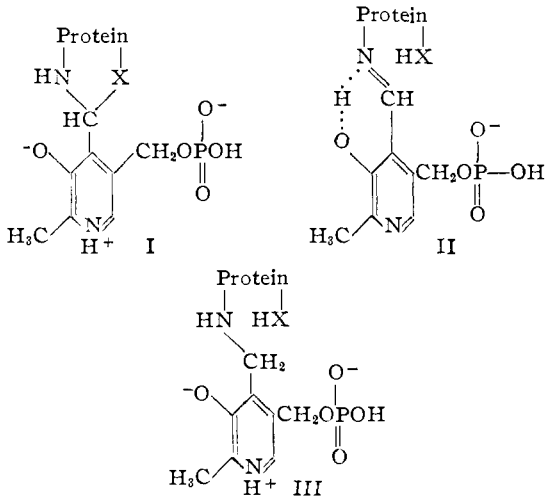
that the enzyme could be freed of PLP with concomitant loss of activity, which could be restored by readdition of the B_6 derivative.

A study of the spectral properties of phosphorylase[4] has indicated that PLP is bound to the enzyme as a substituted aldamine derivative[5] (I) which is converted to a free Schiff base form (II), and eventually split off the enzyme, by treatment with acid, base or urea.



Further evidence in support of structures I and II has been obtained in a study of the reaction of $NaBH_4$ with phosphorylase, which was undertaken with the hope that (II) could be reduced to a stable pyridoxylamine derivative[6] (III) so that the structure of the active site of the enzyme might be investigated.

Treatment of a solution of the enzyme with $NaBH_4$ at *p*H values where the yellow form II is predominant resulted in immediate decolorization, and the B_6 derivative could no longer be liberated from the protein by acid or base. This reaction was not observed between *p*H 5 and 9.5, where the enzyme shows the spectral properties of form I. To obtain a soluble, fully reduced enzyme, crystalline phosphorylase *b* at 0° was precipitated at one third saturation of ammonium sulfate; the *p*H was brought to 4.5 and $NaBH_4$ was added to a final concentration of 0.5 mg./ml. After centrifugation the protein was dissolved in a neutral buffer and dialyzed.

The reduced enzyme showed an absorption maximum at 330 mμ and gave a positive dichloroquinone-chloroimide test.[7] Surprisingly, it was fully active in the phosphorylase reaction, in the conversion of phosphorylase *b* to *a* catalyzed by phosphorylase kinase, and in the reconversion to *b* catalyzed by PR-enzyme. The possibility of a reoxidation of form III back to form I in the activity test was ruled out.

The reduced enzyme was degraded by chymotrypsin and the pyridoxylamine derivative obtained as a pure peptide by column chromatography,[8] high-voltage electrophoresis and paper chromatography. After acid hydrolysis, a fluorescent, positively charged, ninhydrin reacting compound was isolated and compared to pure synthetic pyridoxyl amino acids, including mono α-, mono ϵ-, and di-pyridoxyllysine. On the basis of its quantitative reactions with ninhydrin and dichloroquinone-chloroimide, its behavior on paper chromatography and its characteristic migration in high voltage paper electrophoresis at *p*H 9.6, it was identified as ϵ-N-pyridoxyllysine.

The finding that the bound PLP of phosphorylase can be reduced to form a stable pyridoxylamine derivative without impairing the enzymatic properties of the enzyme poses a serious question as to the role of the B_6 derivative in the phorphorylase system. This problem is being further investigated, together with the action of $NaBH_4$ on other pyridoxal phosphate dependent enzymes.

(5) H. N. Christensen, THIS JOURNAL, **80**, 99 (1958), has assumed the formation of carbinolamine derivatives in the reaction of PLP with peptides and proteins.

(6) D. Heyl, S. A. Harris and K. Folkers, *ibid.*, **70**, 3429 (1948).

(7) M. Hochberg, D. Melnick and B. L. Oser, *J. Biol. Chem.*, **155**, 109 (1944).

(8) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **219**, 623 (1956).

