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PURIFICATION AND PROPERTIES OF ENOLASE OF HUMAN ERYTHROCYTES

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ABSTRACT

1. Human erythrocyte enolase (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11) was purified 1000-fold.

2. The pH-optimum was at pH 6·5. The molecular weight, estimated by gel filtration, was found to be 95,000 ± 5,000.

3. Electrophoresis on agar-agarose at pH 8·5 and 6·4 showed only one band in hemolysate as well as in purified preparations.

4. The enzyme was stabilized by magnesium and ammonium ions.

5. The K_m values for 2-phosphoglyceric acid at pH 6·5 and pH 7·2 were found to be the same: K_m (2-phosphoglyceric acid) = 28 μM. The optimal magnesium concentration was between 1 and 5 mM.

6. Ca^{2+}, at physiological concentrations, inhibited the enzyme. This inhibition depends on the Mg^{2+} concentration.

7. The enzyme was inhibited by fluoride. This inhibition depends on magnesium, fluoride and phosphate concentration. Moreover the F^- inhibition was dependent on 2-phosphoglyceric acid concentration in a competitive way.

ENOLASE (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11) reversibly converts 2-phospho-D-glycerate to phosphoenolpyruvate, the loss of water changing the low-energy phosphate group of 2-phosphoglyceric acid into the high-energy phosphate group of phosphopyruvic acid. The enzyme is present in all tissues or organisms capable of glycolysis or of fermentation. The enzyme was discovered in 1934 by Lohmann and Meyerhof and has been isolated since then from a variety of sources (Wold, 1971).

From human sources, enolase has been purified from muscle (Baranowski, Wolna & Morawiecki, 1968) and erythrocytes (Witt & Witz, 1970). Recently Stefanini (1972) described a patient with a deficiency of enolase in human erythrocytes. He related this deficiency to a chronic hemolytic anaemia, which was exacerbated by ingestion of a drug (nitrofurantoine). It is well known that enolase is inhibited by fluoride. Warburg & Christian (1941) stated that this inhibition was dependent on the concentration of fluoride, magnesium and phosphate. With particular reference to inherited erythrocyte enolase deficiency, we purified the enzyme and determined some molecular and catalytic properties. We also studied a possible influence of low fluoride concentrations on the purified enzyme.

MATERIALS AND METHODS

CHEMICALS

2-Phosphoglycerate (2-PG), NADH, ADP, phosphoenolpyruvate (PEP), lactate dehydrogenase (LDH) and pyruvate kinase (PK) were ob-

tained from Boehringer; nitrobluetetrazolium (NBT) and phenazinemethosulphate (PMS) were obtained from Sigma Chemical Co. The agar used in the electrophoresis was Special Noble Agar from Difco. The agarose was Indubiose A 37, from l'Industrie Biologique Française. Ammonium sulphate (granular) analyzed reagent was from Baker. All other p.a. reagents were from E. Merck. DEAE-Sephadex A-50, capacity 3·5 ± 0·5 meq./g., particle size 40–120 μm., Sephadex G-100 and Sephadex G-150 were purchased from Pharmacia, Uppsala, Sweden. Human erythrocytes were obtained from healthy donors from the Netherlands Red Cross Blood Transfusion Service.

Determination of Activity

The assays were carried out at 25°C (with a Zeiss spectrophotometer PM QII in combination with a Vitatron recorder UR 401) by following the change in absorption at 240 nm. due to the formation of PEP (Warburg & Christian, 1941) and at 340 nm. due to the oxidation of NADH with pyruvate as an acceptor (Witt & Witz, 1970). Enolase activity at 240 nm. was determined in 0·25 M Tris-HCl buffer (pH 6·5) in a final volume of 2·5 ml. containing 1 mM 2-PG, 5 mM $MgCl_2$ and enzyme. The reaction was started by adding enzyme solution to the cuvette. Enolase activity at 340 nm. was determined in 0·045 M triethanolamine buffer in a final volume of 2·5 ml. containing 10 mM $MgCl_2$, 76 mM KCl, 0·3 mM NADH, 1 mM 2-PG, 1 mM ADP, 20 μl. LDH (10 mg./ml., 20 μl. PK (10 mg./ml.) and enzyme. The reaction was started by adding enzyme solution to the cuvette. The determination of enzymatic activity at 340 nm. was only used in the first experiment (determination of pH-optimum in three different ways). A unit of activity is defined as the amount of enzyme required to convert 1 μmole of 2-PG per minute under the conditions mentioned above. The specific activity is defined as units/mg. protein. The protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline human serum albumin as a standard.

