specific biological activity when isolated from different species may differ chemically. The haemoglobins are an example of this (Porter & Sanger, 1948), and it is clear that some variation in structure may occur in such a series of compounds without the specific action being greatly modified. From solubility studies, Desreux & Herriott (1939) postulated the existence of at least two components in swine pepsin, both of which possess enzymic activity. It is probable that the duality of ox ribonuclease is a similar phenomenon. As a similar composition was found for a number of preparations made from small numbers of pancreases obtained over 12 months from different slaughter houses, it would seem that the two forms exist together rather than that individual animals have wholly one or the other type. The structural differences which alter the chromatographic behaviour have not yet been investigated and may well be subtle enough to escape detection by the technique at present available.

From the aspect of protein structure these results would appear to give support to the idea of the existence of families of nearly identical proteins (cf. Synge, 1950).

SUMMARY

1. A partition chromatogram using the two liquid phases produced by mixtures of ammonium sulphate, ethylene glycol monoethyl ether ('cellosolve') and water has been devised with kieselguhr used as the inert support.

2. On such chromatograms crystalline ribonuclease gives without difficulty zones having convenient rates and symmetrical form. It was found to contain two enzymically active components in the approximate ratio 10:1.

3. Evidence is presented which suggests that both forms pre-exist in ox pancreas.

We wish to thank Mrs A. Dovey for her electrophoretic examination of ribonuclease.

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Inhibition of Alkaline Phosphatase by Oestradiol Phosphates

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In a preliminary report Aldman, Diczfalusy & Rosenberg (1948) have shown that kidney alkaline phosphatase is inhibited by some phosphoric acid esters of the oestrogenic steroids. In this paper more detailed data are presented as to the specificity, reversibility and mode of inhibition.

MATERIALS AND METHODS

Enzyme. The enzyme was prepared, as previously, from the kidneys of young rabbits, using with slight modifications the method described by van Thoai, Roche & Sartori (1944). In the course of repeated fractionations it was found that the enzymic activity of the crystalline material decreased simultaneously with increasing activity of the mother liquor, confirming thus the results of Abul-Fadl, King, Roche & van Thoai (1949), according to which the crystals consist of inorganic material with enzyme adsorbed on it. In some

experiments electrophoretically purified enzyme has been used.

Substrates. The following substrates have been used: phenyl phosphate, β -glycerophosphate and glucose-6-phosphate (the last-named compound was kindly supplied by Dr O. Lindberg, Wenner-Gren Institute, Stockholm).

Analytical methods. Phenol was determined with Folin's reagent as described by Buch & Buch (1939), inorganic phosphate according to Lowry & Lopez (1946), and in some instances with a modified Martin & Doty (1949) method. The measurements were carried out in a Beckman spectrophotometer.

Conditions of enzyme experiments. Enzyme activity determinations were carried out within the pH range 7.0–11.0, using a 0.04M-borate-carbonate-acetate buffer. Reaction volume, generally 2.5 or 5.0 ml.; incubation time, 10 or 16 min. at 37°. Enzyme concentration, 1.0 μ g. of protein/ml., or less.

Inhibitors. The following substances were examined as to their inhibitory action: oestradiol 3-phosphate, oestradiol 17-phosphate, oestradiol 3:17-diphosphate and oestrone glucoside. Oestradiol 3-phosphate and oestradiol 17-phosphate were synthesized according to Miescher & Meystre (1946), oestradiol 3:17-diphosphate as described by Claesson, Högberg & Rosenberg (1949). Oestrone glucoside was synthesized by deacetylation of oestrone tetra-acetyl glucoside according to Kongsted (1939).

clear at present. In electrophoretic studies, using acetate buffer at various pH values between 3.5 and 5.9 (ionic strength 0.1), it was revealed that the highly purified preparation showed the presence of only two components, one of which could be identified as the active principle. The partially purified preparation, examined at the same ionic strength and pH, contained at least four components (see Fig. 2).

