REFERENCES

1 D. S. GOLDMAN, *Biochim. Biophys. Acta.*, 27 (1958) 506.
2 D. S. GOLDMAN, *Biochim. Biophys. Acta.*, 27 (1958) 513.
3 I. C. GUNSALUS, in W. D. MCELROY AND B. GLASS, eds, *The Mechanism of Enzyme Action*, The Johns Hopkins Press, Baltimore, Md., 1954, pg. 545.
4 S. OCHOA, *Advances in Enzymology*, 15 (1954) 183.
5 L. J. REED, *Advances in Enzymology*, 18 (1957) 319.
6 S. KORKES, A. DEL CAMPILLO, I. C. GUNSALUS AND S. OCHOA, *J. Biol. Chem.*, 193 (1951) 721.
7 L. P. HAGER AND I. C. GUNSALUS, *J. Am. Chem. Soc.*, 75 (1953) 5767.
8 L. J. REED AND B. G. DEBUSK, *J. Am. Chem. Soc.*, 75 (1953) 1261.
9 I. C. GUNSALUS, L. S. BARTON AND W. GRUBER, *J. Am. Chem. Soc.*, 78 (1956) 1763.
10 L. S. BARTON, Thesis, University of Illinois, 1955.
11 F. LIPMANN AND L. C. TUTTLE, *J. Biol. Chem.*, 159 (1945) 21.
12 R. A. PETERS, H. M. SINCLAIR AND R. H. S. THOMPSON, *Biochem. J.*, 40 (1946) 516.
13 I. C. GUNSALUS, *J. Cell. Comp. Physiol.*, 41 (Suppl. 1) (1953) 113.
14 D. S. GOLDMAN, *J. Bacteriol.*, 71 (1956) 732.
15 D. R. SANADI AND R. L. SEARLS, *Biochim. Biophys. Acta*, 24 (1957) 220.
16 K. BURTON AND T. H. WILSON, *Biochem. J.*, 54 (1953) 86.
17 L. J. REED, *Physiol. Rev.*, 33 (1953) 544.
18 B. KE, *Biochim. Biophys. Acta*, 25 (1957) 650.
19 E. CUTULO, *Arch. Biochem. Biophys.*, 64 (1956) 242.
20 R. M. MACLEOD, I. FRIDOVICH AND P. HANDLER, *Arch. Biochem. Biophys.*, 72 (1957) 239.
21 D. S. GOLDMAN, *Biochim. Biophys. Acta*, 28 (1958) 436.
22 E. R. STADTMAN, in W. D. MCELROY AND B. GLASS, *The Mechanism of Enzyme Action*, Johns Hopkins Press, Baltimore, Md., 1954, p. 581.
23 R. O. BRADY AND E. R. STADTMAN, *J. Biol. Chem.*, 211 (1954) 621.
24 I. C. GUNSALUS AND R. A. SMITH, *Intern. Symp. Enzyme Chem.*, Tokyo, 1957, paper 103.
25 M. I. DOLIN AND I. C. GUNSALUS, *Federation Proc.*, 11 (1952) 203.
26 L. J. REED, *International Symposium on Enzyme Chemistry*, Tokyo, Japan, 1957, paper No. 102.
27 L. J. REED, I. C. GUNSALUS, G. H. F. SCHANAKENBERG, Q. F. SOPER, H. E. BOSS, S. F. VERN AND T. V. PARKE, *J. Am. Chem. Soc.*, 75 (1953) 1267.
28 A. F. WAGNER, E. WALTON, G. E. BOXER, M. P. PRUSS, F. W. HOLLY AND K. FOLKERS, *J. Am. Chem. Soc.*, 78 (1956) 5079.
29 D. S. GOLDMAN, unplublished results.
30 R. S. SCHWEET, B. KATCHMAN, R. M. BOCK AND V. JAGANNATHAN, *J. Biol. Chem.*, 196 (1952) 563.
31 R. S. SCHWEET AND K. CHESLOCK, *J. Biol. Chem.*, 199 (1952) 749.
32 V. MASSEY, *Biochim. Biophys. Acta*, 30 (1958) 205.

Fe++-DEPENDENT ALKALINE PHOSPHATASE OF YEAST

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SUMMARY

The common occurence of a ferrous iron-dependent alkaline phosphatase in crystalline preparations of yeast alcohol dehydrogenase is reported. Some of the properties of the enzyme together with a method for separating it from ethanol dehydrogenase are described.

INTRODUCTION

Brewer's yeast contains a specialized phosphatase that cleaves *p*-nitrophenyl phosphate to equimolar amounts of orthophosphate and *p*-nitrophenol. This activity on

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p-nitrophenyl phosphate was first observed in incubation mixtures to which crystalline ADH had been added to generate $DPNH^+$. The source of the phosphatase proved to be the ADH preparation itself. This catalyst was inactivated by heating and required ferrous iron for activity[§]. A survey of several different commercial preparations of crystalline yeast ADH revealed that although phosphatase activity was not always associated with ADH activity, it was exhibited by a large number of the samples tested. Accordingly, attempts were made to find a reliable method for separation of the enzymes and, also, some additional properties of the phosphatase were ascertained.

