with distilled water. The void volume was found to be 20 ml. The total elution volume for each sample was 300 ml. Purified polypeptides and proteins of known molecular weight were filtered under the same conditions in order to determine the elution volume.

Analysis. Total proteins were determined on an aliquot of the fractions by measuring the optical density at 280 nm and by the LOWRY-FOLIN method[7] using crystalline serum albumin as standard. Galactose was measured with the technique of DISCHE[8] using galactose Sigma as standard. Total hexoses were determined by the method of YEMM and WILLIS[9] using glucose as standard. Total uronic acids were measured by the carbazol method of BITTER and MUIR[10] using glucuronic acid as standard.

Results. The Figure shows the elution curves of the urine concentrates corresponding to 20 ml of filtered urine, on Sephadex G-100 and G-25. The line represents the average values of 6 samples, each of them run in duplicate. The void volume of Sephadex G-25, almost neglegible in most cases, was not represented. The column was eluted with distilled water. Pigments were normally reversibly adsorbed on the columns and appeared in the eluate as shown in the Figure.

In all experiments, authrone analyses gave 3 distinct peaks on both Sephadex G-100 and G-25. These results were in agreement with those of LUNDBALD et al.[3] Peak A varied in height in the different samples, and peaks B and C, always distinct, were almost constant in height. On Sephadex G-100 the small peaks A′ and B′ were not constant, but peak C′ appears in all samples with similar height. Probably most of the hexoses of these peaks were galactose, as can be seen in the corresponding elution curves.

The galactose curve approximately followed the total hexoses curve, with the exception of peaks C an G-25 and A′ on G-100 that are resolved in about 66% of the analyses in 3 peaks. Peak A on this curve has a MW near 1,500; peak B about 3,000 and peak C about 4,000. Peak A′ on Sephadex G-100 has a molecular weight about 6,000 and peaks B′ and C′ are near 22,000 MW.

The total proteins curve shows some similarity with that of total hexoses, but peaks A and A′ are absent. According with the above results, most of the protein would be galactose containing glycoproteins. This is in agreement with NORDEN's[11] findings on the galactose content of normal urine.

The uronic acid recorded on Sephadex G-25 is eluted in 4 peaks ranging from 3,500 to 4,000 MW. Peaks A and B were almost constant and distinct. Peaks C and D varied in height with the different samples. Microcolumn fractionation[11] and IR-spectra were used in order to identify the uronic acid containing glycosaminoglycans present in the fractions. Chondroitin-4-sulfate and heparitin sulfate were the glycosaminoglycans identified. Also traces of hyaluronic acid and heparin have been found. Probably all peaks represent degradation products of glycosaminoglycan-protein complexes of different molecular weights. Peak D was always eluted with a low galactose peak. The uronic acid recorded in G-100 appears in only one peak (A′) (M W about 25,000) being always eluted with galactose containing glycoproteins[12]. Work is in progress with further separation and closer chemical characterization of various fractions described in this paper.

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Repressible Alkaline Phosphatase in *Aspergillus niger*

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Summary. ALP from *A. niger* is a) P_i repressible enzyme; b) stimulated by addition of Zn^{++} to the growth medium, and c) that EDTA inhibits the enzyme reversibly, which could be restored by addition of Zn^{++} and perhaps Mg^{++}. This property is in contrast to the enzyme from *N. crassa*, which is independent of any metal requirement.

Alkaline phosphatase ALP[2] E.C. 3.1.3.1 occurs in *E. coli*[3,4] and in a number of other organisms[5–7]. The enzyme is non-specific, repressed by P_i, is a metalloprotein and requires Zn^{++} for its maximal activity[8]. ALP from *N. crassa*[5] has been separated into 2 components, one of which is repressed by P_i and the other derepressed. The repressed enzyme does not require any metallic ion for its activity but is stimulated by addition of EDTA. This study relates to a P_i repressible ALP from *A. niger*, which is similar to *E. coli* enzyme in its requirement for Zn^{++}. The enzyme is inhibited by both EDTA and 1:10 phenanthroline. The EDTA inhibition is reversed by Zn^{++}.

Aspergillus niger N.R.R.L. 67 was obtained from Northern Regional Research Laboratory, Peoria, Illinois, USA. The organism was grown on a modified Czapek-Dox medium. The modifications were that $NaNO_3$ was replaced by NH_4NO_3 and K_2HPO_4 by KH_2PO_4; 0.7 m*M* $ZnSO_4 7H_2O$ was added as a supplement to the medium unless otherwise noted, and pH adjusted to 2.2–2.3 with dilute HNO_3. Fungal mats grown under stationary culture at 28 °C for 96 h were washed thrice with ice-cold distilled

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[2] Abbreviations: ALP, alkaline phosphatase; EDTA, ethylene diamine tetra acetic acid disodium salt; *p*-npp, *p*-nitrophenyl phosphate; *p*-np, *p*-nitrophenol; P_i, KH_2PO_4; OD_{410}, optical density at 410 nm; SA, specific activity.
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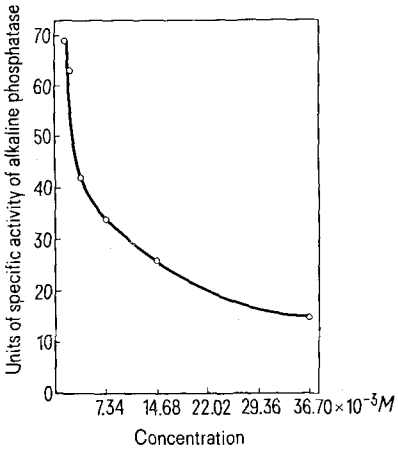

Fig. 1. Effect of varying molar concentrations of KH_2PO_4 on alkaline phosphatase activity.

