia. Lab-trol was a product of Dade Reagents. Cellulose acetate strips for electrophoresis were from Oxo Ltd. ATP, *p*-mercuribenzoate, glyceraldehyde 3-phosphate, DPN<sup>+</sup>, and Tris were purchased from Sigma. Crystalline yeast phosphoglycerate mutase, enolase free of mutase, 3-P-glycerate (free from 2,3-P-glycerate), and 2,3-P-glycerate were prepared as previously described (2, 3, 10, 11). Deionized, distilled water was used throughout the work presented in this paper.

The Gilford model 2000 spectrophotometer or the Beckman DU spectrophotometer with an SRL Sargent recorder was used to measure absorbances.

The enolase-mutase coupled assay (3) was used to determine mutase activity. 2,3-P-glycerate phosphatase and enolase were measured as described before. A phosphatase unit is the amount of enzyme that liberates 1  $\mu$ mole of P<sub>i</sub> from 2,3-P-glycerate in 30 min. One enolase unit is the amount of enzyme that causes an increase of 0.1 absorbance unit at 240 m $\mu$  in 1 min (11, 12). Specific activity is defined as the enzyme units per mg of protein. Proteins were measured by the procedure of Mokrasch, Davidson, and McGilvery (13) with the use of Lab-trol as a protein

standard. Glyceraldehyde 3-phosphate dehydrogenase was assayed by the method of Krebs (14). 2,3-P-glycerate was estimated as previously described (11) and  $P_i$  by the procedure of Gomori (15). Electrophoresis experiments were carried out in a Shandon Universal electrophoresis apparatus for  $2\frac{1}{2}$  hours at 170 volts and 0.5 ma per cm in a 0.04 M potassium phosphate buffer.

Sedimentation velocity experiments were performed in the Spinco model E ultracentrifuge. The sedimentation coefficients were obtained according to Schachman (16). All the experiments were performed at room temperature. The diffusion coefficient was calculated from a synthetic boundary run. The molecular weight was obtained from the approach to equilibrium method of Archibald (17).

Sulfhydryl groups were measured by the spectrophotometric method of Boyer (18). The extinction coefficient ( $E_{255}$ ) for the mercaptide in phosphate buffer, pH 7.0, was found in this laboratory to be  $7.6 \times 10^3$ ; this is in agreement with Pizer (5). However, in guanidine  $E_{255}$  was  $6.2 \times 10^3$ .

The coenzyme-binding capacity of the enzyme was studied by the gel filtration technique of Pfeleiderer and Auricchio (19) as follows. Sephadex G-25 was stirred in water and washed four to five times with 0.063 M ammonium sulfate, pH 7.0, and allowed to stand in the same buffer for about 8 hours at 4°. The gel was packed into a column (25 × 1 cm), and was equilibrated with the eluting solution, which was 0.0001 M 2,3-P-glycerate (pH 7.0)-0.063 M ammonium sulfate (pH 7.0). For some experiments P-glycerate was added in order to inhibit the 2,3-P-glycerate phosphatase activity of the enzyme (10), and in these cases the eluting solution was 0.0001 M 2,3-P-glycerate (pH 7.0)-0.063 M ammonium sulfate (pH 7.0)-0.001 M P-glycerate (pH 7.0). The enzyme was dialyzed for 24 hours at 4° against the ammonium sulfate buffer. The enzymatic activity and protein concentration were determined. The solution, containing 183  $\mu$ moles of phosphoglycerate mutase per ml, was made equimolar with respect to the components of the eluting fluid immediately before use. Then 0.4 or 0.8 ml was taken and added onto the column. The columns were developed at 4° with the eluting solution at a flow rate of 1 ml per min. Aliquots of 1 ml were collected in tubes that contained 0.5 ml of 0.5 M perchloric acid. The tubes with visible precipitates were centrifuged, the supernatant fluids were decanted, and the precipitates were redissolved in water in order to measure the protein.

