mutant of *E. coli* (205–2, Davis) and has been designated as the "205–2" enzyme. The other enzyme contains vitamin B_{12} or a derivative as a prosthetic group and has been designated as the "B_{12}-enzyme." The purpose of this communication is to report the order of action of these two enzymes and to describe an intermediate of the above reaction.

When "205–2" enzyme was incubated with DPNH and N^5,N^{10}-methylene folate-H_4 (labeled in the methylene carbon with C^{14}), a radioactive product was formed which upon acid hydrolysis yielded one mole of glutamate per mole of the carbon atom labeled with C^{14}. This product could be isolated by application to a triethylaminoethyl cellulose column (3.8 cm. in diameter × 22 cm. in height) and by elution with 0.1 *M* ammonium carbonate. The elution of the product was followed by its radioactivity and emerged from the column between 5 and 9 column volumes. A spectrum was taken of a sample eluted at the peak of the curve of radioactivity and represents the absorption of compound (compound I) which had undergone a minimum of oxidative side reactions (Curve A, Fig. 1). The pooled radioactive fractions then were lyophilized at 4° until most of the ammonium carbonate was removed. The residue was taken up in a small volume of 0.02 *M* ammonium carbonate without undue agitation or exposure to air. The spectrum of an aliquot of this material (not shown in Fig. 1) agreed with that of compound I except that the absorption in the region ot 250 mμ was slightly higher than might be expected from the absorption at 290 mμ used as a reference point. Thus, a small but measurable amount of oxidation of the compound probably had taken place. Compound I, which is a folate derivative, has one symmetrical absorption peak with a maximum at a wave length of 290 mμ (*cf.* ref. 3). Based on radioactivity, the intermediate has a molar absorbancy index of about 29×10^6 mole^{-1}cm.^{2}. When this material is shaken in air or oxygen at *p*H 8.7 it is changed to a second compound (Curve B, Fig. 1) which has absorption maxima at 290 and 250 mμ. The ratio of light absorption at these two wave lengths is about 1.28. The spectrum does not change with further exposure to oxygen at this pH. If the pH of the solution containing compound II is changed to 4.3, a third spectrophotometric species (Compound III, Curve C, Fig. 1) is formed which has an absorption maximum at 282 mμ. In the presence of PtO_2 and H_2 at pH 8 Compound II may be reduced with the uptake of one mole of H_2 to yield a material with the spectrum of Compound I. Compound I does not react with the aldehyde binding reagent, 5,5-dimethyl-1,3-cyclohexanedione (dimedon), and upon treatment with HI yields radioactive CH_3I which has been isolated as the quaternary iodide.[7] It serves as a growth factor for *Lactobacillus casei*[8] but not for *Streptococcus faecalis* or *Leuconostoc citrovorum*.

At the pH of the enzymatic incubation, compounds I and II may serve as substrates in the second reaction and both require homocysteine, DPNH, FAD, Mg^{++}, ATP and "B_{12}-enzyme." The products of the reaction were methionine and folate-H_4. The latter was identified by its ability to support growth of *L. citrovorum* and by its migration index (R_f) in paper chromatography.[9] N^{10}-Methyl folate-H_4 prepared by the catalytic reduction of N^{10}-methyl folate could not serve as a substrate of the second reaction.

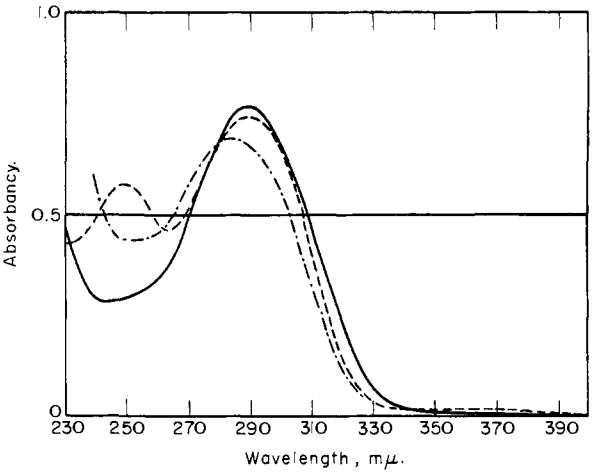

Fig. 1.—Absorption spectra of methyl tetrahydrofolate and oxidized derivatives: Curve A——, Compound I; Curve B - - -, Compound II; Curve C—·—, Compound III. The concentration of folate derivatives was $2.66 \times 10^{-5}M$.