Molecular Weight Determination

The molecular weight was estimated according to Andrews (1965). The chromatography was carried out with Sephadex G-100 (column size 2·5 × 70 cm.) and G-150 (column size 2·5 × 100 cm.), equilibrated with 0·1 M Tris-HCl buffer (pH 7·2) and 20 mM $MgCl_2$. The reference proteins used for calibration of the column were pyruvate kinase (mol. wt. 237,000), lactate dehydrogenase (mol. wt. 140,000), human haemoglobin (mol. wt. 64,500) and cytochrome *c* (mol. wt. 11,700).

Electrophoresis

Electrophoresis was performed at 17°C in an apparatus according to Wieme (1965). The composition of the carrier medium was 0·6% agar and 0·4% agarose. The buffers used were 82 mM Barbital-HCl (pH 8·4) and 82 mM Tris-HCl (pH 6·5). The electrophoresis was carried out for 20 minutes, the current being 25 mA/slide, corresponding with a voltage of 150–200 V.

After the electrophoresis the slides were incubated for 75 minutes at 37°C with a solution containing 0·25 M Tris-HCl buffer (pH 6·5), 76 mM KCl, 10 mM $MgCl_2$, 1·0 mM 2-PG, 1·0 mM ADP, 3·0 mM NADH, 0·08 mg./ml. PK and 0·08 mg./ml. LDH (von Fellenberg, Richterich & Aebi, 1963). After incubation, the gels were rinsed with tap water and incubated for 30 minutes at 37°C without the incubation solution. Staining was performed for 15 minutes at 37°C with the following solution: 0·25 M Tris-HCl buffer (pH 6·5), 0·75 mM NBT and 0·20 mM PMS (Zondag, 1964).

RESULTS

Isolation of Enolase

Approximately 200 ml. erythrocytes were washed three times with 0·9% NaCl and mixed afterwards with an equal volume of 0·4% saponine. This mixture was stirred for one hour at 4°C. To the hemolysate were added 4l. of DEAE-Sephadex suspension prepared according to the instructions of the manufacturers, equilibrated with 0·01M Tris-HCl buffer (pH 8·8) and stirred for one hour at 4°C. Almost all of the haemoglobin was bound to the Sephadex, while most of the enolase activity was found in the eluate. The pH of the eluate was brought to 7·2. Ammonium sulphate (500 g./l.) was added to 2200 ml. of the enzyme solution, maintaining the pH at 7·2. The precipitate was collected by centrifuging (13,000*g* for 40 minutes at 4°C) and was suspended in 0·01M Tris-HCl buffer (pH 7·2) containing 20 mM $MgCl_2$. This suspension (25 ml.) was dialyzed for two hours against 0·01 M Tris-HCl buffer (pH 7·2) containing 20 mM $MgCl_2$. To the dialysate (35 ml.) 3·5 g. ammonium sulphate was added. The enzyme solution was heated for 3 minutes at 55°C and dialyzed overnight against 0·01 M Tris-HCl buffer (pH 7·2) containing 20 mM $MgCl_2$ and 40 mM ammonium sulphate. The dialysate (44 ml.) was centrifuged (38,000 *g* for 30 minutes at 4°C).

The whole purification procedure is summarized in Table I.