All fractions were brought to pH 7.0 with 0.5 M KOH and made up to 2 ml. In each sample the 2,3-P-glycerate and  $P_i$  were determined. The  $P_i$  was used as an index of the 2,3-P-glycerate hydrolyzed because of 2,3-P-glycerate phosphatase activity. One mole of  $P_i$  is liberated per mole of 2,3-P-glycerate hydrolyzed (10).

*Purification of Chicken Breast Mutase*—Unless otherwise specified, all operations were carried out at 0–3°, and centrifugations were at approximately 2000 × *g* with the International centrifuge (model PR-2). Ammonium sulfate solutions were saturated and neutralized to pH 7.0 ± 0.1 by the addition of concentrated ammonium hydroxide solution; pH was determined for a 1:20 dilution. All volumes refer to the initial volume for a particular step. All reagents were measured and added at 0–3°.

*Extraction*—The chicken breasts were thawed, and the fat and bones were discarded. The muscle was ground twice with a meat grinder. Each chicken breast yielded approximately

100 g of ground muscle. The ground meat can be kept frozen for at least 1 week without losing activity. The ground muscle was extracted for 10 min with gentle stirring with 1.5 volumes of cold water and then strained through gauze. The pulp was re-extracted for 10 min with 1 volume of cold water and strained again. The combined extracts were centrifuged for 10 min, and the precipitate was discarded. The supernatant fluid is the crude fraction. An average of 2.3 ml of crude fraction were obtained from each gram of ground muscle.<sup>1</sup>

*Acetone Step*—To each volume of crude fraction was added, with constant stirring, 0.5 volume of acetone. The mixture was centrifuged for 10 min, and the precipitate was discarded. To the supernatant fluid was added 0.15 volume of acetone per volume of crude fraction, and the mixture was centrifuged for 15 min. The precipitate was taken up in water to half the volume of the crude fraction. This acetone fraction is stable at –20° for at least 2 weeks, but repeated freezing and thawing for 2 months resulted in about 70% loss of activity.

*Heating Step*—To the acetone fraction was added 0.1 volume of 0.06 M 2,3-P-glycerate at pH 7.0. The mixture was heated with continuous gentle stirring for 10 min at 62° (marked turbidity and protein precipitation begin when the mixture reaches 50°); it was then cooled rapidly and centrifuged for 15 min, and the supernatant fluid was filtered through coarse filter paper. The filtrate is called the heated fraction.<sup>2</sup>

*First Ammonium Sulfate Fraction*—To each milliliter of the heated fraction was added 0.4 g of ammonium sulfate. The mixture was centrifuged for 10 min at 18,000 × *g*, the supernatant fluid was discarded, and the precipitate was taken up in water to 0.045 the volume of the heated fraction. This is ammonium sulfate Fraction I. This fraction could be kept at 2° for at least 1 week without loss of activity and is stable at –20°; however, repeated freezing and thawing resulted in loss of activity. In addition, the enzyme could be lyophilized and stored as such.

Up to this stage the time required for the purification, starting with 200 g of ground muscle, is about 4 hours.

*Second Ammonium Sulfate Fraction*—To each milliliter of the ammonium sulfate Fraction I, 0.7 ml of saturated ammonium sulfate was added. A small precipitate appeared, and the mixture was centrifuged at 18,000 × *g* for 10 min. To the supernatant fluid saturated ammonium sulfate was added very slowly until a crystalline<sup>3</sup> precipitate appeared; this increased as the mixture stood for 4 hours at 2°. The suspension was centrifuged for 20 min at 18,000 × *g*, and the precipitate was taken up to 1.5 ml of water. This is ammonium sulfate Fraction II. A photomicrograph is shown in Fig. 1.

<sup>1</sup> Extracting the ground muscle in a Waring Blender, repeated freezing and thawing, or storing at –20° of the ground muscle before extraction does not increase the amount of enzyme extracted or the specific activity. The crude extract can be stored frozen for at least 1 month without loss of activity, but repeated freezing and thawing over a 2-month period result in some 40% loss of activity.

