

N, 5.62; CH₃CO, 17.27. Found: C, 57.79; H, 4.71; N, 5.45; CH₃CO, 20.0).

The infrared spectrum of X was consistent with an O,N-diacetyl compound containing an unsaturated lactone structure; the spectrum of XI showed amide, but neither ester nor unsaturated lactone absorption. Hydrolysis of XI in hydrochloric acid-dioxane gave XII. Titration data ($pK_a = 2.9$) and a fluorescein chloride color test⁵ both indicated that XII is an aromatic amine. Acetylation of compounds XI and XII gave X. Thus, no rearrangement during the deacetylation of X had occurred. 2,4-Dihydroxy-3-methylbenzoic acid,⁶ m.p. 220–224°, and 2,4-dihydroxy-3-methylphenylglyoxylic acid, m.p. 139–140°, were formed by alkaline degradation of cyclonovobiocic acid.^{1,3} The glyoxylic acid was identical with a sample prepared by reaction of 2-methylresorcinol with ethyl oxalyl chloride followed by hydrolysis.

The desmethyl analog XIII was synthesized for comparison with X. 4,7-Dihydroxycoumarin⁷ was converted by nitrous acid into the oximino derivative which was hydrogenated. The product was acetylated to XIII, m.p. 256–260°. The infrared absorption spectra of XIII and X were essentially identical between 5 and 6.5 μ .

It has been shown that the sugar portion of the molecule is attached to the coumarin moiety.² The 4-hydroxy-3-(3-methyl-2-butenyl)-benzoic acid^{1,2} may be attached to XII only through an ester or amide linkage. Absence of ester-carbonyl absorption in the infrared spectrum of IX shows that the *p*-hydroxybenzoyl moiety is attached by an amide linkage.

(5) F. Feigl, "Qualitative Analysis by Spot Tests," 3rd ed., Elsevier Publishing Company, Inc., Houston, Texas, 1946, p. 371.

(6) Identical with synthetic sample prepared by procedure of R. C. Shah and M. C. Laiwalla, *J. Chem. Soc.*, 1828 (1938).

(7) A. Sonn, *Ber.*, **50**, 1299 (1917).

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ZINC, A COMPONENT OF RABBIT MUSCLE LACTIC DEHYDROGENASE

Sir:

Zinc has been found as a functional component in the apoenzyme molecules of glutamic¹ and alcohol dehydrogenases.^{2,3,4,5} DPN dependent glutamic dehydrogenation takes an exceptional place in metabolism, among the known pyridine nucleotide dependent reactions, since it dehydrogenates an amino acid with an accompanying

(1) B. L. Vallee, S. J. Adelstein and J. A. Olson, *THIS JOURNAL*, **77**, 5198 (1955).

(2) B. L. Vallee and F. L. Hoch, *ibid.*, **77**, 821 (1955).

(3) B. L. Vallee and F. L. Hoch, *Proc. Natl. Acad. Sci. (U. S.)*, **41**, 327 (1955).

(4) H. Theorell, A. P. Nygaard and R. Bonnichsen, *Acta. Chem. Scand.*, **9**, 1148 (1955).

(5) B. L. Vallee, in "Advances in Protein Chemistry," Vol. 10, p. 317 (1955).

deamination. It seemed important to enlarge upon the implications of the presence of zinc in alcohol dehydrogenases by investigating a second dehydrogenation reaction devoid of concomitant deamination.

Crystalline Lactic Dehydrogenase (LDH) was prepared from rabbit muscle by the method of Beisenherz, *et al.*⁶ Quantitative emission spectrography and microchemical analysis² established the presence of significant quantities of zinc in the crystals of this enzyme. The concentration of all other metals was found to be small and variable as compared to that of zinc in the many preparations examined. Zinc was the only element consistently present in high concentrations, the exact amount being a function of purity of the preparation. Both spectrographic and microchemical data for zinc are shown in Table I. The differences between them are within the limits of the methods when milligram quantities of enzyme are analyzed but their zinc concentration is expressed per gram of protein.

