Résumé

On a étudié l'effet de divers fixateurs histologiques sur l'activité de la ribonucléase et discuté la signification des résultats pour le travail cytologique.

The Effect of Phosphate on Cream Xanthine Oxidase

Metalloflavo-protein enzymes can be activated under certain conditions by orthophosphate ions. This fact, and the suggested interaction of phosphate and these enzymes[1] prompted an investigation of the effect of phosphate on cream xanthine oxidase.

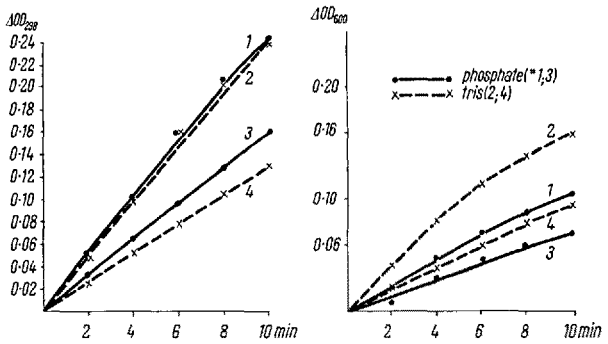

Fig. 1.—The effect of heating and of phosphate on cream xanthine oxidase. 0·1 μM xanthine, 0·3 ml xanthine oxidase (1:100 in Tris), (at right) 0·1 ml of 2,6-dichlorophenol indophenol (30 mg/100 ml). Completed to 3·0 ml with 0·1 M phosphate, pH 7·8; or 0·05 M Tris, pH 7·8 (as indicated). Temperature 24°C. Curves # 1 and 2: freshly diluted enzyme; curves # 3 and 4: heated 6·5 h at 37°C.

Cream xanthine oxidase (Nutritional Biochemical Co., lot # 9493) was kept frozen, and was diluted for use 1:100 with Tris (Sigma) buffer, pH 7·8, 0·05 M. All other reagents were dissolved in the same buffer or in water. Xanthine oxidase activity was assayed spectrophotometrically in the Beckmann DU spectrophotometer. Oxygen reduction, as measured by uric acid formation, was determined by recording the changes of optical density at 298 mμ, the reduction of dye (2,6-dichlorophenol-indophenol; Fisher) was determined at 600 mμ, xanthine being used as substrate in both cases. All assays were carried out in (a) 0·1 M orthophosphate, pH 7·8, or (b) 0·05 M Tris, pH 7·8.

When freshly diluted enzyme was used, no difference was observed in oxygen reduction, between reactions carried out in phosphate or Tris buffers. However, when the reaction was run in phosphate, the reduction of dye was inhibited about one third in relation to the reaction rate observed with Tris buffer (Fig. 1). When the diluted enzyme (working solution) was aged by standing in the freezer, enzymic activity decreased, both for the reduction of oxygen as well as of dye. Under these conditions, phosphate caused a partial restitution of activity, when oxygen was used as electron acceptor; on the other hand the inhibition of dye reduction disappeared, phosphate causing even some stimulation of the activity of the aged enzyme in some experiments.

The inactivation of xanthine oxidase could also be produced by heating the working solution in water bath at 37°C. Incubation at 37°C for 6·5 h in the Dubnoff shaker caused no turbidity, nor changes in optical density at 260 or 280 mμ. Heating the enzyme in Tris buffer caused greater loss of activity than heating the enzyme dissolved in phosphate. The activity of xanthine oxidase was reduced to the same degree with both oxygen as well as dye as electron acceptor. The longer the enzyme was heated, the greater was the stimulation of xanthine oxydase activity caused by phosphate, when oxygen was used as electron acceptor; on the other hand, with dye as electron acceptor, the inhibition caused by phosphate decreased with progressive heating. The effect of phosphate on enzymic reduction of oxygen varied with the experimental conditions: Phosphate caused a partial reactivation when the enzyme was heated for 6·5 h at 37°C, but no reactivation could be observed when the enzyme was heated for 10 min at 55°C.

