Pyruvate Carboxylase. VIII. The Subunit Structure as Examined by Electron Microscopy*

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ABSTRACT: Examination in the electron microscope of pyruvate carboxylase isolated from chicken liver mitochondria shows that the enzyme molecules outlined by negative staining appear primarily as tetramers with four subunits arranged at the corners of squares. Studies correlating changes in structure as observed in the electron microscope with alterations in catalytic activity and sedimentation behavior support the hypothesis that the tetramer is the catalytically active form of this enzyme.

Exposure of pyruvate carboxylase at 2° results in loss of catalytic activity, dissociation of the protein into subunits as observed by sedimentation analysis, and virtual disappearance of the tetrameric molecules observed in the electron microscope. Rewarming of the cold-inactivated enzyme to 23° causes regain of catalytic activity and restoration of both the original sedimentation pattern and tetramer content. The molecular weight as calculated from the diameter of the subunits of the tetramer observed in the electron microscope is consistent with the value obtained for pyruvate carboxylase from sedimentation studies.

Pyruvate carboxylase (pyruvate:CO_2 ligase (ADP)[1] EC 6.4.1.1) purified from chicken liver mitochondria (Utter and Keech, 1963) catalyzes the reaction

$$\text{pyruvate} + \text{ATP} + HCO_3^- \xrightleftharpoons[Mg^{2+}]{\text{acetyl CoA}} \text{oxalacetate} + \text{ADP} + P_i \quad (1)$$

The enzyme has been obtained in a highly purified state and is a large protein ($s^0_{20,w}$ = 14.8 S, $\bar{M}_w$ = 660,000) (Scrutton and Utter, 1965), which contains both bound biotin (Scrutton and Utter, 1965) and bound manganese (Scrutton *et al.*, 1966) each in amounts approaching 4 moles/mole of enzyme. The enzymatically active protein has therefore been proposed to contain four subunits with an average molecular weight in the range of 165,000 (Scrutton and Utter, 1965) and each containing one biotin and one manganese site. This proposal for the subunit structure of pyruvate carboxylase is further supported by changes in the sedimentation properties of the enzyme which accompany inactivation at 2° as will be described below.

Electron microscope studies of some other large enzyme molecules as outlined by negative staining procedures have been sufficiently satisfactory to permit the proposal of models describing the arrangement of the constituent subunits. Suggestions have been made concerning the structure of glutamate dehydrogenase from bovine liver (Horne and Greville, 1963), pyruvate oxidase from *Escherichia coli* (Fernandez-Moran *et al.*, 1964), catalase from bovine liver (Valentine, 1964; Horne and Greville, 1964), fraction I protein from Chinese cabbage leaves (Haselkorn *et al.*, 1965), ribonucleic acid (RNA) polymerase from *E. coli* (Colvill *et al.*, 1966), and acetyl Co-A carboxylase from chicken liver (Gregolin *et al.*, 1966). Analogous studies on pyruvate carboxylase from chicken liver are reported here and provide further evidence for the presence of four subunits in the molecule of this enzyme.

Methods

Pyruvate carboxylase was purified through the 22–70% $(NH_4)_2SO_4$ precipitation step (stage 5) (Scrutton and Utter, 1965), and was assayed spectrophotometrically in the direction of CO_2 fixation (Utter and Keech, 1963). Specific activities are expressed as micromoles per minute per milligram of protein at 25°. Protein was estimated spectrophotometrically (Warburg and Christian, 1941).

Pyruvate carboxylase (10–15 mg/ml) in 1.5 M sucrose containing 0.1 M phosphate, pH 7.0, and 0.06 M $(NH_4)_2SO_4$ was prepared for examination in the electron microscope by dilution to 50 μg of protein/ml in 0.05 M phosphate, pH 7.0, at 23°. Diluted enzyme (1 drop) was immediately applied with a platinum loop to a carbon-coated nitrocellulose film on an electron microscope grid. The grid was blotted at the edge with filter paper to give a thin film which was permitted to evaporate for a few seconds. Just before the film dried, drops of the negative stain (4% sodium silicotungstate, pH 7) were added and blotted off. The grids were

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[1] Abbreviations used: ADP, adenosine diphosphate; ATP, adenosine triphosphate.