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RECEIVED APRIL 5, 1958

MICROWAVE EXCITATION AS A SYNTHETIC TOOL: THE PREPARATION OF DIBORON TETRACHLORIDE[1]

Sir:

Despite numerous efforts to find more efficient methods for the preparation of diboron tetrachloride (B_2Cl_4), *e.g.*, by the reaction of metal borides with chlorine,[2] by the reduction of boron trichloride with metals, metal borides and other reducing agents,[3] none have been as satisfactory as the electrical discharge method first reported by Stock, Brandt and Fischer[4] and improved by Wartik, Moore and Schlesinger.[5]

Comparable yields have now been obtained by microwave excitation[6] of gaseous boron trichloride at less than 4 mm. pressure. The boron trichloride, maintained at $-78.5°$ is transferred with pumping through the microwave cavity into a $-111.9°$ trap. The free chlorine produced was continuously removed from the $-111.9°$ trap, along with small amounts of boron trichloride and collected in a liquid nitrogen trap.

The diboron tetrachloride retained in the $-111.9°$ trap was purified by fractional distillation of the boron trichloride at $-78.5°$. The diboron tetrachloride was identified by its vapor tension and by infrared spectrum.[7] The determination of the

(1) This work was performed under the auspices of the U. S. Atomic Energy Commission.

(2) E. Apple, Ph.D. Thesis, The Pennsylvania State University, 1955.

(3) G. Urry, T. Wartik, R. E. Moore and H. I. Schlesinger, THIS JOURNAL, **76**, 5293 (1954).

(4) A. Stock, A. Brandt and H. Fischer, *Ber.*, **58**, 855 (1925).

(5) T. Wartik, R. Moore and H. I. Schlesinger, THIS JOURNAL, **71**, 3265 (1949).

(6) Baird Associates' Mercury 198 Exciter operating at a wave length of 12.2 cm. in the 2400–2500 megacycle band.

(7) M. J. Linevsky, E. R. Shull, D. E. Mann and T. Wartik, THIS JOURNAL, **75**, 3287 (1953).

free chlorine recovered from the liquid nitrogen trap agreed, within experimental error, with the diboron tetrachloride produced.

$$2BCl_3 \longrightarrow B_2Cl_4 + Cl_2$$

Only trace amounts, if any, of lower chlorides of boron were observed.

Various parameters which might increase the concentration of active species and at the same time inhibit recombination of chlorine with diboron tetrachloride are under investigation. The attractiveness of this procedure lies in its simplicity and probable adaptability to other systems.

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RECEIVED APRIL 19, 1958

REQUIREMENT FOR BICARBONATE IN FATTY ACID SYNTHESIS[1]

Sir:

While studying a highly purified enzyme system[2] from avian liver which catalyzes the synthesis of palmitic acid from acetyl CoA in the presence of Mn^{++}, ATP[3] and TPNH we have observed an absolute requirement for bicarbonate ion (or its equilibrium forms). Table I shows the characteristics of this requirement. The half-maximal rate of fatty acid synthesis is attained at a HCO_3^- concentration of less than 2×10^{-3} *M*. HCO_3^- is not replaceable by phosphate, sulfate, chloride, formate, acetate, malonate, α-ketoglutarate, isocitrate or succinate. This absolute requirement for HCO_3^- applies to the system at all stages of purification[4] providing that (a) HCO_3^- is removed from enzyme and reagent solutions, and (b) systems which generate HCO_3^- are eliminated. Radio-labelled HCO_3^- is not incorporated into long-chain fatty acid. Hence HCO_3^- cannot be considered a substrate for fatty acid synthesis. We postulate a catalytic role for this component.