As shown in Table I, employment of the conventional purification procedure for yeast ADH (1) results in the simultaneous enrichment, to the same extent, of both enzymes. This explains why most crystalline yeast ADH preparations exhibit phosphatase activity.

TABLE I

SIMULTANEOUS PURIFICATION OF AN ALKALINE PHOSPHATASE AND ADH FROM YEAST

Protein	*mg*	*Specific activity*		
		*Phosphatase**	*ADH***	*ADH/phosphatase*
Heated crude extract***	13,800	0.83	9,280	11,200
Dialyzed acetone ppt.	6,000	1.88	15,350	8,170
0.286 sat. $(NH_4)_2SO_4$ supernatant solution	855	5.0	39,300	7,850
First crystalline preparation	312	6.15	58,700	9,550

* The specific activity of the phosphatase is defined as μmoles *p*-nitrophenol liberated/mg protein/h. The reaction mixtures contained TRIS buffer, pH 7.9, 20 μmoles; disodium *p*-nitrophenyl phosphate, 20 μmoles: $FeSO_4$, 5 μmoles; AMP, 5 μmoles and enzyme, 0.5 to 3 units in a total vol. of 0.5 ml. Samples were incubated in an atmosphere of helium or hydrogen in 10 × 75 mm stoppered test tubes for 30 min at 31°. $HClO_4$ [3%, v/v] was added and aliquots of the deproteinized solutions assayed for *p*-nitrophenol[2].

** ADH units are those defined by RACKER except that 0.1 μmole rather than 0.05 μmole DPN/ml was employed in the assay. Specific activity equals units/mg protein.

*** Prepared from 200 g of dried Fleischmann's baker's yeast and fractionated according to the procedure of RACKER[1].

Adsorption steps were tested as a means of separating the two proteins, since in the ordinary procedure for ADH isolation, these techniques are not employed. Calcium phosphate gel[3], DEAE-cellulose[4] and hydroxy apatite[5] were added in varying amounts to enzyme solutions in 10^{-3} *M* K phosphate, pH 6.8. At this pH the hydroxy apatite proved most suitable. Accordingly, a crystalline enzyme preparation containing ADH and phosphatase was diluted with the pH-6.8 buffer and aliquots were treated with increasing amounts of hydroxy apatite. A selective adsorption of ADH was observed and the phosphatase tended to remain in the supernatent solution (Table II). The ADH was quantitatively eluted from the hydroxy apatite with 0.1 *M* K pyrophosphate, pH 8.5. Therefore, use of a gradient-elution system with this adsorbent would undoubtedly yield ADH preparations devoid of phosphatase activity.

The following abbreviations are employed: ADH, alcohol dehydrogenase; $DPNH^+$, reduced diphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; ATP, adenosine-5′-triphosphate; ADP, adenosine-5′-diphosphate; AMP, adenosine-5′-phosphate; TRIS, tris(hydroxymethyl)aminomethane-hydrochloride; IMP, inosine-5′-phosphate; GMP, guanosine-5′-phosphate; DEAE-cellulose, diethylamino ethyl cellulose (Eastman).

§ Initially ferrous iron had been generated by a DPN-linked iron reductase present in one of the bacterial extracts added to the incubation mixtures.

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TABLE II

SEPARATION OF YEAST ALKALINE PHOSPHATASE AND ADH ACTIVITIES

Treatment	*Protein remaining in supernatent solution mg/ml*	*Enzyme remaining in supernatent solution**		
		Phosphatase units/ml	*ADH units/ml*	*ADH/phosphatase*
None**	1.65	6.55	570	87
OH-apatite (A**)	1.07	5.83	370	63.5
OH-apatite (B**)	0.86	5.40	300	55.5
OH-apatite (C**)	0.276	1.96	approx. 20	approx. 10.3

* See footnotes Table I.

** Twice-crystallized ADH (Sigma) was diluted in 10^{-3} *M* K phosphate, pH 6.8, and aliquots were treated with hydroxy-apatite; Sample A, 6.4 mg; Sample B, 8.5 mg and Sample C, 12.7 mg OH-apatite/mg protein.

Aging is probably the simplest procedure for obtaining phosphatase preparations with little or no ADH activity since ADH decays much more rapidly at 0°, especially in dilute solution. Commercial preparations of twice-crystallized yeast ADH exhibited phosphatase activities ranging from 12 to 0 units/mg protein.

Conditions for assay of phosphatase activity on *p*-nitrophenyl phosphate are described in a footnote of Table I. At pH 7.9, the phosphatase is saturated with *p*-nitrophenyl phosphate at a concentration of 0.04 *M*. Under similar conditions, the enzyme is 2 to 3 times more active on phosphoramidate (0.02 *M*) as substrate. However, creatine phosphate, acetyl phosphate, phospho*enol*pyruvate, glucose-1-phosphate, glucose-6-phosphate, β-glycerol phosphate, ATP and AMP are not hydrolyzed. The preparation resembles, somewhat, beef-spleen phosphoramidase[6] but is readily distinguishable from a number of non-specific alkaline phosphomonoesterases[7-9]. The identity of the natural substrate has not been further investigated.