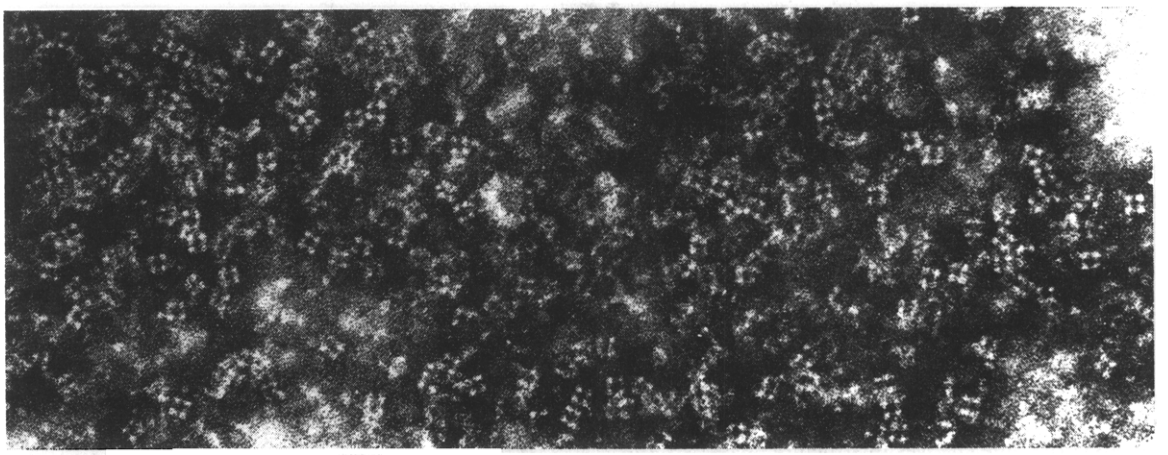

PLATE I: Molecules of pyruvate carboxylase (sp act. 33.2) in a loose aggregate stained as described in Methods; magnification, 250,000×.

PLATE II: A typical electron micrograph of a preparation of pyruvate carboxylase (sp act. 35.0). The intact molecules show four subunits arranged at the corners of a square; magnification, 500,000×.

examined in a Philips EM 200 electron microscope and plates (Ilford N.60) were exposed to the image at a magnification of 55,000. The microscope was calibrated by use of the 163-A spacing of catalase crystals.

For the electron microscopic studies of the effect of cold inactivation on the enzyme structure, the enzyme was diluted to 500 μg of protein/ml with 0.01 M phosphate or 0.01 M Tris–Cl, pH 7.0, and incubated at 2° for the time indicated. Cold phosphate buffer was then added to give a final concentration of 0.05 M potassium phosphate, pH 7.0, and 50 μg of protein/ml immediately prior to preparation of the grids at 2° as described above. Reactivation of cold-inactivated enzyme (500 μg of protein/ml) was accomplished by warming to 23° in the presence of 2.5×10^{-3} M ATP and 0.05 M phosphate, pH 7.0, for 20–30 min.

For the sedimentation studies of cold inactivation pyruvate carboxylase was equilibrated with 0.01 M potassium phosphate, pH 7.2, containing 0.2 M KCl by gel filtration on Sephadex G-50 columns (15 × 1 cm), and the protein concentration was adjusted to 1.7 mg/ml with the same buffer. Sedimentation analysis was conducted in a Spinco Model E analytical ultracentrifuge at 50,740 rpm using the AN-E rotor and a 30-mm aluminum cell.

Sodium dodecatungstosilicate (sodium silicotungstate) was obtained from Hopkin and Williams (Chadwell Heath, England). All other chemicals were obtained or prepared as described previously (Scrutton and Utter, 1965).