<sup>2</sup> A smaller amount of 2,3-P-glycerate does not protect as well. A temperature of 62° gave a higher specific activity than 60°, and at 65° the enzyme losses were very high. More than 15 min at 62° gave losses of about one-half of the enzyme activity.

<sup>3</sup> Since crystallization is not a trustworthy criteria for purity, and since all other tests indicated homogeneity, in only two cases were the preparations recrystallized; the specific activity remained unchanged.

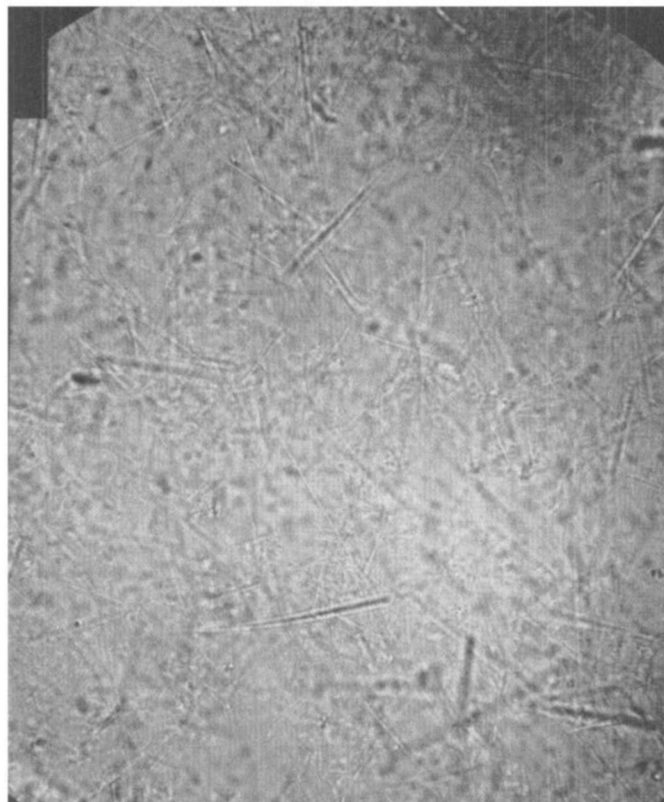


FIG. 1. Photomicrograph of chicken breast phosphoglycerate mutase ammonium sulfate Fraction II.  $\times 300$ .

Table I presents a resume of the purification<sup>4</sup> procedure.

**Other Enzyme Activities Present**—Samples of ammonium sulfate Fraction II were assayed for different enzymatic activities. With the use of 4  $\mu\text{g}$  of protein per assay neither enolase nor triosephosphate dehydrogenase activity was detected. With the same amount of protein 2,3-P-glycerate phosphatase activity was calculated to be 57  $\mu\text{moles}$  of  $\text{P}_i$  liberated per mg of protein per min, which is about  $\frac{1}{1000}$  the mutase activity. This mutase to phosphatase ratio is similar to that found for other mutases (3).

The ATPase activity was investigated under the same conditions as for the 2,3-P-glycerate phosphatase assay (10) except that 5  $\mu\text{moles}$  of ATP were used in place of 2,3-P-glycerate. The enzyme liberated 1.06  $\mu\text{moles}$  of  $\text{P}_i$  per mg of protein per min.