From these data we wish to propose N^5-methyl tetrahydrofolate as the tentative structure of compound I. On the basis of the hydrogenation experiments, compound II would be an N^5-methyl dihydrofolate and compound III, another partially oxidized form of compound I.[10]

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THE USE OF A PROTON-PROTON SPIN DECOUPLING METHOD FOR THE DETERMINATION OF NUCLEAR MAGNETIC RESONANCE CHEMICAL SHIFTS

Sir:

The complexities of high-resolution n.m.r. spectra of molecules can in many cases be simplified drastically by the use of double resonance spin decoupling techniques.[1] Until recently the application of spin decoupling techniques to proton–proton systems was limited by the complexity of the required instrumentation.[2] A new technique for accomplishing proton–proton spin decoupling with relatively simple instrumentation has been described recently by Kaiser[3] and Freeman[4]; this is the audio side band phase detection technique.[5] We now describe a new application of

(1) See J. A. Pople, W. G. Schneider and H. J. Bernstein, "High-resolution Nuclear Magnetic Resonance," McGraw-Hill Book Co., New York, N. Y., 1959, pp. 160–161, 229–230, 298–305 and 370–371, for discussions and leading references.

(2) W. A. Anderson, *Phys. Rev.*, **102**, 151 (1956).

(3) R. Kaiser, *Rev. Sci. Instr.*, **31**, 963 (1960).

(4) R. Freeman, *Molecular Phys.*, **3**, 435 (1960).

(5) J. Itoh and S. Sato, *J. Phys. Soc. Japan*, **14**, 851 (1959), previously described an audio side band technique without phase detection;

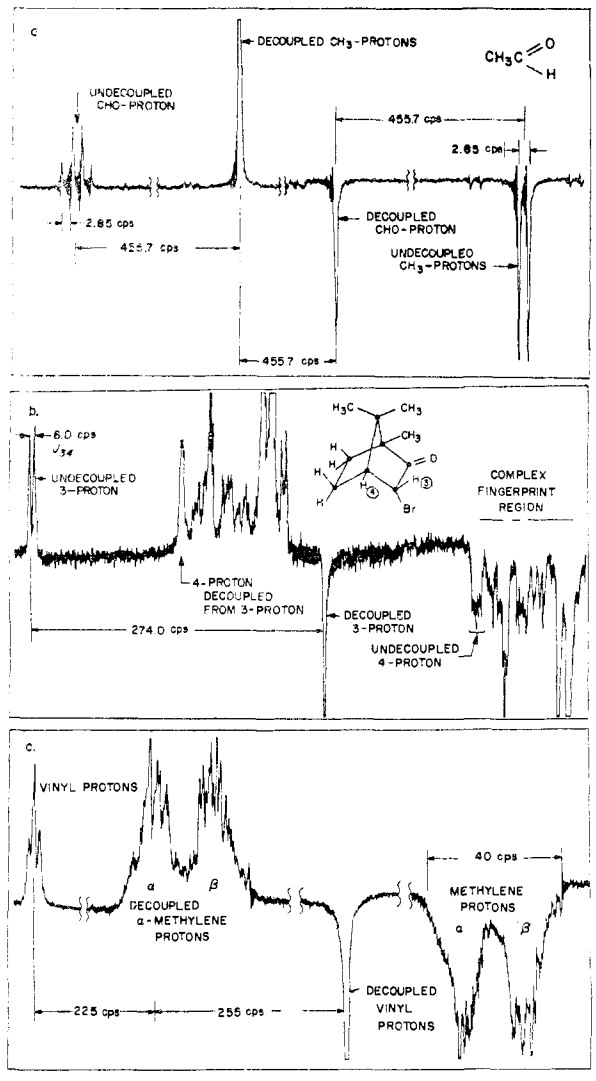

Fig. 1.—Audio side band phase detection decoupled nuclear magnetic resonance spectra: a, protons of acetaldehyde (neat), $\gamma H_1/2\pi$ = 13 cps. and Ω = 455.7 ± 1.0 cps., δ from decoupling = 455.7 ± 1.0 cps.; b, protons of α-bromocamphor (nearly saturated solution in $CDCl_3$), $\gamma H_1/2\pi$ = 6 cps. and Ω = 137.0 ± 0.5 cps., δ from decoupling = 137.0 ± 0.5 cps.; c, protons of cyclohexene (neat), $\gamma H_1/2\pi$ = 20 cps. and Ω = 225 ± 1 cps., δ from decoupling = 225 ±1 cps. Increasing field in all cases is from left to right.

this technique for the determination of certain proton chemical shifts which could not be unambiguously or accurately measured heretofore.