Results

The Structure of Pyruvate Carboxylase. Preparations of pyruvate carboxylase prepared for examination in the electron microscope as described above always showed amorphous debris suggesting that considerable denaturation of the protein had occurred during the staining and drying procedures employed. This denaturation can be reduced by partial evaporation of the preparations in the presence of 0.05 M phosphate buffer prior to staining with sodium silicotungstate. Under these conditions extensive areas of preserved molecules are seen (Plate I), in which the molecules show a clear tetramer structure with four subunits arranged at the corners of squares (Plate II).

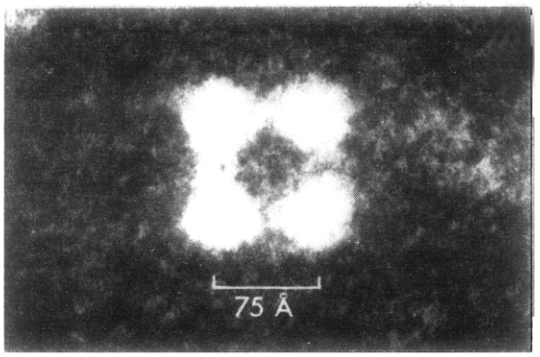

PLATE III: The superimposed images of five intact molecules. The center-to-center spacing of adjacent subunits is 70–75 A; magnification, 2,000,000×.

Superimposition of the images of five molecules at higher magnification (Plate III) permits the measurement of the distance between the centers of adjacent subunits as 70–75 A. This is probably the best estimate of the diameter of the subunits since only their centers show clearly in the negative stain. The diameter of a spherical subunit with a molecular weight of 165,000 may be calculated as 73 A if the partial specific volume is taken as 0.75 ml/g. Therefore the size of the observed tetramers is consistent with their identification as the molecules of pyruvate carboxylase. Plates I and II suggest that the molecules normally absorb flat on the supporting film and although the other possible orientations of a square tetramer (linear dimers and trimers) may be found, they are not sufficiently common or characteristic to be identified definitively.

Effect of Cold Inactivation of Pyruvate Carboxylase and of Reactivation of Cold-Inactivated Enzyme on the Sedimentation Properties and Structure of Pyruvate Carboxylase. The proposed identification of the catalytically active form of pyruvate carboxylase as the tetramer has been confirmed by correlation of the effects of cold inactivation on the sedimentation pattern of the enzyme and on the structure as observed in the electron microscope. Pyruvate carboxylase has been shown to be reversibly inactivated by exposure at 2° (Scrutton and Utter, 1965). The inactivation is accompanied by dissociation of the active enzyme ($s^0_{20,w}$ = 14.8 S) to yield subunits ($s^0_{20,w}$ = 6.75 S) which do not catalyze reaction 1. On rewarming the cold-inactivated enzyme to 23° a marked or complete recovery of catalytic activity occurs accompanied by restoration of the sedimentation pattern. The reactivation process is enhanced by addition of 2.5×10^{-3} M ATP just prior to rewarming. It should be noted that in contrast to certain other large enzymes, *e.g.*, glutamate dehydrogenase (Olson and Anfinsen, 1952), dilution of pyruvate carboxylase at room temperature and pH ranges of 6.8–7.5 does not cause dissociation of the enzyme into subunits when examined over a range of 50 μg–10 mg of protein/ml. Since 0.05