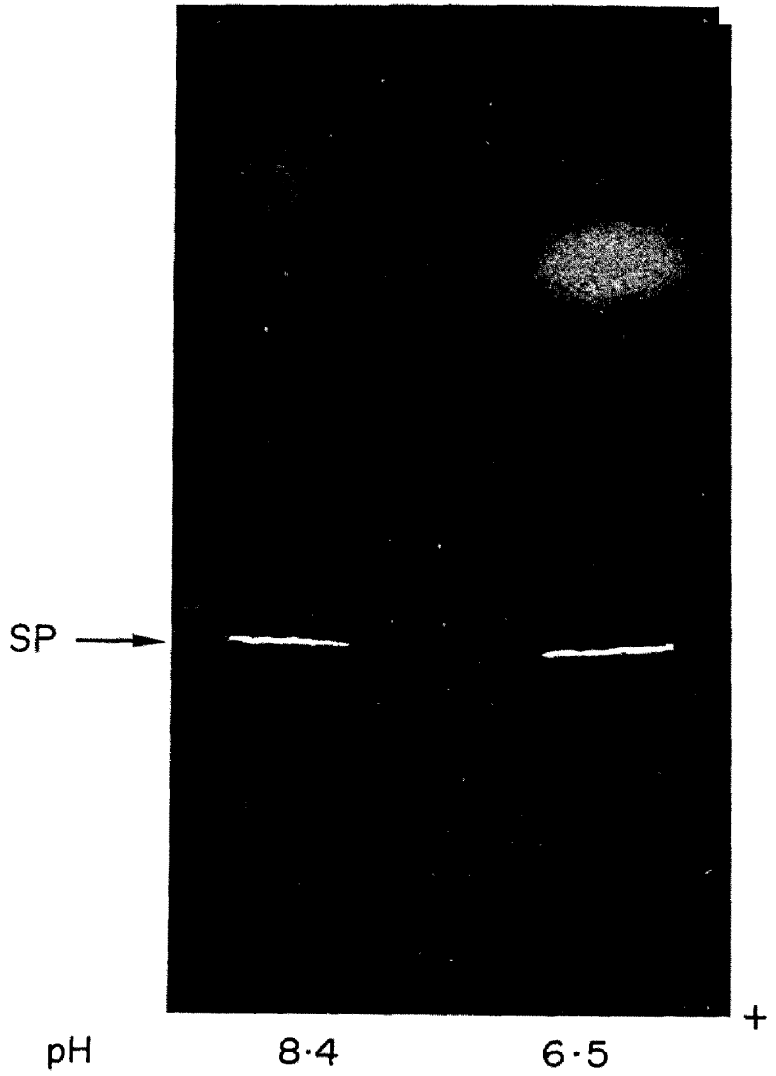

FIG. 2.—Electrophoresis of enolase with agar-agarose at pH 8·4 and pH 6·5. SP=starting point. Further conditions are given under ' Materials and Methods '.

Table I.—Purification of Enolase from Human Erythrocytes

Step of Purification	Total Volume (ml.)	Total Protein (mg.)	Total Activity (Units)	Specific Activity (Units/mg. protein)	Yield (%)	Cumulative Purification (-fold)
1	380	$76{\cdot}5 \times 10^3$	340	$4{\cdot}45 \times 10^{-3}$	100	1
2	2200	286	190	0·66	56	148
3	35	34	76	2·23	21	502
4	44	11·4	52	4·55	15	1020

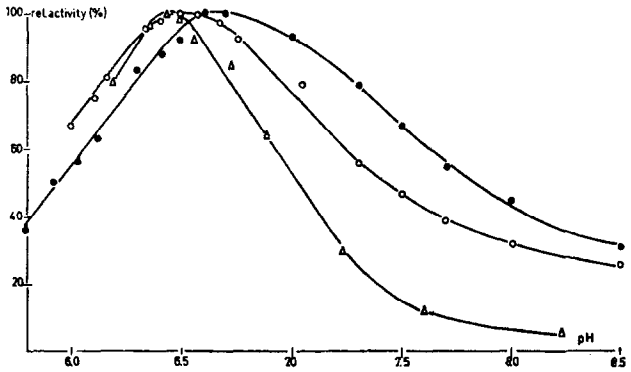

Fig. 1.—Influence of pH on enolase activity. Purified enzyme preparation (○), and hemolysate (△), determined by PEP-absorption measurement at 240 nm.; purified enzyme preparation (●) determined by measurement of NADH disappearance at 340 nm. (The molecular absorbence coefficient of PEP at 240 nm. was corrected for pH dependence). Further conditions are given in ' Materials and Methods '.