There are many molecules whose high-resolution proton n.m.r. spectra have one proton or group of equivalent protons which are chemically shifted considerably downfield from most of the other protons. As is usually the case for only moderately complex molecules, the signals from the latter protons may form a very complex fingerprint region. Any multiplet structure of the downfield protons generally can *not* be analyzed completely to give the relevant coupling constants and chemical shifts.

In order to spin decouple two groups of chemically shifted protons, it is necessary that the condition $\gamma H_1/2\pi > J$(γ = gyromagnetic ratio, H_1 = rotating magnetic rf. field, J = spin-spin coupling constant in cps.) must be met.[6] If a resonance signal occurs at frequency ν (cps.) and a weak audio frequency Ω (cps.) is applied to the sweep coils of the spectrometer, the first side bands will occur at $\nu \pm \Omega$ if H_1 is small.[3,6] However, for large H_1, as required in the spin decoupling of two groups of chemically shifted protons, the first side bands do not occur at $\nu \pm \Omega$.[3,6] If δ is the position of the first side band (measured in cps. from the center band) then

$$\delta = \pm(\Omega^2 - \gamma^2 H_1^2/4\pi^2)^{1/2} \qquad (1)$$

In the audio side band phase detection method for proton–proton spin decoupling, δ is the chemical shift between the two groups of protons being decoupled. Since in a decoupling experiment all the parameters on the right of eq. 1 are known, the chemical shift can be computed easily.

To test this technique, we studied the n.m.r. spectrum of acetaldehyde at 60 Mc. (Fig. 1a). The chemical shift determined in the usual manner by a side band technique[7] was 455.7 ± 0.2 cps. The value determined by optimizing Ω and minimizing $\gamma H_1/2\pi$ was 455.7 ± 1.0 cps. This agreement seems excellent. This technique was applied to the n.m.r. spectrum of α-bromocamphor[8] in order to determine the unknown chemical shift between the 3- and 4-protons. Fig. 1b summarizes our results. The chemical shift was determined as 137.0 ± 0.5 cps. (at 60 Mc.). As is evident from Fig. 1b the resonance signal due to the 4-proton can be unambiguously assigned to the downfield end of the complex fingerprint region.

We also have applied this technique to the determination of the chemical shift between the vinyl protons and the α-methylene group protons of cyclohexene and the results are summarized in Fig. 1c. The signals of the two types of methylene protons in cyclohexene give two broad (about 20 cps. wide), very complex asymmetrical regions approximately two hundred cps. up field from the vinyl protons (at 60 Mc). The shape and overlap of this multitude of lines make it unsafe to assume that the visual center of gravity of these two broad bands corresponds to the chemical shift positions of the α- and β-methylene protons. By the double resonance technique we have determined the chemical shift between the α-methylene protons and the vinyl protons as 225 ± 1 cps.

The application of the audio side band phase detection decoupling technique to the determination of chemical shifts as described by us should be an extremely valuable means for the analysis of the n.m.r. spectra of many molecules.

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(6) A. L. Bloom and J. N. Shoolery, *Phys. Rev.*, **97**, 1261 (1955).

(7) The first side band of one group was symmetrically superimposed on the center band of the other group; the audio frequency (Ω) was measured by counting its period with a Hewlett-Packard Model 524-C frequency counter.

(8) The high-resolution n.m.r. spectrum of this molecule at 40 Mc. was reported previously by W. D. Kumler, J. N. Shoolery and F. V. Brutcher, *J. Am. Chem. Soc.*, **80**, 2533 (1958).

the decoupled signals were observed by their d.c.-component. The disadvantages of this method have been discussed in ref. 3.