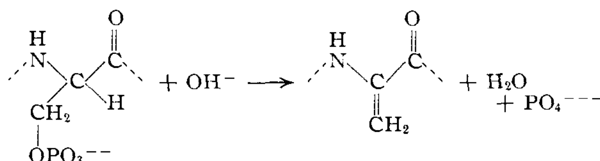
was essentially no incorporation of isotope into the inorganic phosphate.

TABLE I

	Dephosphorylation with Potassium hydroxide	Dephosphorylation with Barium hydroxide
Atom per cent. excess O^{18} in water	4.3	4.6
Casein concentration, % w./v.	5	15
Alkali concentration, *N*	0.32	0.50
Time of incubation, at 37°, hr.	15	12
Dephosphorylation, %	100	85
Atom per cent. excess O^{18} in phosphate oxygen	0.04	0.01

The β-elimination of phosphate from a serine residue in a protein would be formulated as



Clearly, isotopic oxygen from the medium would not be incorporated into the phosphate during this reaction. If, on the other hand, the reaction were one of hydrolysis, and if the hydrolysis proceeded by P–O bond fission, the isolated phosphate would contain one-fourth as much O^{18} as the water of the medium. The hydrolysis of phosphate esters in alkali has not been studied as thoroughly as might be desirable. However, it has been demonstrated that alkali cleavage is at the P–O bond in trimethyl phosphate,[5] in the cyclic phosphodiesters of nucleosides,[6] and in methyl α/β-D-glucofuranoside 3-phosphate and 1,2-*O*-isopropylidene-D-glucofuranose 3-phosphate.[7] Furthermore, the enzymatic hydrolysis[8] and the lanthanum hydroxide-promoted hydrolysis[9] of phosphomonoesters, as well as the cleavage of the monoanions of these substances by water,[9] all take place with P–O fission. Hydrolyses of phosphate esters at C–O bonds have so far been observed only in neutral or acid media. The present experiments thus support the hypothesis that the removal of phosphate from phosphoproteins by mild alkali proceeds by β-elimination. Further experiments to test the hypothesis are in progress.

Our results also would seem to rule out the existence of appreciable numbers of N–P bonds[2] in casein. It is difficult to write a plausible mechanism for the cleavage of such bonds which would not lead to the incorporation of oxygen from water into the phosphate.

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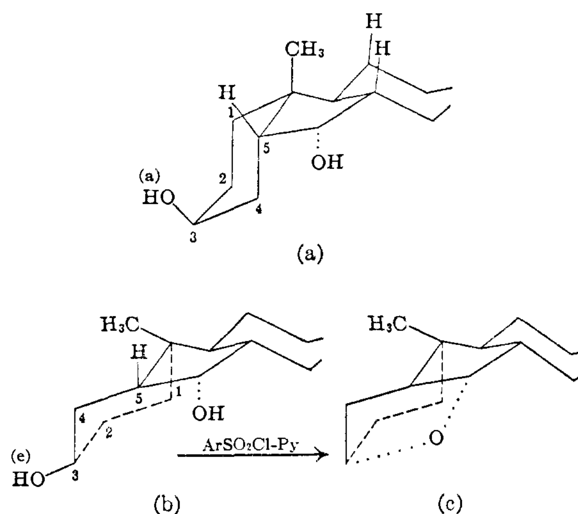
(5) E. Blumenthal and J. B. M. Herbert, *Trans. Faraday Soc.*, **41**, 611 (1945).
(6) D. Lipkin, P. T. Talbert and M. Cohn, THIS JOURNAL, **76**, 2871 (1954).
(7) E. E. Percival and E. G. V. Percival, *J. Chem. Soc.*, 874 (1945).
(8) M. Cohn, *J. Biol. Chem.*, **180**, 771 (1949); S. S. Stein and D. E. Koshland, Jr., *Arch. Biochem. Biophys.*, **39**, 229 (1952).
(9) W. W. Butcher and F. H. Westheimer, THIS JOURNAL, **77**, 2420 (1955).

