It is not oxidized by mammal cytochrome oxidase preparation[5] as demonstrated by manometric and spectrophotometric experiments.

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Riassunto

Una emoproteina è stata estratta e parzialmente purificata dall'epatopancreas di *Sepia officinalis*. Essa è ossidata dal ferricianuro e ridotta dall'acido ascorbico; gli spettri di assorbimento delle due forme, ossidata e ridotta, sono quelli tipici del citocromo c di mammifero.

In comune con quest'ultimo il citocromo estratto dall'epatopancreas di sepia ha diverse altre proprietà come per esempio quella di non dare un emocromogeno con la piridina e di non perdere il gruppo prostetico dopo trattamento con acetone acido. A differenza del citocromo c di mammifero, il pigmento di sepia non resiste al calore e agli acidi; è precipitato dal solfato di ammonio al 75% di saturazione e sedimenta dopo prolungata centrifugazione a 105000 × *g*. Non è ossidato dalla citocromossidasi di mammifero.

[5] E. F. HARTREE, in K. PEACH and M. V. TRACEY, Modern Methods of Plant Analysis *4*, 197 (1955).

The Effect of some Common Fixatives on the Enzymatic Activity of Ribonuclease

While studying the action of ribonuclease on living oocytes of starfish, it was found that after freeze substitution basophily disappeared from the cytoplasm even though the oocytes were treated with ribonuclease only for 5 min. On the other hand, there was no loss in basophily when they were fixed in Zenker. These results are similar to what BRACHET[1] observed in the case of onion root tips. In later experiments, it was observed that the enzymatic activity of ribonuclease was not destroyed by freeze substitution. (A detailed account of this work will be published later.)

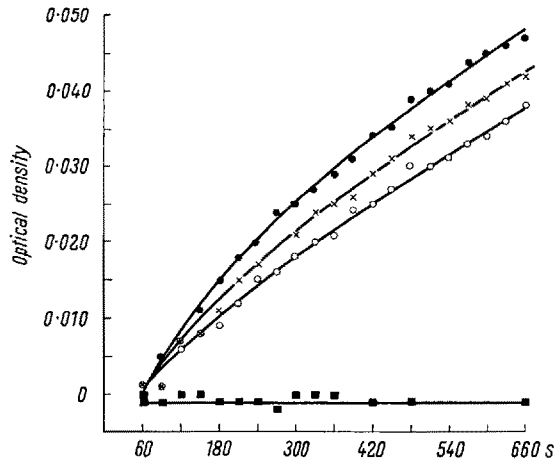
GHOSH[2] has reported that after freezing and thawing there is no loss in activity of DNase, as determined by its capacity to reduce Feulgen stainability. LAGERSTEDT[3] has suggested that RNase activity might be present in sections of pancrease fixed in Carnoy's fluid, and, after the addition of McIlvaine's Buffer it may initiate RNA digestion.

When this paper was under preparation, JONSSON and LAGERSTEDT[4] reported that RNase activity was retained in the sections of pancreas fixed in Carnoy, while measurable RNase activity could not be demonstrated in the formaldehyde fixed material.

The present work was undertaken with a view to ascertaining the effects of some common fixatives like alcohol–acetic acid (3:1), Serra and Zenker.

Methods.—Four tubes were taken and into each 2 ml of RNase (Armour Crystalline, 2 mg/ml) were pipetted. To the first tube was added 1 ml of alcohol-acetic acid, to the second 1 ml of Serra, to the third 1 ml of Zenker and to the fourth 1 ml of distilled water. After thorough mixing, the respective mixtures were allowed to stand for 1 h at room temperature (22°C). The mixtures were then dialysed in separate cellophane bags against distilled water for 24 h. When Zenker was added to the RNase solution, the latter became cloudy, probably due to the precipitation of RNase by the mercuric salts present in the fixative.

After the dialysis, the contents of the bags were centrifuged, and the supernatant in each was adjusted to equal volumes with distilled water. The enzymatic activity of these solutions was determined according to the method of KUNITZ[5].



Enzymatic activity of the solutions after dialysis.
• Control × Alcohol-acetic acid ○ Serra ■ Zenker

It is evident from the results obtained (Figure) that only the solution which was mixed with Zenker lost all its enzymatic activity, while the rest retained almost all of it. The RNase activity per minute calculated for each of these solutions is of the value of 0·006 for the control, 0·005 for the solution mixed with alcohol-acetic acid and 0·004 for the one mixed with Serra.

According to JONSSON and LAGERSTEDT, formaldehyde-fixation destroys the enzymatic activity of RNase. The results obtained by me show that the fixative, Serra, though it contains formalin, does not inactivate RNase. This may be due to the fact that the inactivation of RNase by formalin is reversible on dialysis.

These results indicate that RNase does not lose its enzymatic property after fixation in certain fixatives, and when these fixatives are completely removed during the process of washing and dehydration, the enzyme can digest the RNA of the sections after hydration of them.

Acknowledgment.—I wish to express my grateful thanks to Prof. J. BRACHET for suggesting the lines of investigation, his unfailing guidance and constant help, and also for the facilities afforded to me in his laboratory. My thanks are also due to Mme. Y. THOMAS for her generous help.

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Laboratoire de Morphologie animale, Université libre de Bruxelles (Belgique), le 28 novembre 1957.

[1] J. BRACHET, Biochem. biophys. Acta *19*, 583 (1956).
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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.

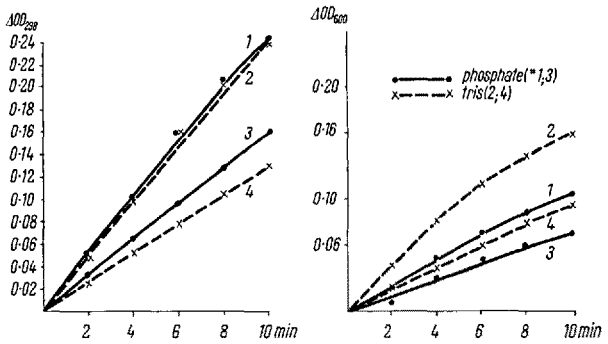

Fig. 1.—The effect of heating and of phosphate on cream xanthine oxidase. 0·1 μM xanthine, 0·3 ml xanthine oxidase (1:100 in Tris), (at right) 0·1 ml of 2,6-dichlorophenol indophenol (30 mg/100 ml). Completed to 3·0 ml with 0·1 M phosphate, pH 7·8; or 0·05 M Tris, pH 7·8 (as indicated). Temperature 24°C.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

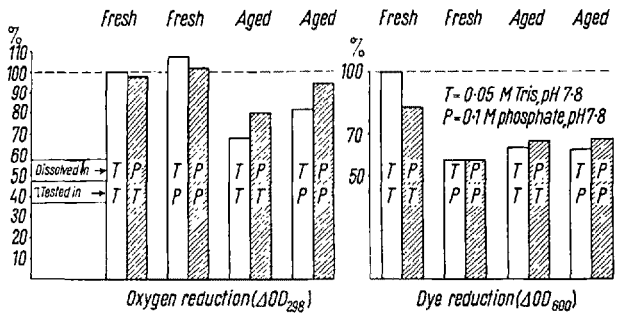

Fig. 2.—The effect of phosphate and of aging on cream xanthine oxidase. Assay conditions as in Figure 1. The diluted enzyme had been kept in 0·05 M Tris, pH 7·8 (T), or in 0·1 M phosphate, pH 7·8 (P), for 14 days at + 4°C.

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.

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[2] R. Fried, in manuscript.