<sup>4</sup> If the specific activity of the ammonium sulfate Fraction I is less than 1,900, another heating step can be carried out after adding 0.1 volume of 0.06 M 2,3-P-glycerate, pH 7.0, heating for 10 min at 62°, centrifuging for 15 min, and filtering the supernatant fluid. Alternatively, the protein concentration is adjusted to 14 mg per ml. One volume of saturated ammonium sulfate is added, and the mixture centrifuged for 10 min at 18,000  $\times g$ . The precipitate is discarded. To the supernatant fluid saturated ammonium sulfate is added (0.4 ml for each milliliter of the "adjusted" ammonium sulfate Fraction I, and the suspension is centrifuged for 20 min at 18,000  $\times g$ . The bulky precipitate is taken up in water to a concentration of 0.8 the volume of the adjusted ammonium sulfate Fraction I. Saturated ammonium sulfate is then added until a very slight opalescence appears. The solution is stored at 2°. After 2 days a precipitate appears. After centrifugation for 20 min at 18,000  $\times g$ , the precipitate is dissolved in a minimum amount of water.

The ATPase activity was not stimulated by the addition of up to  $3 \times 10^{-3}$  M  $\text{Mg}^{++}$ .

**Effect of Coenzyme Concentration**—The chicken breast phosphoglycerate mutase is a 2,3-P-glycerate-dependent enzyme (1). There was no activity without added 2,3-P-glycerate. With the enolase coupled method and ammonium sulfate Fraction II dialyzed against 0.1 M potassium phosphate buffer, pH 7.0, the  $K_m$  of the enzyme for 2,3-P-glycerate at pH 7.3 and 30° was calculated to be  $5 \times 10^{-6}$  M. A Lineweaver-Burk plot (20) is presented in Fig. 2.

TABLE I

*Purification of chicken breast muscle phosphoglycerate mutase*

Steps 1 to 3 have been repeated 15 times with less than 5% difference in yield and 10% in specific activities. Steps 4 and 5 have been repeated 12 times with a maximal variation of 15% in yield and in specific activity.

Step	Fraction	Volume	Activity	Protein	Specific activity	Recovery
		ml	units	mg	units/mg	%
1	Crude <sup>a</sup>	400	650,000	6,488	100	100
2	Acetone	200	500,000	3,600	138	77
3	Heated	185	416,250	463	900	64
4	Ammonium sulfate I	8	360,000	189	1,900	55
5	Ammonium sulfate II	1.5	187,500	75	2,500	28

<sup>a</sup> From 200 g of ground muscle.

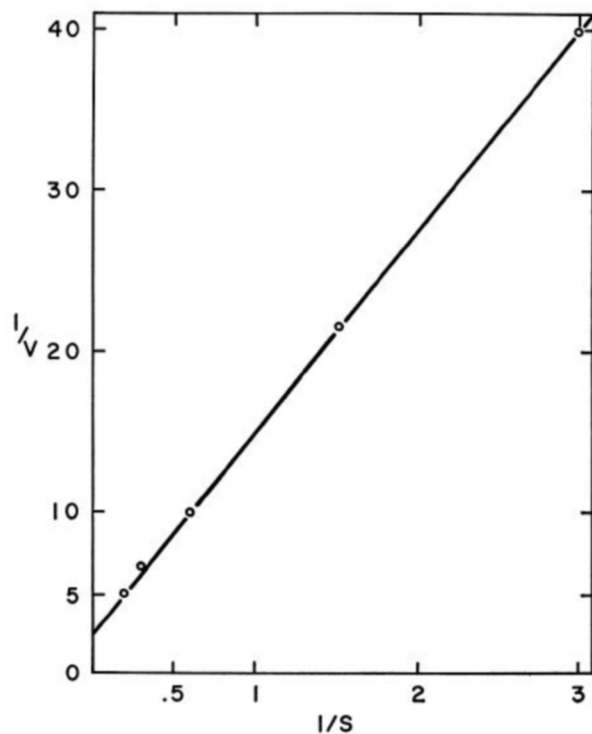


FIG. 2. Effect of coenzyme concentration. The incubation volume was 3.0 ml, and the temperature was 30°. The additions were as follows: P-glycerate, 50  $\mu\text{moles}$ ;  $\text{MgSO}_4$ , 10  $\mu\text{moles}$ ; Tris, pH 7.3, 100  $\mu\text{moles}$ ; 2,3-P-glycerate, from 10 to 200  $\mu\text{moles}$ ; enolase, 15 units; and chicken breast muscle mutase, 0.072  $\mu\text{g}$ .  $S$  is expressed as millimicromoles of 2,3-P-glycerate per ml,  $V$  as mutase units.