It then was of interest to verify, whether phosphate could not only restore the activity of xanthine oxidase, but could also protect the enzyme against inactivation due to aging. For this purpose, xanthine oxidase was diluted 1:100 with (a) Tris buffer, pH 7·8, 0·05 M, or (b) 0·1 M phosphate, pH 7·8. Both solutions were kept at + 4°C for 14 days, and then tested for reduction of dye and of oxygen, in both buffers (Fig. 2). The results parallel those reported above. The enzyme was inactivated to a lesser degree when dissolved in phosphate than when dissolved in Tris, if oxygen was used as electron acceptor. The aged enzyme was stimulated when the assay was carried out in phosphate. With dye as electron acceptor, the inhibition caused by phosphate disappeared on aging, and the presence or absence of phosphate during assay had little effect.

It is known that folic acid and other pteridines are potent inhibitors of xanthine oxidase[1]. The inhibition caused by folic acid (Folvite, Lederle) is much less when dyes are used as electron acceptor, than when oxygen is used as electron acceptor[2]. It was found (Table), that the degree of inhibition caused by folic acid was inversely related to the enzymic activity observed in absence of inhibitor. Phosphate afforded a partial protection against the inhibitory effect of folic acid.

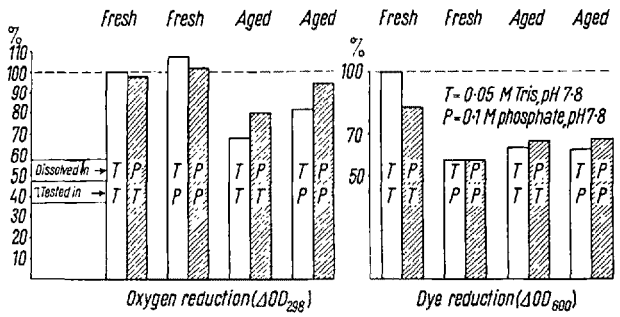

Fig. 2.—The effect of phosphate and of aging on cream xanthine oxidase. Assay conditions as in Figure 1. The diluted enzyme had been kept in 0·05 M Tris, pH 7·8 (T), or in 0·1 M phosphate, pH 7·8 (P), for 14 days at + 4°C.

Other buffers tested (in absence of folic acid) had the same effect on cream xanthine oxidase as Tris buffer. When cream xanthine oxidase was stored for several months, even when kept frozen, the described effects of phosphate could no longer be observed with freshly diluted working solution. This probably indicates that after prolonged storage, the enzyme is irreversibly altered, so that phosphate causes always stimulation with oxygen as electron acceptor, and has no effect when dye is used as electron acceptor.

[1] H. R. Mahler, Adv. Enzymol. *17*, 233 (1956).
[2] R. Fried, in manuscript.

The effect of phosphate and folic acid on cream xanthine oxidase. 0·1 μM xanthine, 0·005 μM folic acid (FA), xanthine oxidase (Diluted 1:100 in Tris), as indicated. For dye reduction, 0·1 ml of 2,6-Dichlorophenol indophenol (30 mg/100 ml). Completed to 3·0 ml with 0·05 M Tris, pH 7·8, or with 0·1 M phosphate, pH 7·8. Values correspond to the change in optical density ($\times 1000$) at 298 mμ—in assays # 6 and 7 change in optical density at 600 mμ—for the first 10 min after the addition of substrate. All reactants, except xanthine, were preincubated for 5 min at 24°C. Assay temperature: 24°C. All values are averages of three determinations.

Assay #	Enzyme	Tris		Phosphate	
		−FA	+FA	−FA	+FA
1	0·5 ml fresh	258	154	258	191
2	0·5 ml aged	203	86	244	139
3	0·5 ml aged	168	44	222	128
4	0·3 ml fresh	207	106	237	126
5	0·3 ml fresh	160	79	190	99
6	0·5 ml fresh	201	175	140	126
7	0·3 ml fresh	150	129	103	94

Several other instances of the effect of orthophosphate on metalloflavo-proteins have been reported. MACKLER *et al.*[3] stated phosphate to be required for reduction of one-electron acceptors by cream xanthine oxidase. DPNH-peroxidase is inhibited by phosphate[4]; this is also true for DPNH-cytochrome c reductase[5]. MAHLER *et al.*[5] attribute this inhibition to a 'coordination of phosphate with the iron of the enzyme during the catalytical process'.

The present data indicate that the processes of loss of enzymic activity due to aging or mild heating, the inhibition by folic acid, and the effect of orthophosphate, are all concerned with the same site of cream xanthine oxidase; it is likely, that the site affected is the molydenum or iron, or both metals. The data indicate further, that the level of orthophosphate determines, which pathway of electron transport is favored for xanthine oxidase activity in a given system. Phosphate shunts enzymic activity away from the 'dye pathway' in fresh enzyme. The aging process can be interpreted as a gradual removal of endogenous phosphate from an active site, an effect which can be partially reversed by the addition of orthophosphate.