The pH optimum of the phosphatase, with *p*-nitrophenyl phosphate as substrate, is in the range 7.5 to 8.0 (0.04 *M* TRIS buffers). Omission of Fe^{++} from the reaction mixture prevents the hydrolysis of both *p*-nitrophenyl phosphate and phosphoramidate by the purified enzyme. Activity of the phosphatase is completely destroyed by heating at 100° for 10 min.

As would be suspected from their behavior during purification, the two yeast proteins that catalyze ethanol oxidation and *p*-nitrophenyl phosphate cleavage are remarkably similar in a number of their properties. Like yeast ADH, the phosphatase is sensitive to SH-binding agents. The enzyme was found to be inhibited 80% by preincubation for 15 min at 0° with 10^{-4} *M* *p*-chloromercuriphenyl sulfonate and 50% by 10^{-5} *M*. In similar experiments with iodoacetate, 80% inhibition was observed at 10^{-2} *M* and 40% inhibition at 10^{-3} *M* concentrations. Preparations of the phosphatase at all stages of purity after the acetone step are markedly dependent upon the addition of pyrophosphate or various nucleotides for activity on either *p*-nitrophenyl phosphate or phosphoramidate. The activating effects of these substances are even more striking on the crystalline enzyme preparations (Fig. 1) and are similar to those reported by VAN EYS *et al.* for yeast ADH[10]. ATP and ADP stimulate the phosphatase equally as well as does pyrophosphate; DPN (4×10^{-3} *M*) is about 2/3 as effective on a molar basis. In contrast to ADH, a pyrophosphate

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grouping does not appear to be obligatory for activation of the phosphatase, since AMP and to a lesser degree IMP and GMP also serve as activators.

In view of the ease of crystallization of yeast ADH after a relatively small overall purification, it is not surprising that the crystalline material frequently contains more than one enzyme. The experiments reported here illustrate that the conventional steps for ADH preparation can result in the purification to a comparable degree and, presumably, the cocrystallization of a very similar protein possessing phosphatase activity.

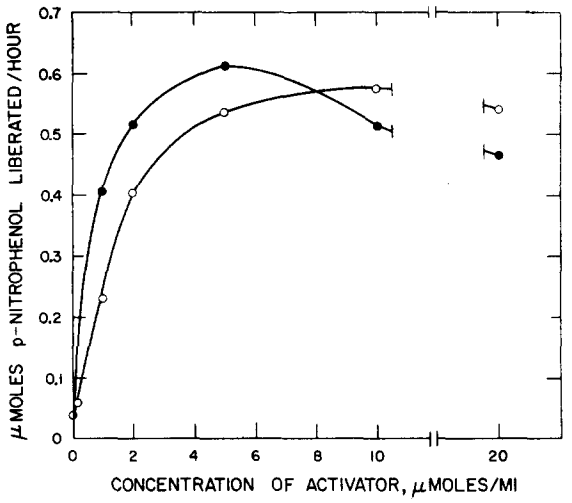

Fig. 1. The effect of increasing concentrations of AMP (open circles) and pyrophosphate (closed circles) on the rate of reaction of yeast alkaline phosphatase. Each sample (0.5 ml) contained 53 μg crystalline yeast ADH (Sigma) and the indicated amount of activator. Other reactants and conditions are listed in Table I.

REFERENCES

1 E. RACKER, in *Methods in Enzymology*, Vol. I, p. 500 (Colowick and Kaplan, eds), Academic Press, Inc., New York, 1955.
2 O. A. BESSEY AND R. H. LOVE, *J. Biol. Chem.*, 196 (1952) 175.
3 M. KUNITZ, *J. Gen. Physiol.*, 35 (1952) 423.
4 A. E. PETERSON AND H. A. SOBER, *J. Am. Chem. Soc.*, 78 (1956) 751.
5 A. TISELIUS, S. HJERTEN AND O. LEVIN, *Archiv. Biochem. Biophys.*, 65 (1956) 132.
6 M. F. SINGER AND J. S. FRUTON, *J. Biol. Chem.*, 229 (1957) 111.
7 L. A. HEPPEL, in *Methods in Enzymology*, Vol. II, p. 530 (COLOWICK AND KAPLAN, eds), Academic Press, Inc., New York, 1955.
8 R. K. MORTON, in *Methods in Enzymology*, Vol. II, p. 533 (COLOWICK AND KAPLAN, eds), Academic Press, Inc., New York, 1955.
9 E. VOLKIN, in *Methods in Enzymology*, Vol. II, p. 539 (COLOWICK AND KAPLAN, eds), Academic Press, Inc., New York, 1955.
10 J. VAN EYS, M. M. CIOTTI AND N. O. KAPLAN, *J. Biol. Chem.*, 231 (1958) 571.