**Effect of pH**—With the use of the enolase-coupled assay method 10  $\mu$ l of ammonium sulfate Fraction II containing 0.38  $\mu$ g of protein were assayed for phosphoglycerate mutase activity at different pH values at 30°. As illustrated in Fig. 3, the optimum pH is 7.3.

**Molecular Weight**—The enzyme used in these studies was the ammonium sulfate Fraction II. A portion containing 20 mg of protein per ml was dialyzed for a 24-hour period against a 0.1 M

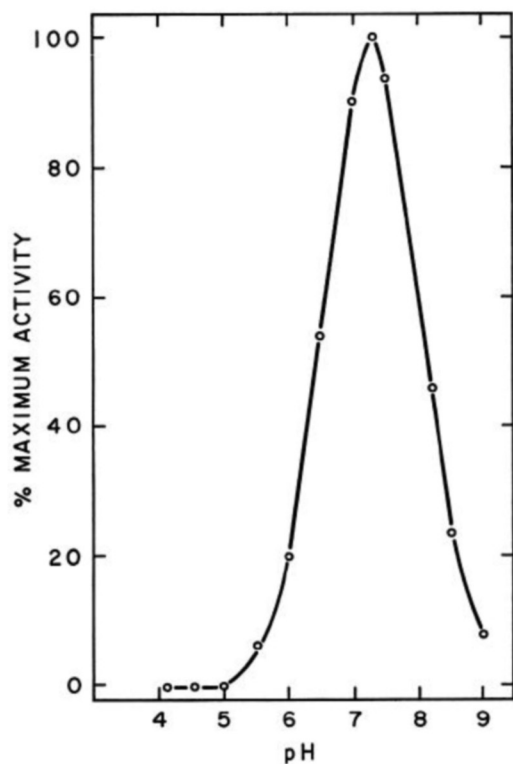


FIG. 3. The effect of pH upon mutase activity. Aliquots of a reaction mixture containing 50  $\mu$ moles of P-glycerate, 0.2  $\mu$ mole of 2,3-P-glycerate, and 10  $\mu$ moles of  $MgSO_4$  were adjusted to various pH values by the addition of Tris, HCl, and KOH and diluted to 3 ml with the further addition of 10 enolase units per assay. Each reaction was started by the addition of 0.38  $\mu$ g of chicken breast mutase.

NaCl-0.1 M potassium phosphate buffer, pH 7.0. The enzyme activity was the same after dialysis. Finally the protein was diluted with the dialysis solution to a concentration of 10 mg per ml.

In the sedimentation velocity experiments the protein sedimented as a single symmetrical peak. A picture is shown in Fig. 4. The observed sedimentation coefficient was  $3.917 \times 10^{-13}$ , which is in agreement with the values obtained for the rabbit muscle enzyme (4). As in this case, a partial specific value of 0.74 was assumed. The calculated molecular weight, 65,690, is also very close to that of the rabbit muscle mutase (4, 5). The diffusion coefficient was  $6.1 \times 10^{-7}$ .

**Electrophoresis**—An electrophoretic study of ammonium sulfate Fraction II at pH 5.4 revealed only one component. It migrated 2.2 cm toward the cathode in 2 hours.