Fig. 1. Relationship between pH and phosphatase activity at different stages of purification. *A*, partially purified enzyme (identical with the initial product of van Thoai *et al.* 1944); *B*, highly purified enzyme (obtained after six reprecipitations by 50% (v/v) acetone). Activity is expressed as a percentage of the activity at the pH optima. Substrate, 0.005M-phenyl phosphate. Acetate-carbonate-borate buffer, 0.04M 16 min.; incubation at 37°.

RESULTS

The relationship between enzymic activity and pH is influenced by impurities as shown in Fig. 1, where the activity of a preparation at two different degrees of purity is plotted against pH. The activity is expressed as a percentage of the activity at the pH optima.

Fig. 2. Electrophoretic patterns of partially purified (*A*) and highly purified (*B*) kidney alkaline phosphatase. Acetate buffer, pH 5.9, ionic strength 0.1 at 0.5°. (*A*), 2.5 hr. electrolysis at 7.7 V./cm., 1.2% solution. (*B*), 3.5 hr. electrolysis at 7.9 V./cm., 0.5% solution.

It is of interest that preparation *B* has about 25% of the maximal activity even at pH 7.5. The less pronounced second peak at pH 7.5, shown in curve *B*, was invariably observed in experiments with highly purified preparations and phenyl phosphate as a substrate. The significance of this second peak is not

From electrophoretic mobilities at different pH values the isoelectric point of kidney alkaline phosphatase was estimated to be approximately pH 4.8–4.9, as shown in Fig. 3.

Fig. 3. Electrophoretic mobility of kidney alkaline phosphatase as a function of pH. Acetate buffer, ionic strength 0.1, temp. 0.5°. Highly purified enzyme, 0.35–0.5% solutions.

Inhibitors. The relative inhibitory powers of the various compounds at pH 9.3 are compared in Table 1. In the concentrations used monophosphates inhibit phosphatase only slightly. Among the different substances tested oestradiol diphos-

Table 1. *Inhibition of kidney alkaline phosphatase by some derivatives of the oestrogenic hormones*

(Acetate-carbonate-borate buffer, pH 9.3, 16 min. incubation at 37°. Substrate: 0.004M-phenyl phosphate. Enzyme-protein concentration, 0.81 $\mu\text{g./ml.}$)

Inhibitor	Concn. (M)	Phosphatase activity ($\mu\text{g. phenol liberated}$)		Inhibition (%)
		Individual exp.	Mean	
Nil	—	113	113	—
Nil		113		
Nil		114		
Oestradiol diphosphate	10^{-5}	70	70	38
Oestradiol diphosphate		69.5		
Oestradiol 3-phosphate	2×10^{-4}	107	107	5
Oestradiol 3-phosphate		108		
Oestradiol 17-phosphate	10^{-4}	92	91	19
Oestradiol 17-phosphate		90		
Oestrone glucoside	2×10^{-4}	114	115	0
Oestrone glucoside		117		

phate is the most powerful inhibitor. The same results are obtained in the presence of magnesium or manganese ions, which in a concentration of 10^{-3}M do not interfere with the inhibition by oestradiol diphosphate.

Fig. 4. Relationship between pH and inhibition of phosphatase by 10^{-5}M -oestradiol diphosphate. Acetate-carbonate-borate buffer, 37°, 16 min. incubation. Substrate, 0.004M-phenyl phosphate. Enzyme protein concentration, 0.9 $\mu\text{g./ml.}$ $\times - \times - \times$, without inhibitor; $\bigcirc - - - \bigcirc$, with 10^{-5}M -oestradiol diphosphate.

Type of inhibition. The inhibition of alkaline phosphatase by oestradiol diphosphate, using phenyl phosphate as a substrate, is presented in Fig. 4. The inhibition is limited to the acid side of the pH optimum, the greatest inhibition occurring at pH 7.5–9.0. An inhibition of kidney alkaline phos-

phatase of this type by various ions has been described by Aebi & Abelin (1948).