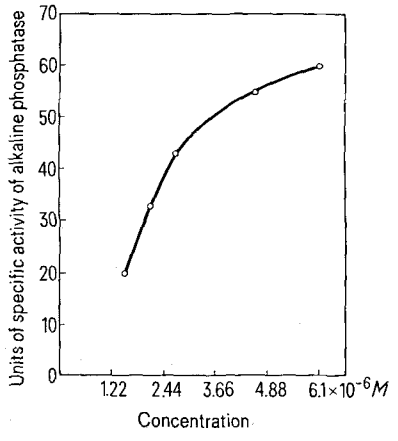

Fig. 2. Effect of varying molar concentrations of Zn^{++} on alkaline phosphatase activity.

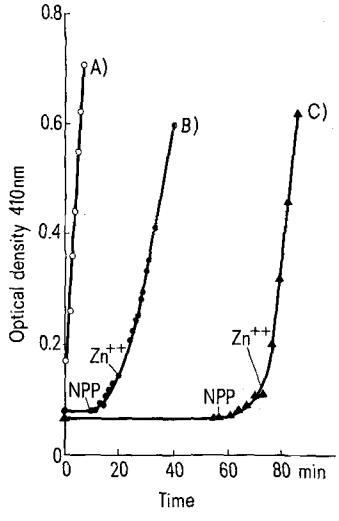

Fig. 3. Inhibition of alkaline phosphatase by EDTA and its reversal by Zn^{++}.

water, macerated in a glass homogenizer and ALP was extracted with 0.01 *M* $MgCl_2$. The enzyme was partially purified by collecting the protein precipitate obtained between 0.5–0.8 saturation of ammonium sulphate. The precipitate was redissolved in 0.01 *M tris*-maleate buffer of pH 7.6 and dialyzed against the same buffer. The 10-fold purified enzyme was employed for EDTA inhibition and its reversal studies.

The enzyme assay reaction mixture contained in a cuvette in 3.0 ml 5 μmoles *p*-npp 1 μmole mercaptoethanol, 100 μmoles *tris*-maleate buffer of pH 7.6 and 100–150 μg protein. Other additions, if any, were added in small volumes and correction made for dilution effects. The OD_{410} arizing from *p*-np[9] liberation at 25 °C during the initial period of enzymic hydrolysis was measured. The increase in OD_{410} against time in min are plotted or SA calculated as μmoles *p*-np hydrolyzed/mg protein/h. Protein was determined colormetrically[10].

ALP activity in fungal mats grown with varying concentrations of P_i is given in Figure 1. The SA of the enzyme decreased as the concentration of P_i was increased. At the concentration of 1.46 m*M* P_i, the SA was 69, which was reduced to 15, (a value less than a $^1/_4$th) when P_i concentration was increased to 36.7 m*M*. In other words, P_i repressed ALP activity. The extent of repression, however, is not as steep as in the case of *E. coli*[4], and the level of enzyme activity remains low and somewhat steady at higher concentration of P_i (between 22.02–36.7 m*M*). This low and constant level of enzyme, which is not markedly affected by P_i, may represent a derepressed enzyme, a distinction which has been made in the case of *N. crassa*[5].

A 3-fold stimulation in enzymic specific activity is obtained when the concentration of Zn^{++} was varied from 1.22 μ*M* to 6.1 μ*M* in the medium (Figure 2). This stimulatory effect is further reflected in the reversal of EDTA inhibition by Zn^{++}.

The rate of enzymic hydrolysis of *p*-npp without EDTA treatment is shown in Figure 3A, and that by enzyme treated with 1.0 μmole and 2.0 μmole EDTA in the lower part of the Figure 3B and 3C. The inhibition by EDTA treatment at the above concentrations is almost complete, between 92–98%. Following the addition of 5 μmoles and 10 μmoles of Zn^{++} to 3B and 3C respectively at the arrow points indicated, there is a reversal of inhibition or increase in OD_{410} due to renewed hydrolysis and release of *p*-np. The extent of reversal is 28% and 52% respectively. Addition of a further 10 μmoles Mg^{++} (not shown in the figure) completely reverses the inhibition.

ALP from yeast[6] which occurs in association with alcohol dehydrogenase is stimulated by Fe^{++} and that from *Anabaena*[7] by Ca^{++}. A number of metallic ions are therefore involved in the function of ALP from different sources. The role suggested for the active metal is in promoting the nucleophilicity of water molecule which attack phosphorus[11]. *A. niger* ALP, which requires Zn^{++} and perhaps Mg^{++} differs from *N. crassa* in its specific metal ion requirement.

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