**Sulfhydryl Groups**—Fraction II dialyzed against 0.1 M phosphate buffer, pH 7.0, for 24 hours was used for —SH measurements. In 0.05 M potassium phosphate buffer, pH 7.0, at 30° we found  $4 \pm 0.1$  —SH groups per mole of enzyme. In order to test for possible buried —SH groups, 0.35 mg of the mutase in 50  $\mu$ l was added to 0.5 ml of 5 M guanidine. After 15 min at 30°, 2 ml of potassium phosphate buffer, pH 7.0, water, and 0.1

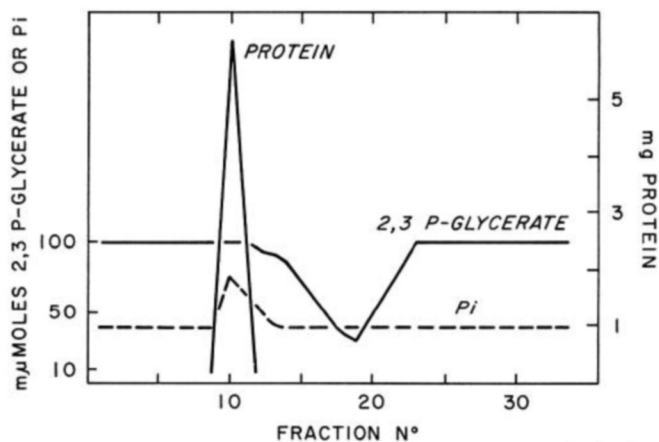


FIG. 5. Elution profile of binding experiments with chicken breast mutase. Enzyme, 146  $\mu$ moles, was passed through a Sephadex column under the conditions described (see "Material and Methods"). The 2,3-P-glycerate bound was 306  $\mu$ moles.

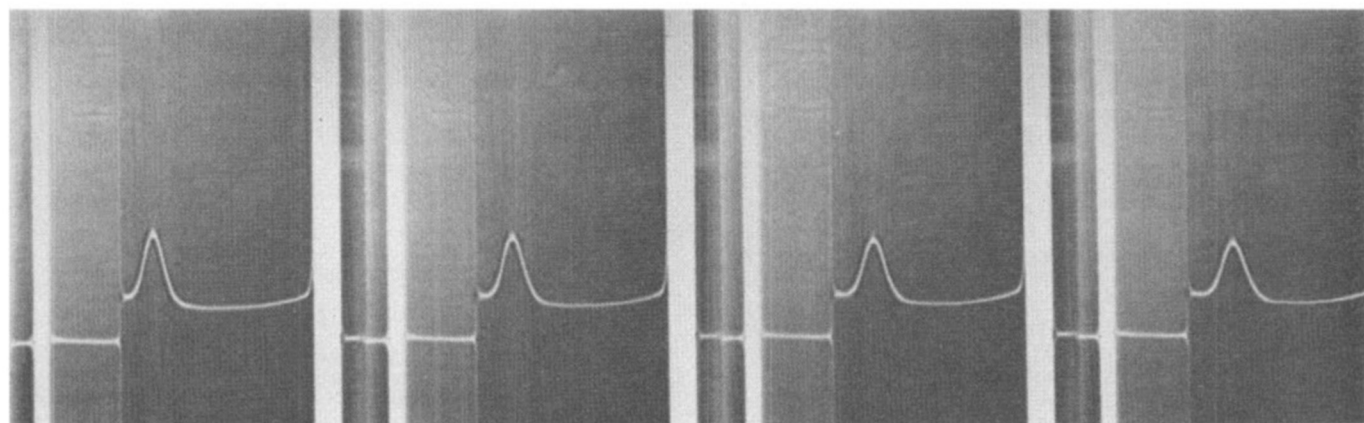


FIG. 4. Sedimentation pattern of chicken breast muscle mutase. Protein concentration, 10 mg per ml; 0.1 M sodium chloride-0.1 M potassium phosphate buffer, pH 7.0. Average temperature during centrifugation, 20°; speed, 60,000 rpm. Bar angle, 70 degrees; exposures (from left to right) at 28, 32, 36, and 40 min after speed equilibration.