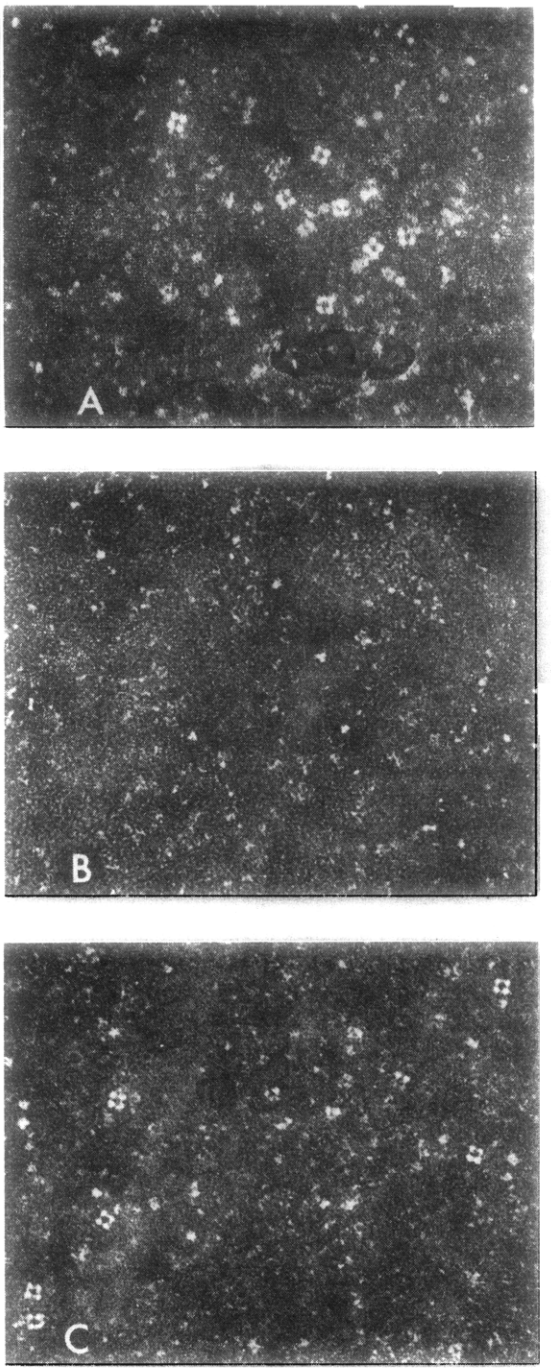

PLATE IV: The effect of cold inactivation and subsequent thermal reactivation on the structure of pyruvate carboxylase molecules as observed by electron microscopy. Pyruvate carboxylase (sp act. 29.2) was prepared for electron microscopy as described in Methods. The enzyme was initially diluted with 0.01 M Tris–Cl, pH 7.0. The plates shown are (A) at 23° for 20 min (active enzyme); (B) at 2° for 20 min (inactive enzyme); and (C) at 2° for 20 min followed by 23° for 20 min (reactivated enzyme); magnification, 200,000×.

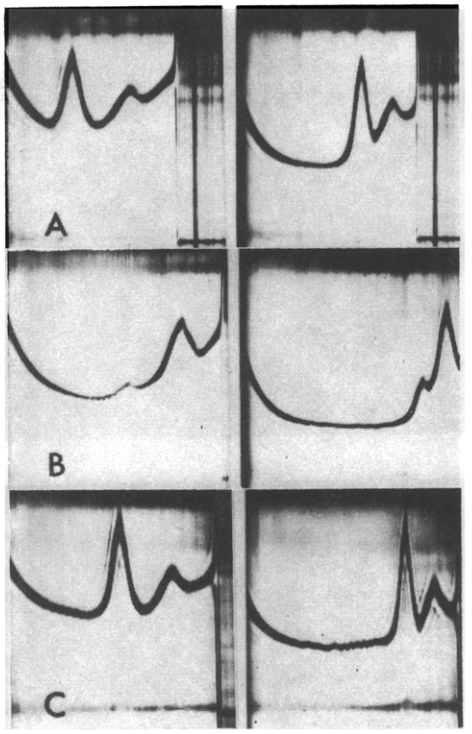

PLATE V: The effect of cold inactivation and subsequent thermal reactivation on the sedimentation properties of pyruvate carboxylase. The same preparation of pyruvate carboxylase (sp act. 29.2) as used for the electron microscope studies (plate IV) was equilibrated with 0.01 M phosphate, pH 7.2, containing 0.2 M KCl as described in Methods to give a final protein concentration of 1.7 mg/ml. Sedimentation patterns are shown for pyruvate carboxylase incubated at (A) 23° for 30 min (active enzyme); (B) 2° for 30 min (inactive enzyme); (C) 2° for 30 min followed by 23° for 30 min (reactivated enzyme). Sedimentation was from right to left at 22.6° (A), 2.9° (B), and 20.0° (C). The schlieren photographs were taken at 16 and 32 min (A and C), and 24 and 56 min (B). The phase plate angle was 50° for the earlier time and 45° for the later time in all cases.