Properties of the Enzyme

pH-*optimum*

Fig. 1 shows the pH-optimum of the purified enolase and enolase from the hemolysate, determined by the method of Warburg & Christian (1941). This figure also shows the pH-optimum of the purified enolase as determined by the method of Witt & Witz (1970). From this figure it can be concluded that there is little or no difference in pH-optimum determined by these two methods and also that there exists no difference in pH-optimum before and after purification. The pH-optimum is found to be at pH 6·5.

Molecular weight

The molecular weight was estimated by means of gel filtration with Sephadex G-100 and Sephadex G-150. The mean molecular weight found by these two procedures was 95,000 ± 5,000 daltons. This corresponds well with the molecular weight of enolase from human muscle (95,000 ± 3,000) determined by Baranowski, Wolna & Morawiecki (1968). However Witt & Witz (1970) reported a molecular weight of 80,000 for enolase from human erythrocytes.

Electrophoresis

Fig. 2 shows the agar-agarose electrophoresis at pH 8·4 and pH 6·5 of the purified enzyme. In both cases, one band is obtained, indicating that there are no isoenzymes of enolase in human erythrocytes. Electrophoresis of the hemolysate (not given in a figure) also showed a single band with enolase activity with the same electrophoretic mobility. From this it can be concluded that during the purification no isoenzymes are lost.

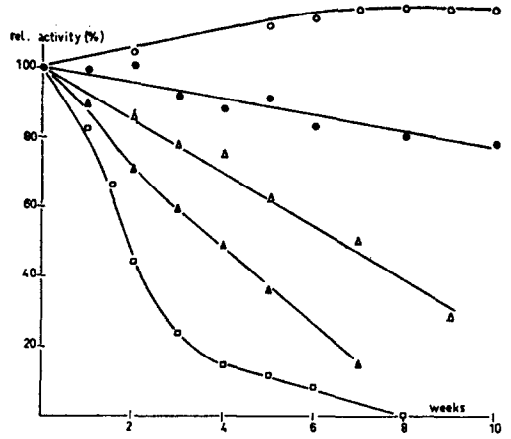

FIG. 3.—Changes in activity of enolase during storage at 4°C at different [$(NH_4)_2SO_4$] and [$MgCl_2$]. ○, 0·75 M $(NH_4)_2SO_4$, 20 mM $MgCl_2$; ●, 40 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$; △, 40 mM $(NH_4)_2SO_4$, without $MgCl_2$; ▲, without $(NH_4)_2SO_4$, 20 mM $MgCl_2$; □, without $(NH_4)_2SO_4$, without $MgCl_2$.

Stability of the enzyme

The change in activity of the enzyme during storage at 4°C under different circumstances is shown in FIG. 3. These experiments clearly demonstrate that ammonium and magnesium ions are necessary for the maintenance of enzyme activity. No loss of activity could be observed at high ammonium sulphate concentration (0·75 M) and magnesium chloride concentration of 20 mM during a period of nine weeks. Moreover it can be concluded that ammonium ions stabilize the enzyme more effectively than magnesium ions (see lowest lines in FIG. 3). In another experiment the enzyme was heated for 3 minutes at different temperatures in the presence of 20 mM $MgCl_2$, and in the presence or absence of ammonium sulphate (FIG. 4). From this figure it can be concluded again that ammonium ions stabilize the enzyme. In the presence of ammonium sulphate, the heat denaturation process starts at about 50°C and is completed at about 65°C. Without ammonium sulphate, the denaturation process proceeds much faster.

In order to study the stability of the enzyme with respect to the protein concentration of the solution and its dependence on magnesium ions, we diluted the enzyme preparation with buffer in the presence or absence of Mg^{2+} ions and measured the activity ten minutes after dilution. The results (FIG. 5) indicate that enzyme stability does not depend on the protein concentration up to 15 μg./ml. However dilution in the absence of Mg^{2+} ions results in a decrease of enzyme activity at about 0·5 mM Mg^{2+}. This effect is more pronounced at lower Mg^{2+} concentrations ($[Mg^{2+}] < 0{\cdot}2$ mM).