TABLE II

*Binding of 2,3-P-glycerate to chicken breast muscle mutase*

All experiments were carried out as indicated in the text. For Experiments 1 to 3, the column was eluted with the ammonium sulfate-2,3-P-glycerate mixture. For Experiment 4, the column was eluted with the mixture containing 3-P-glycerate (see the text).

Experiment	2,3-P-glycerate bound	Enzyme used	Calculated molar <sup>a</sup> ratio of 2,3-P-glycerate bound to enzyme
	$\mu\text{moles}$		
1	159	73	2.178
2	137	73	1.876
3	306	146	2.095
4	270	146	1.849

<sup>a</sup> Average  $2.0 \pm 0.14$ .

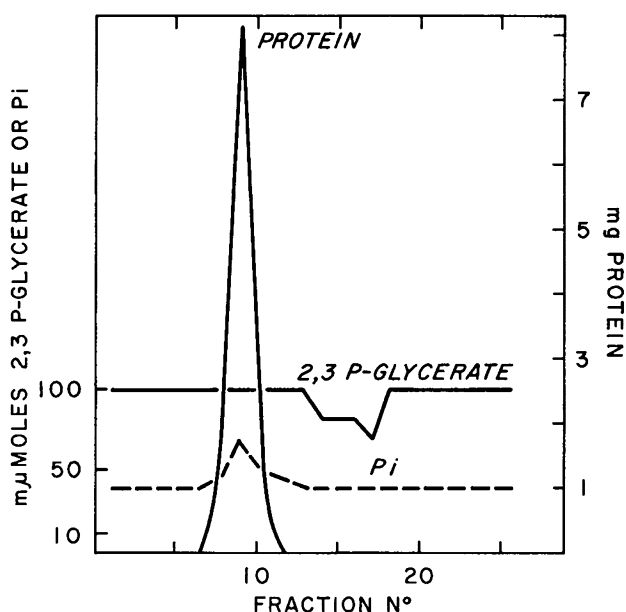


FIG. 6. Elution profile of binding experiments with yeast mutase. Enzyme, 100  $\mu\text{moles}$ , was passed through a Sephadex column under the conditions described (see "Material and Methods"). The 2,3-P-glycerate under the base line is equivalent to the  $P_i$  above it.

ml of 0.001 M *p*-mercuribenzoate were added. The final volume was 3 ml. A value of  $4.2 \pm 0.1$  —SH groups per mole of enzyme was found. This indicated lack of buried —SH groups and in addition agrees with the number of —SH groups found for the rabbit muscle enzyme by Pizer (5).

*Coenzyme Binding*<sup>5</sup>—The chicken breast muscle mutase, like

<sup>5</sup> We have chosen to use the term "coenzyme binding" since extensive experiments conducted with P-glycerate mutase from rabbit muscle and 2,3-P-glycerate labeled with <sup>32</sup>P and <sup>14</sup>C have indicated that there are two types of binding of the coenzyme to the enzyme. That is to say, there is phosphoenzyme formation and also a lesser binding of the carbon chain of 2,3-P-glycerate. Phosphoryl transfer occurs to about 6 times greater extent and rate than that of the carbon chain of 2,3-P-glycerate. The phosphoryl moiety of the P-enzyme, as well as the carbon chain of 2,3-P-glycerate enzyme bound, exchanges with 2,3-P-glycerate and with 3-P-glycerate; again the exchange is 6 to 7 times faster with the phosphoryl moiety. Also, we found that 3-P-glycerate labeled with <sup>32</sup>P is incorporated into 2,3-P-glycerate at about one-tenth the rate of incorporation into 2-P-glycerate.

the rabbit muscle enzyme,<sup>6</sup> binds <sup>32</sup>P-2,3-P-glycerate. Since it was then of interest to study the binding of the cofactor to the chicken breast muscle by a different method, the technique of Pfeleiderer and Auricchio (19) (see "Material and Methods") was used. Fig. 5 shows a typical elution curve.