The inhibition is independent of the nature of the substrate used; with phenyl phosphate, β -glycero-

Fig. 5. Relationship between pH and inhibition of the enzymic hydrolysis of β -glycerophosphate by oestradiol diphosphate. Acetate-carbonate-borate buffer, 37°, 16 min. incubation. Substrate concentration, 0.005M. Enzyme-protein concentration, 1.2 $\mu\text{g./ml.}$ $\times - \times - \times$, without inhibitor; $\bigcirc - - - \bigcirc$, with $2 \times 10^{-5}\text{M}$ -oestradiol diphosphate.

phosphate and glucose-6-phosphate, essentially the same results are obtained. In Fig. 5 the inhibition by oestradiol diphosphate of the enzymic hydrolysis of β -glycerophosphate by alkaline phosphatase is presented.

To test whether the inhibition was competitive or non-competitive, the concentrations of the substrate (phenyl phosphate) and inhibitor (oestradiol diphosphate) were varied at constant concentration of the enzyme. In Fig. 6 the reciprocal of the rate of

Fig. 6. Relation between enzyme activity and substrate concentration with different concentrations of oestradiol diphosphate. Activity is expressed as the reciprocal of the amount of P liberated in 10 min. at 37°. Enzyme-protein concentration: 0.81 $\mu\text{g./ml.}$ Substrate: phenyl phosphate. Acetate-carbonate-borate buffer, pH 8.5. x—x—x, without inhibitor; O—O—O, 10^{-6}M -oestradiol diphosphate; Δ — Δ — Δ , $2 \times 10^{-6}\text{M}$ -oestradiol diphosphate.

Fig. 7. Relation between enzyme activity and inhibitor concentration $[I]$ at pH 8.5. Substrate: phenyl phosphate, acetate-carbonate-borate buffer, inhibitor: oestradiol diphosphate. 10 min. incubation at 37°. Enzyme activity expressed as the reciprocal of the rate of hydrolysis extrapolated for $[S] = \infty$. Enzyme-protein concentration: 0.81 $\mu\text{g./ml.}$

hydrolysis (v , expressed as $\mu\text{g. P}$ liberated after 10 min. incubation at 37°) is plotted as ordinate against the reciprocal of the substrate concentration (S) following the procedure of Lineweaver & Burk (1934). It appears that the inhibition is not substrate-competitive, in contrast to what might be expected, since the inhibitor itself is a phosphoric acid ester. The dissociation constant, K_s , of the enzyme-substrate complex is 6×10^{-3} .

In Fig. 7 the reciprocal values of the rate of hydrolysis extrapolated for $[S] = \infty$ are plotted against the concentration of the inhibitor. It appears that the relationship is linear.

The curves in Figs. 6 and 7 are consistent with the equation

$$\frac{1}{v} = \frac{1}{v_{\infty(0)}} \left[1 + \frac{K_s}{[S]} + \frac{[I]}{K_I} \right],$$

where $[I]$ is the concentration of the inhibitor, $v_{\infty(0)}$ is the rate of hydrolysis extrapolated for $[S] = \infty$ and $[I] = 0$, and K_I the dissociation constant of the complex of enzyme, substrate and inhibitor (ESI) according to the reaction $ESI \rightleftharpoons ES + I$.

$$K_I = 1.4 \times 10^{-6}.$$

Reversibility of inhibition. The inhibition of the enzyme by oestradiol 3:17-diphosphate could be quantitatively reversed by small amounts of basic proteins, such as protamine sulphate (0.5 g./l.). These substances do not activate the alkaline phosphatase preparations used.