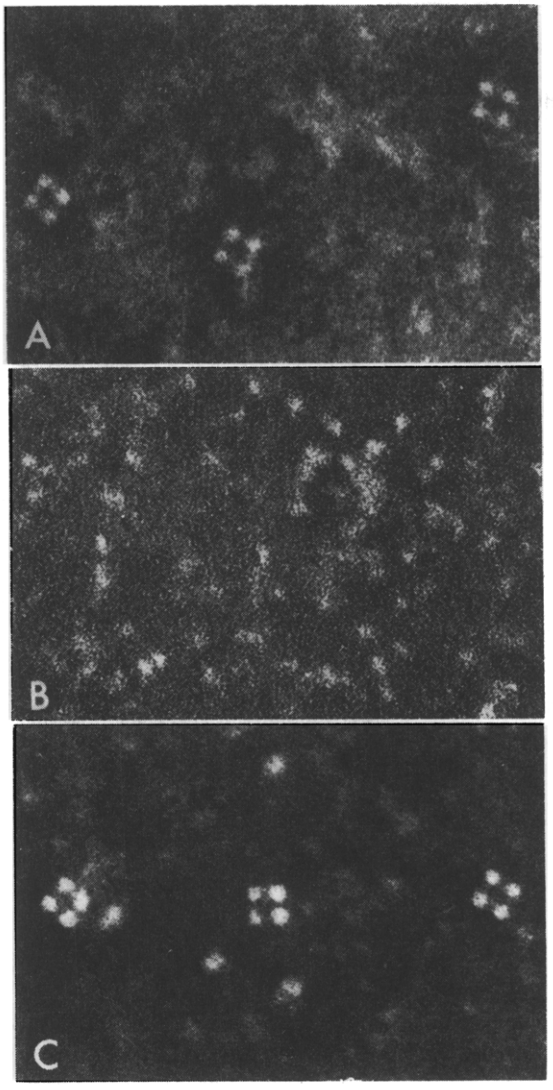

PLATE VI: The effect of cold inactivation and subsequent thermal reactivation on the structure of pyruvate carboxylase molecules as observed by electron microscopy. Conditions were as described for Plate IV except pyruvate carboxylase (sp act. 35.0) was diluted in 0.01 M phosphate, pH 7.0, and all incubations were for 30 min; magnification, 500,000×.

M phosphate causes marked protection of pyruvate carboxylase against cold inactivation, the enzyme preparations used for such studies were diluted in 0.01 M phosphate, pH 7.0, or 0.01 M Tris–Cl, pH 7.0, prior to incubation at 2° and examination in the electron microscope. After incubation for 20 min at 2° in 0.01 M Tris–Cl, pH 7.0, very few tetramers are visible and a marked increase in the number of smaller irregular objects (presumably isolated subunits) is observed (Plate IVB) as compared with a control held at 23° (Plate IVA). Although estimates are difficult to make due to variation in the extent of denaturation which occurs during specimen preparation, assessment of several specimens shows at least 80% of the tetramers have dissociated in the sample held at 2°. Sedimentation analysis of the same pyruvate carboxylase preparation in 0.01 M phosphate, pH 7.2, containing 0.2 M KCl at 23° (Plate VA) and at 2° (Plate VB) demonstrates that incubation at 2° had caused a 80–90% conversion of the 14.8S species to the 6.75S subunit. On rewarming the cold-inactivated enzyme to 23° for 20 min in the presence of 2.5×10^{-3} M ATP a restoration of the tetramer content (Plate IVC) and sedimentation pattern (Plate VC) is observed as compared with the control sample (Plates IVA and VA). Measurement of the catalytic activity