The total amount of 2,3-P-glycerate which disappeared, as represented by the "trough" below the basal level, and the total amount of  $P_i$  liberated, as represented by the "peak," were calculated. The total  $P_i$  liberated served to calculate the 2,3-P-glycerate split because of phosphatase action. The amount of 2,3-P-glycerate that disappeared (corrected for phosphatase action) should then be bound to the enzyme. On this basis and from four experiments, an average of 2 moles of 2,3-P-glycerate (see Table II) are bound per mole of enzyme.

It should be noted that there was no liberation of bound 2,3-P-glycerate; that is, no excess cofactor (above base line) was found in the supernatant fluid after precipitation of the protein with perchloric acid. In other experiments each milliliter of eluate was collected in tubes containing 0.4 g of solid ammonium sulfate. The precipitate was centrifuged, and the measurement of 2,3-P-glycerate in the supernatant fluid gave data similar to those found with acid precipitation. It seems then that the 2,3-P-glycerate bound to the enzyme was not separated from it or, if separated, was not released as 2,3-P-glycerate.

2,3-P-glycerate binding experiments with 100  $\mu\text{moles}$  of yeast phosphoglycerate mutase demonstrated that there was no 2,3-P-glycerate bound to the enzyme inasmuch as the total amount that disappeared was equal to the amount of  $P_i$  liberated (see Fig. 6).

## DISCUSSION

The procedure described here is extremely rapid and convenient. It should be mentioned that either frozen or fresh chicken breasts purchased from any commercial source thus far tried have yielded satisfactory preparations. A conservative estimate is that it takes one-fifth to one-tenth the effort and expense necessary for the preparation of the rabbit enzyme (3). The rabbit muscle mutase was purified in this laboratory to an estimated 85% purity. Later on the enzyme was crystallized by Pizer (5). All phosphoglycerate mutases, while varying in molecular weight from about 112,000 to 30,000, appear to have about equal activity per mg of protein, *i.e.* about 2,500 units; and, as shown in this paper, the chicken breast enzyme is not an exception. It should be noted in this regard that the available commercial preparations reported to be crystalline are, in our estimation and based on specific activity, less than 10% pure. The chicken breast enzyme seems to be very similar to the rabbit muscle mutase. Indeed, as exemplified in this paper, all physical and molecular parameters are essentially the same. Moreover, while this has not been further documented for compactness of presentation, other parameters measured have indicated extreme similarity of the rabbit and chicken muscle enzymes. On the other hand, the yeast enzyme has no —SH groups, and yet, as shown by Pizer (6) and by ourselves, the muscle enzymes have —SH groups and require intactness of —SH groups for activity. Further, as reported previously, evidence for phosphoenzyme formation could be obtained with the rabbit muscle, but not with the yeast enzyme. As noted above, the chicken enzyme binds <sup>32</sup>P-2,3-P-glycerate so that there seems to be phosphoenzyme formation.

<sup>6</sup> R. Jacobs and S. Grisolia, unpublished experiments.

Again, as shown in this paper by an independent method, the binding of 2,3-P-glycerate occurs with the chicken breast muscle enzyme, but not with the yeast enzyme.

As pointed out by Pizer (6), in spite of the ready availability of pure substrates and enzymes, we have but a superficial knowledge of the mutase reaction. Indeed, the differences in 2,3-P-glycerate requirement, together with the differences in binding and in phosphoenzyme formation, indicate that more work is necessary and that comparative studies with P-glycerate mutases may be of much general interest for enzymology. The ease of preparation of chicken breast P-glycerate mutase may facilitate comparative studies, particularly with the yeast enzyme.

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#### CORRECTION

In the paper by Norio Hayashi, Yutaro Motokawa, and Goro Kikuchi (Vol. 241, No. 1, issue of January 10, 1966, page 79), in line 9, column 1, page 81, "...remained far lower..." should read "...remained far higher..."; in the legend under Fig. 5, page 82, the symbols ●—● and ⊙—⊙ should be transposed.