The molecular weight and the DPN-binding characteristics of this enzyme are not on record. The calculation of stoichiometric proportionality of zinc and LDH are contingent upon the isolation of an enzyme, homogeneous by physical-chemical criteria. This has not been achieved for this enzyme as yet, though the quantities of zinc present are significant. The molecular weight of LDH obtained from beef heart muscle is known to be 137,000⁷ and preliminary analytical and kinetic data on this enzyme also indicate that it is a zinc metalloenzyme.⁸

The zinc is thought to be a functional component of LDH of rabbit muscle. In enzymatic preparations of comparable purity, activity and zinc content, both the oxidation of lactate at *pH* 8.8 and the reduction of pyruvate at *pH* 7.0 are inhibited markedly by metal-binding agents such as sodium diethyldithiocarbamate, 1,10-phenanthroline, sodium azide, sulfide, 8-hydroxyquinoline and

TABLE I

EMISSION SPECTROGRAPHIC ANALYSIS OF TWICE-CRYSTALLIZED LACTIC DEHYDROGENASE OF RABBIT MUSCLE

All values are given as $\mu\text{g.}$ of metal per gram of protein.

	Prepn. 1	Prepn. 2	Prepn. 3
Zinc	368	746	576
Zinc ^a	491	554	746
Aluminum	29	16	15
Magnesium	1100	72	71
Calcium	230	251	185
Strontium	7	0	trace
Barium	9	12	6
Manganese	^b	^b	24
Iron	109	344	232
Cobalt	^b	^b	^b
Copper ^a	27	^c	^c
Lead	^b	^b	trace

^a Chemical analysis. ^b Not detected; and also beryllium, cadmium, lithium, molybdenum, nickel, potassium, silver, tin. Sodium was present but not determined. ^c Lost.

(6) G. Beisenherz, H. J. Boltze, T. Bucher, R. Czok, K. H. Garbade, E. Meyer-Arendt and G. Pfeiderer, *Z. Naturforsch.*, **8b**, 555 (1953).

(7) J. B. Neilands, *J. Biol. Chem.*, **199**, 373 (1952).

(8) B. L. Vallee and W. E. C. Wacker, unpublished observations.

thiourea. Inhibition is prevented when zinc is added simultaneously with one of these agents.

The present data constitute a further test of the hypothesis that pyridine nucleotide linked dehydrogenases may prove to be metalloenzymes.¹ The results here presented give impetus to continued exploration in this direction.

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EXISTENCE OF RING PHOSPHATES HIGHER THAN THE TETRAMETAPHOSPHATE

Sir:

We have shown recently that spots attributable to pentametaphosphate and perhaps higher ring phosphate anions are obtained in the two-dimensional paper chromatography introduced by Ebel.¹ These results are part of a study of the hydrolysis of Graham's salt which has been under way in our laboratories for about three and one-half years.

A number of chromatograms have been made on a partially hydrolyzed sample of Graham's salt having a number average chain length of 117 phosphorus atoms per molecule ion, as determined by end-group titration corrected for the presence of rings. Schleicher and Schuell paper No. 589, Orange Ribbon, was employed, with over-loading by the use of 45 μg . of total P in the drop placed at the origin instead of the optimum amount of 15 μg . which we have recommended elsewhere.² In the series of ring phosphates on the chromatogram, there are separate and distinct spots for trimetaphosphate, tetrametaphosphate, pentametaphosphate, and perhaps hexametaphosphate.³ The spot attributed to hexametaphosphate partially overlaps a continuous band which we attribute to unresolved higher ring phosphates. In this sample, there is also a continuous, but less intense, band which curves upward so as to form a continuum at the high-molecular-weight end of the series of chain phosphates. Since the continuum attributed to higher chains and the continuum attributed to higher rings branch apart so as to form a Y, we feel that there is relatively good evidence for ring phosphates containing as many as eight phosphorus atoms. Indeed, the appearance of the chromatogram would lead one to suspect that

(1) J. P. Ebel, *Bull. Soc. Chim. France*, **20**, 991 (1953).