Kinetics

K_m values for 2-PG at the pH-optimum (pH 6·5) and at physiological pH (pH 7·2)

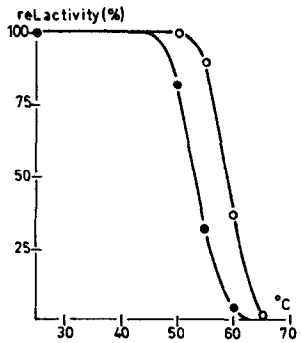

FIG. 4.—Heat inactivation of enolase. The enzyme preparation was heated for 3 minutes at different temperatures in 0·01 M Tris-HCl buffer (pH 7·2) and 20 mM $MgCl_2$, in the presence of 0·2 M $(NH_4)_2SO_4$ (○), and without $(NH_4)_2SO_4$ (●).

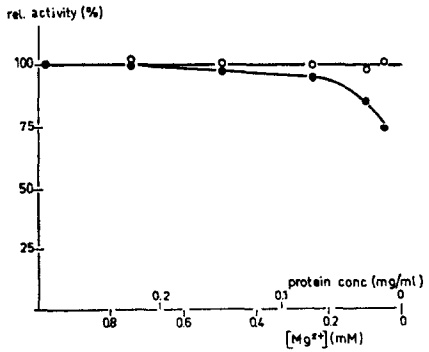

FIG. 5.—Changes of relative activity of enolase after dilution. The enzyme preparation was diluted with 0·01 M Tris-HCl buffer (pH 7·2) in the presence of 20 mM $MgCl_2$ (○), and without $MgCl_2$ (●).

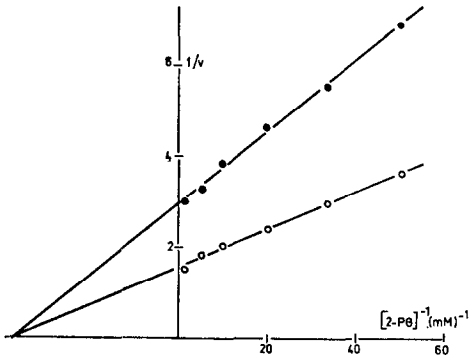

FIG. 6.—$1/v$ *vs* $1/[2\text{-PG}]$ plot at pH 6·5 (○) and pH 7·2 (●); $[Mg^{2+}] = 5$ mM. v is expressed as △E/minute/ml. enzyme solution.

were obtained by the Lineweaver-Burk plots (FIG. 6). From this figure it can be concluded that the K_m values at both pHs are equal (28 μM). No substrate inhibition could be detected.

In order to determine the K_m for Mg^{2+}, the enzyme preparation was dialyzed overnight against 0·01 M Tris-HCl buffer (pH 7·2) without Mg^{2+} and with 0·1 mM Mg^{2+}. In both cases there was a considerable loss of activity and K_m values for Mg^{2+} were found to be between 3×10^{-3}M and 2×10^{-4}M (not given in a figure). So it seems impossible to determine the K_m for Mg^{2+}. Maximal enzyme activities were found at Mg^{2+} concentrations between 1 and 5 mM. At 10 mM Mg^{2+}, we observed some inhibition of enzyme activity.

Since Bszormenyi-Nagy (1955) reported Ca^{2+} inhibition of enolase from hemolyzed erythrocytes, we studied the effect of physiological Ca^{2+} concentrations on the purified enzyme at two different Mg^{2+} and 2-PG concentrations (see FIG. 7). This figure shows that the Ca^{2+} inhibition is dependent on the Mg^{2+} concentration and is independent of the 2-PG concentration.