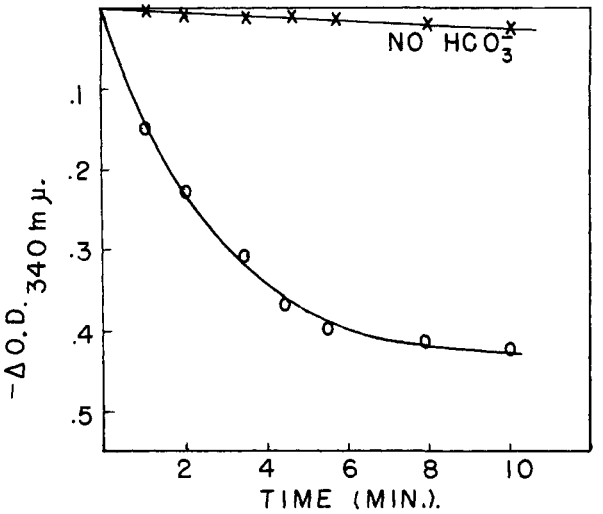

Fig. 1.—Oxidation of TPNH with and without added HCO_3^-: the conditions for these experiments are identical to those in Table I except that 32 mμmoles of acetyl-1-C^{14} CoA was added in each cuvette; 4.0 μmoles of $KHCO_3$ was added as indicated. In the complete system 17 mμmoles of acetyl CoA was incorporated into fatty acids, while there was no incorporation in the system without HCO_3^-.

(1) This investigation has been supported by a research grant, MN-3, from the American Cancer Society; by a research grant H-2236(C2) and postdoctoral training grant, HTS-5006(C8), from the National Heart Institute of the National Institutes of Health, Public Health Service; and Contract Nonr-1202 from the Office of Naval Research.

(2) S. J. Wakil, E. B. Titchener and D. M. Gibson, *Biochim. Biophys. Acta*, in press.

(3) The following abbreviations are used: ATP, adenosine triphosphate; and TPNH, reduced triphosphopyridine nucleotide.

TABLE I

BICARBONATE REQUIREMENT FOR FATTY ACID BIOSYNTHESIS

$KHCO_3$ added (μmoles)	0	1.0	2.0	4.0	5.0
Acetyl CoA incorporated into fatty acids (mμmoles)	1.0	8.0	19.0	25.0	24.0

Each experimental tube contained the following reagents (in a final volume of 0.50 ml.): 50 μmoles of potassium phosphate buffer (*p*H 6.5); 1.0 μmole of ATP; 0.3 μmole of $MnCl_2$; 0.05 μmole of TPNH; and 50 mmoles of acetyl-1-C^{14} CoA. The reaction was started by addition of the purified liver enzymes[2,4]: 0.7 mg. R_{1g} and 0.4 mg. R_{2g}. All samples were incubated five minutes at 38°.

The oxidation of TPNH in the complete system provides an alternative measure of biosynthesis.[2] The absolute requirement for HCO_3^- is also readily demonstrable in this spectrophotometric assay (*cf.* Fig. 1).

The requirement for HCO_3^-, as well as ATP,[2] have suggested to us the possible participation of still another cofactor. In this regard it is significant that one of the purified enzyme fractions contains a considerable concentration of protein-bound biotin.[2]

(4) S. J. Wakil, J. W. Porter and D. M. Gibson, *Biochim. Biophys. Acta*, **24**, 453 (1957); J. W. Porter, S. J. Wakil, A. Tietz, M. I. Jacob and D. M. Gibson, *ibid.*, **25**, 35 (1957).

(5) This work was carried out during the tenure of an Established Investigatorship of the American Heart Association, Inc.

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THE PRESENCE OF N_3^+ AND N_4^+ IN THE MASS SPECTRA OF MOLECULAR NITROGEN

Sir:

We have used two 60° sector instruments equipped with viscous and molecular leaks to study the mass spectra of purified tank nitrogen[1] at high source pressures. A 42^+ and a 56^+ ion current were observed in addition to those currents normally produced by the ionization and fragmentation of nitrogen. At first, it was suspected that the two unusual currents resulted from an increase in the background due to the high operating pressures. High purity argon was used to check this suspicion. It was found that the small 42^+ and 56^+ background ion currents remained constant when argon was run at the same pressures as those used to determine the nitrogen spectra. We also found that nitrogen prepared by the hypobromite oxidation of reagent grade $(NH_4)_2SO_4$ gave the same 42^+

(1) Prepurified grade obtained from the Matheson Co., Joliet, Illinois.