TABLE I: Measurements of Enzymic Activity during Inactivation of Pyruvate Carboxylase at 2° and Subsequent Thermal Reactivation.[a]

Incubn, Deg (min)	Cat. Act. (units/ml)
23 (30)	43.7
2 (30)	6.37
2 (30) followed by 23 (30)	41.0

[a] The same preparation of pyruvate carboxylase (1.7 mg/ml, sp act. 29.2) as used for the electron microscope studies (Plate IV) and the sedimentation studies (Plate V) was equilibrated with 0.01 M phosphate, pH 7.2, containing 0.2 M KCl as described in Methods. Residual catalytic activity was measured spectrophotometrically at 25° after gel filtration, incubation, and treatment as described for Plate V. Previous studies (Scrutton and Utter, 1965) have shown that cold inactivated pyruvate carboxylase is not reactivated on dilution into the spectrophotometric assay mixture at 25°.

(Table I) showed a decrease of 85% on incubation at 2° and restoration to 94% of the original value on rewarming. The sedimentation coefficients ($s_{20,w}$) of the two components observed in Plate V have been calculated as 14.2 and 7.2 (Plate VA), 13.5 and 7.0 (Plate VB), and 14.1 and 7.0 S (Plate VC).

Electron microscope examination of another preparation of pyruvate carboxylase diluted in 0.01 M phosphate, pH 7.0, showed similar loss of tetramer content on incubation at 2° and restoration on rewarming to 23° in the presence of ATP. This is seen at higher magnification in Plate VI.

Discussion

The parallel changes demonstrated in catalytic activity, sedimentation properties, and structure as observed in the electron microscope during exposure at 2° and subsequent rewarming provide convincing evidence for the identification of the tetramer as the enzymatically active form of pyruvate carboxylase. The size of the tetramer as calculated from the center-to-center spacing of the subunits and the fourfold symmetry demonstrated by the electron microscope further support this conclusion. While cold inactivation causes dissociation of pyruvate carboxylase, inactivation of this enzyme under some other conditions occurs without change in the sedimentation properties and it is probable that enzymatically inactive tetramers can exist under some conditions. The electron microscope studies are consistent either with a tetrad molecule having a single fourfold axis of symmetry or with a molecule possessing three dyad axes, but these possibilities cannot be distinguished.

Although the subunits of pyruvate carboxylase appear similar to each other in the electron microscope, this method is not sufficiently sensitive to demonstrate whether or not they are identical. These subunits are seen as coherent, probably spherical bodies when present in the tetramer but are less well defined when present as isolated species, *e.g.*, after inactivation at 2°. Analyses[2] based on the observed $s^0_{20,w}$ of 6.75 S and an assumed molecular weight of 165,000 also indicate that the subunit produced by cold inactivation is considerably more asymmetric than its appearance in the tetramer would suggest. Both these findings are consistent with the proposal that cold inactivation may involve a partial deformation of the subunit in addition to dissociation of the tetramer. Additionally the dimensions of the tetrameric molecules observed in the electron microscope, which permit the calculation of the axial ratio as approximately 3, show an unexplained discrepancy as compared with calculations[2] of the axial ratio based on the observed sedimentation coefficient ($s^0_{20,w}$ = 14.8 S) and molecular weight ($\overline{M}_w$ = 655,000). The latter calculations suggest a molecule with an axial ratio in the range of 10. The possibility therefore cannot be excluded that the molecule has dynamic features in solution not revealed by the negative staining and which account for the high frictional ratio. Electron microscopy only certainly establishes the symmetrical tetrameric nature of the molecule. In particular it should be noted that the subunits observed in the molecule of pyruvate carboxylase by electron microscopy are themselves composed of smaller subunits. Thus in the presence of 1% sodium dodecyl sulfate pyruvate carboxylase dissociates to yield subunits ($s^0_{20,w}$ = 2.7 S) (Scrutton and Utter, 1965) with a molecular weight in the range of 45,000.[3] These smaller subunits are not resolved in the electron microscope.