(2) E. Karl-Kroupa, *Anal. Chem.*, submitted for publication.

(3) The hexametaphosphate described here bears no relation to the term "hexametaphosphate" which has been so often misused in both the technological and scientific literature as the name for vitreous sodium phosphates having $\text{Na}_2\text{O}/\text{P}_2\text{O}_5$ mole ratios lying between 1.0 and 1.2. The hexametaphosphate described herein is a single molecular species consisting of a 12-membered ring of alternating phosphorus and oxygen atoms; whereas the "hexametaphosphate" of commerce is a mixture consisting predominantly of long-chain phosphates.

there are ring phosphates even larger than the octametaphosphate.

The total ring content in this partly hydrolyzed sample of Graham's salt is not large, being about 10% of the total P. Most of the phosphate applied to the chromatogram has not moved from the origin (this allows overloading of the paper).

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SELF-DIFFUSION IN MOLTEN SODIUM NITRATE¹

We have measured the self-diffusion coefficients of both sodium ion and nitrate ion as a function of temperature in fused sodium nitrate. The method employed involved a determination of the rate at which tracer sodium or nitrate ion diffused from a *ca.* 0.65 mm. i.d. quartz capillary tube into a stirred bath of pure sodium nitrate. The method was originally used by Anderson and Saddington² in aqueous solution and has since been employed by others, particularly Wang.³ Fairly detailed investigations have proved that the method is susceptible of good accuracy.^{3,4} Self-diffusion coefficients of liquid sodium⁵ and mercury⁶ have been measured by the capillary method. The only previous published work on self-diffusion in molten salts appears to be that of Berne and Klemm,⁷ who obtained the self-diffusion coefficient of thallos ion in molten thallos chloride; we know of no prior work in which diffusion has been determined for both cation and anion.

In our experiments radioactive Na^{22} was the sodium tracer which was followed by standard counting techniques. The nitrate tracer was labelled with stable O^{18} which was determined mass spectrographically on the oxygen liberated when the nitrate was decomposed quantitatively to nitrite and oxygen. Some rather severe difficulties had to be overcome in filling and calibrating the capillaries because of the elevated temperatures. The temperatures were controlled during diffusion to ± 0.1 to 0.2° .

The self-diffusion coefficients, \mathcal{D} , may be expressed by rate equations of the form $\mathcal{D} = A \exp(-\Delta H^\ddagger/RT)$ where A is a constant, ΔH^\ddagger is the energy of activation for the diffusion, R is the molar gas constant and T is absolute temperature. The self-diffusion coefficient of the sodium ion in fused sodium nitrate between 315 and 375° is given by

$$\mathcal{D}_{\text{Na}} = 12.88 \times 10^{-4} \exp(-4970/RT) \text{ cm.}^2/\text{sec.}$$

while that for the nitrate ion similarly is

$$\mathcal{D}_{\text{NO}_3} = 8.97 \times 10^{-4} \exp(-5083/RT) \text{ cm.}^2/\text{sec.}$$

In each case the probable error in ΔH^\ddagger is 80 cal.

(1) This work was performed for the Atomic Energy Commission.

(2) J. S. Anderson and K. Saddington, *J. Chem. Soc.*, S381 (1949).

(3) J. H. Wang and J. W. Kennedy, *THIS JOURNAL*, **72**, 2080 (1950); J. H. Wang, *ibid.*, **74**, 1182 (1952), and others.

(4) R. Mills, *ibid.*, **77**, 6116 (1955), and previous papers.

(5) R. E. Meyer and N. H. Nachtrieb, *J. Chem. Phys.*, **23**, 1851 (1955).

(6) R. E. Hoffman, *ibid.*, **20**, 1567 (1952).

(7) E. Berne and A. Klemm, *Z. Naturforsch.*, **8a**, 400 (1953).