The author gratefully acknowledges the interest and helpful discussions of Dr. C. A. COLWELL and Dr. J. OTT. This investigation was supported by a research grant (# E–915) from the National Institutes of Health, U. S. Public Health Service, Department of Health, Education and Welfare, to Dr. COLWELL.

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Research Division, Veterans Administration Hospital, Hines (Illinois), and Department of Biochemistry, Northwestern University Medical School, Chicago (Illinois), January 9, 1958.

Zusammenfassung

Die Einwirkung von Orthophosphat auf die Milch-Xanthinoxydase wurde unter verschiedenen experimentellen Bedingungen untersucht. Phosphat hemmt einerseits die Übertragung des mobilisierten Wasserstoffes auf 2,6-Dichlorophenol-indophenol mit frischen Enzym-Präparaten und aktiviert andrerseits die Akzeptorfunktion des Sauerstoffes bei gealterten, oder anderweitig inaktivierten Präparaten.

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[4] M. DOLIN, J. biol. Chem. *225*, 557 (1957).

[5] H. R. MAHLER, A. S. FAIRHURST, and B. MACKLER, J. Amer. chem. Soc. *77*, 1514 (1955).

Heme-Heme Interaction in the Oxygen Equilibrium of Reconstituted Hemoglobins[1]

Object of this note is to demonstrate effective heme-heme interaction in the O_2 equilibrium of reconstituted hemoglobin (Hb) and to investigate the role played in the interaction by the vinyl side chains of the porphyrin.

Apohemoglobin was prepared by acid acetone splitting of human Hb, as reported elsewhere[2]. Protohemin IX was furnished by BDH; crystalline deuterohemin IX and mesohemin IX were prepared according to FISCHER[3]. The recombination of hemes with the globin and other physicochemical properties of reconstituted Hbs will be described elsewhere. The O_2 dissociation curves were determined by the spectrophotometric method developed by us[4]. The O_2 capacity of reconstituted ferro-Hbs is the same as that of native Hb.

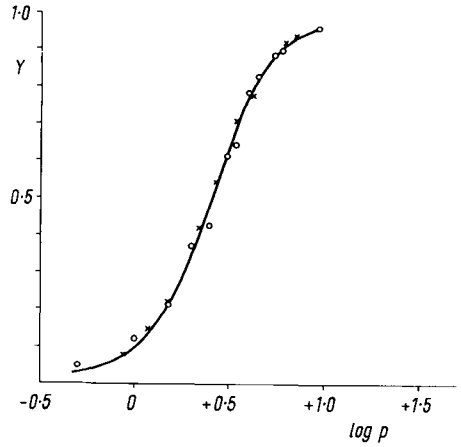

Fig. 1.—Oxygen equilibrium of reconstituted = o and native = × protoHb (2×10^{-4} M as Fe). 20°C; Tris (trishydroxymethyl-aminomethane) buffer 0·1 M, pH 7·4. Y = fractional saturation with oxygen. log p = log oxygen pressure (at 20°) in mm Hg.

Reconstituted protoHb. It can be seen from Figure 1 that the O_2 dissociation curves of native and reconstituted Hb are almost identical to each other with n = 2·6–2·7 (in the Hill empirical equation).

In the plot of Y versus log p the curve obtained for the two proteins appears to be symmetrical. In reconstituted Hb (as in native Hb) there is a slight decrease of interaction in the absence of salts (in phosphate buffer 0·016 M, pH 7·6 n = 2·2). For both proteins we found with urea and para-chloromercurybenzoate respectively n = 1·7 and 1·5.

Reconstituted deuteroHb. The O_2 dissociation curve of deuteroHb (Fig. 2) is still sigmoid in the shape but it is much less inflected than that of protoHb, with n = 1·7 (instead of 2·7 as in reconstituted protoHb). In different conditions of temperature, pH and concentration of the pigment, the value of n was found ranging between 1·5–1·9 (most frequent value 1·7). The salt effect on the shape of the curve is analogue to that observed for protoHb (in phosphate buffer 0·016 M pH 7·6 n=1·4). The

[1] Aided by a grant of the Rockefeller Foundation.

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