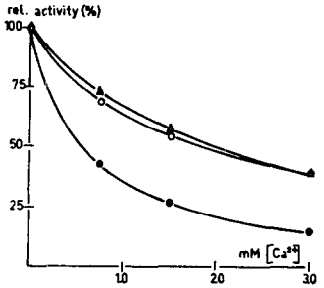

FIG. 7.—Influence of increasing $[Ca^{2+}]$ on enolase activity at different [2-PG] and $[Mg^{2+}]$. ○, 1 mM 2-PG, 5 mM Mg^{2+}; △, 0·1 mM 2-PG, 5 mM Mg^{2+}; ●, 1 mM 2-PG, 0·5 mM Mg^{2+}.

Influence of fluoride on enolase activity

Warburg & Christian (1941) were the first to investigate the influence of fluoride on yeast enolase. They observed that fluoride inhibition only took place in the presence of phosphate ions. They stated that the extent of inhibition depends also on the concentration of Mg^{2+}, phosphate and fluoride.

From our experiments (see FIG. 8) it can be concluded that enolase from human erythrocytes is also inhibited by sodium fluoride. This inhibition is only observed in the presence of phosphate ions. The extent of inhibition depends on the concentration of Mg^{2+}, phosphate and fluoride: an increase of the concentration of these compounds results in a decrease of enzyme activity. Our experiments, however, indicate that the extent of inhibition is also dependent on the concentration of 2-PG.

In order to investigate the kind of inhibition of fluoride we plotted $1/v$ *vs* $1/[Mg^{2+}]$ at $[F^-] = 3 \times 10^{-4}$M and $[PO_4^{3-}] = 10^{-2}$M (not given in a figure). No conclusions could

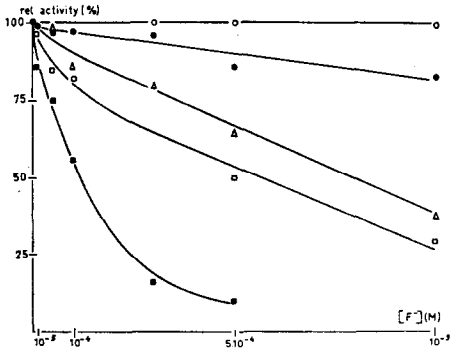

FIG. 8.—Influence of increasing $[F^-]$ on enolase activity at different [2-PG], $[Mg^{2+}]$ and $[PO_4^{3-}]$. ○, 1 mM 2-PG, 5 mM Mg^{2+}, without PO_4^{3-}, ●, 1 mM 2-PG, 5 mM Mg^{2+}, 1 mM PO_4^{3-}, △, 1 mM 2-PG, 2 mM Mg^{2+}, 10 mM PO_4^{3-}; □, 1 mM 2-PG, 5 mM Mg^{2+}, 10 mM PO_4^{3-}; ■, 0·1 mM 2-PG, 5 mM Mg^{2+}, 10 mM PO_4^{3-}.

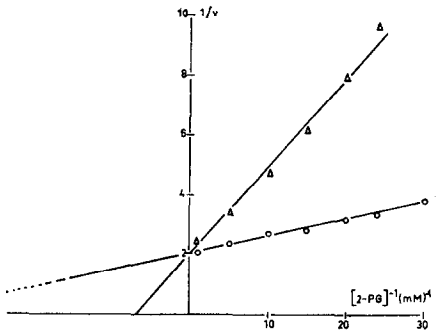

FIG. 9.—$1/v$ *vs* $1/[2\text{-PG}]$ plot with (△) and without (○) F^-. $[F^-] = 5 \times 10^{-5}$ M; $[Mg^{2+}] = 5$ mM. v is expressed as △E/minute/ml. enzyme solution.

be drawn about the kind of inhibition. However, plotting $1/v$ *vs* $1/[2\text{-PG}]$ at pH 6·5 (FIG. 9), leads to the conclusion that fluoride is a competitive inhibitor of 2-PG. The same effect has been observed at pH 7·2 (not given in a figure).