DISCUSSION

Since the inhibition of phosphatase by oestradiol diphosphate is non-competitive, it appears that the attachment between enzyme and inhibitor is not at the same place as the contact between enzyme and substrate. The reversibility, together with the non-competitive type of inhibition, may suggest a displacement of a possible coenzyme, suggested by others (Albers, Beyer, Bohnenkamp & Müller, 1938; Kutscher & Wüst, 1942; Abul-Fadl & King, 1949), though the evidence for the existence of such a coenzyme is doubtful (Schales & Mann, 1948). The most recent evidence of Kutscher & Sieg (1950) suggests that the prosthetic group of alkaline phosphatase is probably choline pyrophosphate. The complex of enzyme, substrate and inhibitor described in this paper may be analogous to the enzymically inactive complex of the enzyme with two molecules of substrate, described and discussed by others (Folley & Kay, 1935; Aebi, 1949), but the affinity of the enzyme-substrate complex for another substrate molecule is much less than the affinity of the complex for a molecule of the inhibitor (oestradiol diphosphate) as it appears from their respective dissociation constants. The corresponding standard free energies (37°) calculated from the dissociation constants are -430 cal. for the reaction $ES + S \rightarrow ES_2$, and -8400 cal. for the reaction $ES + I \rightarrow ESI$.

SUMMARY

1. Purified kidney alkaline phosphatase is partly inhibited by oestradiol 3:17-diphosphate in concentrations lower than 10^{-5}M . This inhibition is not

appreciably affected by the presence of magnesium or manganese ions.

2. The monophosphates of oestradiol are much weaker inhibitors than oestradiol 3:17-diphosphate, while oestrone glucoside does not exert any inhibitory effect in the concentrations investigated.

3. The inhibitory effect of oestradiol 3:17-diphosphate on alkaline phosphatases is greatest in

the region of pH 7.5-9.3, and is not demonstrable on the alkaline side of the pH optimum.

4. The inhibition is independent of the nature of the substrate; using phenyl phosphate, β -glycerophosphate, or glucose-6-phosphate, essentially the same degree of inhibition is observed.

5. The inhibition of alkaline phosphatase by oestradiol 3:17-diphosphate is reversible and non-competitive.

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The Fate of Radioactive Potassium Chloride Administered Intraperitoneally or Intravenously to Rats

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It is known that when potassium salts are administered intravenously to animals the small increase found in the concentration of potassium in the serum can only be explained by assuming that the injected potassium is distributed in a volume of body water greater than that of the extracellular fluid. At least some of the cells of the body, therefore, are readily permeable to potassium. Hahn, Hevesy & Rebbe (1939), Joseph, Cohn & Greenberg (1939), Noonan, Fenn & Haege (1941), Fenn, Noonan, Mullins & Haege (1941) and Hevesy (1942) have studied the distribution of injected radioactive potassium salts. Noonan *et al.* (1941) administered radioactive potassium chloride intraperitoneally to rats and found that equilibrium between the extracellular and intracellular potassium of liver, heart, lung, kidney, gut and skeletal muscle was reached in 4-6 hr. The doses of potassium chloride varied from 6 to 59 mg. (0.3-4.1 m-equiv. potassium/kg.). Within 0.5-1 hr. after the injection, the liver, heart,

lungs, kidneys and gut were so highly radioactive as to suggest that radioactive potassium had, at least, in part entered the cells as a result of a process other than exchange. They remarked that it was not possible, on the basis of their experiments, to separate the two processes.

In the experiments described below, the distribution of large doses of ^{42}KCl after a number of hours and of doses of 0.01 and 1 mg. of ^{42}KCl 5 min. after injection was studied.

METHODS

Female albino rats weighing between 192 and 230 g. were used.

The potassium salts used were irradiated KCl or K_2CO_3 . The latter was neutralized to pH 8 by N-HCl and used in some experiments since the irradiation of KCl in the pile of the Atomic Energy Research Establishment, Harwell, yields ^{42}K and traces of ^{36}S and ^{32}P . We are grateful for information from Harwell that the activity due to NaCl present