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[2] These calculations utilized the assumption of the partial specific volume as 0.75 ml/g, the hydration term as 0.2 mg of water/mg of protein, and the representation of the molecule as ellipsoid.

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Influence of Ionic Strength on Apparent Reaction Mechanism of Phosphoglycerate Mutase*

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ABSTRACT: The salt environment markedly affects both the yeast and muscle phosphoglycerate (P-glycerate) mutases which are 2,3-P-glycerate dependent and were believed to catalyze the reaction 3-P-glycerate + 2,3-P-glycerate ⇄ 2,3-P-glycerate + 2-P-glycerate. At low ionic strength 2-P-glycerate is labeled from 3-P-glycerate-^{32}P much faster than 2,3-P-glycerate; this is theoretically impossible according to the reaction outlined above. Increased ionic strength favors ^{32}P fixation on 2,3-P-glycerate. At 0.5–1 ionic strength negligible ^{32}P appears on 2-P-glycerate; the bulk is now on 2,3-P-glycerate. It appears that 2,3-P-glycerate does not function as a *free obligatory intermediate*, but is more likely enzyme bound.

Our findings do not reflect a change in activity but in mechanism, *i.e.*, increased rate for the reaction; enzyme + 2,3-P-glycerate ⇄ enzyme-bound 2,3-P-glycerate, and/or a new enzyme conformation, yielding a mechanism resembling the non-2,3-P-glycerate-dependent P-glycerate mutases. These effects are best demonstrated with cations which likely change the elastoplastic protein behavior. They may also apply to other cases, *e.g.*, some *isotope experiments* using "pools" in metabolic studies with *in vitro* systems in which ionic strength has not been controlled and/or tested may be misleading. The cationic effects do not fit the pattern expected for lytropic series; the optimum pH is 8 which is far from the optimum pH for activity of yeast and muscle P-glycerate mutase (5 and 6), respectively.

There are two types of P-glycerate[1] mutases, 2,3-P-glycerate-independent and -dependent enzymes (Grisolia, 1962), which catalyze reactions 1 and 2, respectively.

$$\text{3-P-glycerate} \rightleftharpoons \text{2-P-glycerate} \quad (1)$$

$$\text{3-P-glycerate} + \text{2,3-P-glycerate} \rightleftharpoons \text{2,3-P-glycerate} + \text{2-P-glycerate} \quad (2)$$

Pizer (1962) pointed out that Sutherland *et al.* (1949) found that in every conversion of 3- to 2-P-glycerate, 2,3-P-glycerate must be simultaneously a donor and an acceptor as depicted by reaction 2. Recent investigations (Torralba and Grisolia, 1966) have demonstrated binding of 2,3-P-glycerate to chicken breast muscle P-glycerate mutase, extending previous findings with rabbit muscle mutase (Grisolia *et al.*, 1961). While these findings together with those of Zwaig and Milstein (1966) indicate coenzyme binding to muscle P-glycerate mutase, similar evidence is lacking for the yeast enzyme (Torralba and Grisolia, 1966; Grisolia *et al.*, 1961). Recent experiments (Grisolia and Cascales, 1966) have demonstrated a change in the extent of incorporation of 3-glycerate-^{32}P into 2,3-glycerate-^{32}P induced by an increase in ionic strength with both muscle and yeast mutases. This finding requires the postulation of such an enzyme cofactor complex for the yeast mutase also, with a change in mechanism *via* an increase in exchange resulting from either a shielding or a buttressing salt effect. Alternatively, these findings may indicate a more complex type of change in mechanism; perhaps the enzyme acquires its

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[1] Abbreviations: 3-PGA, D-3-phosphoglyceric acid; 2-PGA, N-2-phosphoglyceric acid; 2,3-PGA or DPGA, D-2,3-diphosphoglyceric acid; P-glycerate, phosphoglycerate; PEP, phosphoenolpyruvate; GTP, guanosine triphosphate; ADP, adenosine diphosphate.