DISCUSSION

The human erythrocyte enolase purified by this method had a specific activity of 4·55 units/mg. protein, corresponding to a 1000-fold purification of the original hemolysate. Witt & Witz (1970) reported a 150-fold purification, with a specific activity of 0·71 units/mg. protein. The best step in our purification procedure seems to be the adsorption of the haemoglobin to DEAE-Sephadex at pH 8·8, while enolase is not bound to the resin at this pH. Some properties of enolase from human origin are compared in Table II.

We found a pH-optimum at pH 6·5. Baranowski *et al.* (1968) reported a value of 6·8 for human muscle enolase. Witt & Witz (1970), however, found a value of 7·5 for human erythrocyte enolase (see Table II). Determination of pH-optimum by the method used by Witt & Witz (1970) and determination of pH optimum in the hemolysate both resulted in values not significantly different from 6·5. We cannot explain the differences in pH-optimum found by Witt & Witz (1970) and by us. It seems reasonable to measure enolase activity in the hemolysate at pH 6·5 instead of pH at 8·0 in standard methods (Beutler, 1971). Enolase activity in the hemolysate at pH 8·0 is about 7% of its maximal value (FIG. 1). The stability experiments clearly demonstrate the necessity of magnesium and ammonium ions for optimal maintainance of enzyme activity.

Magnetic resonance studies (Cohn, 1963), and dissociation and re-association studies (reviewed by Wold, 1971) indicate that enolase is composed of two identical subunits (monomers). The binding of two moles of magnesium ion to two monomers results in a catalytically-active metalloenzyme. Addition of two substrate molecules creates two new Mg^{2+} binding sites, activating the enzyme. Studies of Oh & Brewer (1973) indicate that yeast enolase monomers are active under certain conditions, but lose all activity within a few hours.

From these data it is quite clear that Mg^{2+} is necessary for the maintainance of the active dimer. Dialysis of the enzyme preparation against buffer without Mg^{2+}, or a low concentration of Mg^{2+} (0·1 mM), will therefore result in a partial dissociation of the monomers, combined with a fall in activity. Our dilution experiments indicate that at an Mg^{2+} concentration of less than 0·2 mM

Table II.—COMPARISON OF SOME PROPERTIES OF ENOLASE OF HUMAN ORIGIN

ENZYME PROPERTIES	MUSCLE*	ERYTHROCYTES†	ERYTHROCYTES (this paper)
pH optimum	6·8	7·5	6·5
Molecular weight	95,000	80,000	95,000
K_m (2-PG)	0·3 mM	0·055 mM	0·028 mM

* Baranowski *et al.*, 1968.
† Witt & Witz, 1970.

there will be a considerable loss of enzyme activity.

The inhibition of enolase at physiological Ca^{2+} concentrations indicates that Ca^{2+} ions might play a role in the regulation of enolase activity. Studies on the influence of Ca^{2+} ions on pyruvate kinase, a regulatory enzyme of glycolysis, indicate that Ca^{2+} ions might also play an important role in controlling this enzyme activity (results of this study will be published elsewhere).

As already shown by Warburg & Christian (1941), fluoride inhibition takes place by the formation of a magnesium-fluoride-phosphate complex (with the stoichiometry 1:2:1). This complex inhibits the enzyme by reversible replacement of magnesium from enolase. Our experiments indicate that 2-PG can replace this magnesium-fluoride-phosphate complex from the active centre of the enzyme. From studies on substrate specificity (Wold, 1971) it has been obvious that a phosphate group plays an important role in the binding of the substrate in the active centre of the enzyme. Thus it might be possible that the phosphate group from the magnesium-fluoride-phosphate complex is bound to this binding site, in this way competitively inhibiting the binding of 2-PG.

In incubation experiments with erythrocytes in a medium containing 10^{-2}M fluoride ions, Feig, Shohet & Nathan (1971) found a lowering of enolase inhibition when they increased the substrate concentration. They explained this effect by the mass action of 2-PG. From our experiments it seems more reasonable to describe this phenomenon by a competitive replacement of the magnesium-fluoride-phosphate complex by 2-PG.

Physiological fluoride concentrations in serum are maximal at 5×10^{-6}M (Jardillier & Desmet, 1973). Adding fluoride to drinking water (at concentrations up to 1 mg. F^- per ml.) in order to prevent caries, results in a maximal serum concentration of about 10^{-5}M. From Fig. 8 it can be concluded that at [2-PG] = 0·1 mM, $[Mg^{2+}]$ = 5 mM and $[F^-] = 10^{-5}$M, enolase is inhibited by about 15%. It seems reasonable to assume that at $[F^-] = 10^{-5}$M and physiological concentrations of 2-PG and Mg^{2+}, fluoride inhibition of enolase cannot be neglected.

This inhibition might be more pronounced in patients with red cell enolase deficiency and in patients receiving drugs which cause oxidative stress on the erythrocyte. In other cases of inherited enzyme deficiency of red cell glycolysis (such as pyruvate kinase deficiency) this fluoride inhibition might cause exacerbation of the hemolytic anaemia.

REFERENCES

Andrews, P. (1965), 'The gel filtration behaviour of proteins related to their molecular weights over a wide range', *Biochem. J.*, **96**, 595–605.

Baranowski, T., Wolna, E., & Morawiecki, A. (1968), 'Purification and properties of crystalline 2-phospho-D-glycerate hydro-lyase from human muscle', *Eur. J. Biochem.*, **5**, 119–123.

Beutler, E. (1971), 'Red cell metabolism', in *A Manual of Biochemical Methods*, p. 56. New York and London: Grune & Stratton.

Bszormenyi-Nagy, I. (1955), 'Formation of phosphopyruvate from phosphoglycerate in hemolyzed human erythrocytes', *J. Biol. Chem.*, **212**, 495–499.

Cohn, M. (1963), 'Magnetic resonance studies of metal activation of enzymic reactions of nucleotides and other phosphate substrates', *Biochemistry*, **2**, 623–629.

Feig, S. A., Shohet, S. B., & Nathan, D. G. (1971), 'Energy metabolism in human erythrocytes. I. Effects of sodium fluoride', *J. Clin. Invest.*, **50**, 1731–1737.

Fellenberg, R. von, Richterich, R., & Aebi, H. (1963), 'Electrophoretisch verschiedenen wandernde Pyruvat-Kinasen aus einigen Organen der Ratte', *Enzymol. biol. clin.*, **3**, 240–250.

Jardillier, J. C., & Desmet, G. (1973), 'Etude du fluor sérique et de ses combinaisons par une technique utilisant une électrode spécifique', *Clinica Chim. Acta*, **47**, 357–363.

Lohman, K., & Meyerhof, O. (1934), 'Ueber die enzymatische Umwandlung von Phosphoglycerinsäure in Brenztraubensäure und Phosphorsäure', *Biochem. Z.*, **273**, 60–72.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951), 'Protein measurements with the Folin phenol reagent', *J. Biol. Chem.*, **193**, 265–275.

Oh, S. K., & Brewer, J. M. (1973), 'Purification and properties of enolase from swine kidney', *Archs. Biochem. Biophys.*, **157**, 491–499.

Stefanini, M. (1972), 'Chronic hemolytic anaemia associated with erythrocyte enolase deficiency exacerbated by ingestion of nitrofurantoin', *Am. J. Clin. Path.*, **58**, 408–414.

WARBURG, O., & CHRISTIAN, W. (1941), 'Isolierung und Kristallisation des Gärungsferments Enolase', *Biochem. Z.*, **310,** 384–421.

WIEME, R. J. (1965), *Agar gel electrophoresis.* Amsterdam: Elsevier.

WITT, I., & WITZ, D. (1970), 'Reinigung und Charakterisierung von Phosphopyruvat-Hydratase (= Enolase; EC 4.2.1.11) aus Neugeborenen-und Erwachsenen-Erythozyten', *Hoppe-Seyler's Z. Physiol. Chem.*, **351,** 1232–1240.

WOLD, F. (1971), in *The Enzymes*, (ed. Boyer), Vol. V, 3rd ed., pp. 499–538. New York: Academic Press.

ZONDAG, H. A. (1964), Thesis, Groningen, The Netherlands.

Key Word Index: Enolase, erythrocytes, fluoride.