B-NORCOPROSTANE DERIVATIVES

Sir:

One of us[1] established that the Butenandt ketone is a B-norstane-3,6-dione and tentatively assigned the 5β-configuration to this substance and to the parent B-norstane. Dauben and Fonken[2] obtained the same hydrocarbon from a B-norstanol[3] derived from B-norcholesterol[3] and inferred that the 3β-hydroxyl group of the stanol is equatorial because lithium aluminum hydride reduction of the 3-ketone (unhindered) afforded the original B-norstanol. They assumed that the 5β-stanol would have the conformation shown in (a) (for a 3,6-diol), in which the 3β-hydroxyl group is axial, and concluded that the B-norstanol belongs to the 5α-series, in which the hydroxyl group would be equatorial. On the other hand, the rotatory dispersion curve indicates that the ketone probably is B-norcoprostane-3-one.[4]

In seeking to reconcile the seemingly conflicting evidence, we noticed that ring A of form (a) can undergo a flip to produce the conformation (b) in



which the 3β-hydroxyl is equatorial. Since this flip tilts the methyl group away from ring B and so relieves a severe $10\text{-CH}_3{:}11\beta\text{-H}$ interaction (and a lesser $10\text{-CH}_3{:}8\beta\text{-H}$ interaction), (b) should be more stable than (a). We have now prepared the $3\beta,6\alpha$-diol (b) and converted it into the oxide (c) and so proved that the diol and the related stanol are in fact B-norcoprostane derivatives. Sodium borohydride reduction of B-norcoprostane-3,6-dione gave diol (b), m.p. 140–141°, $[\alpha]_D$ −2° and an isomeric diol, m.p. 170–172° $[\alpha]_D$ +35° (found C, 79.91; H, 11.95, and C, 80.04; H, 11.97), separated by chromatography of the diacetates; acetate (b), an oil, and the isomeric diacetate, 93–94°, $[\alpha]_D$ +38° (C, 75.77; H, 10.60). Both diols on oxidation gave the original dione. Heated in pyridine with benzenesulfonyl chloride, diol (b) afforded in 40% yield the oxide (c), m.p. 85.5–86.5°, $[\alpha]_D$ +31° (C, 83.64; H, 11.85; mol

(1) L. F. Fieser, THIS JOURNAL, **75**, 4386 (1953).
(2) W. G. Dauben and G. J. Fonken, *ibid.*, **78**, 4736 (1956).
(3) F. Šorm and H. Dykova, *Coll. Czech.*, **13**, 407 (1948).
(4) C. Djerassi, D. Marshall and T. Nakano, THIS JOURNAL, **80**, 4853 (1958).

wt., 363); TNM and Br_2 tests negative; no absorption at 205–300 mμ; no infrared hydroxyl or carbonyl bands; characteristic bands (CS_2) at 8.90, 9.22, 9.85, 10.22, 10.65, 11.53, and 12.54 μ; proton resonance peaks at 82 and 94 cps. in $CDCl_3$ (referred to benzene resonance peak; no other peaks in the range 0–160 cps.) indicating two hydrogens attached to different oxygen-linked carbons. The oxide is stable to hydrochloric acid in refluxing methanol–dioxane. That cyclization is not attended with rearrangement of the ring system was established as follows. When heated with *p*-toluenesulfonic acid in acetic anhydride the oxide afforded an oily unsaturated acetate which on hydrogenation and hydrolysis yielded B-norcoprostane-6α-ol, m.p. 102.5–103.5°, $[\alpha]_D$ −20° (C, 83.32; H, 12.30). Oxidation gave the 6-ketone, m.p. 98–98.5°, $[\alpha]_D$ +34°, λ^{CS_2} 5.77 μ (C, 83.59; H, 11.79). An identical ketone was obtained from B-norcoprostane-3,6-dione by partial reduction with sodium trimethoxyborohydride to an oil containing predominantly the 3-ol-6-one. Conversion to an ethyleneketal mixture, oxidation, and chromatography on alumina gave B-norcoprostane-3,6-dione 6-ethyleneketal, m.p. 120–121°; $[\alpha]_D$ +50°, λ^{CS_2} 5.83 μ (C, 77.76; H, 10.83). Wolff–Kishner reduction of the ketol and hydrolysis gave B-norcoprostane-6-one, identical with the above sample (m.p., $[\alpha]_D$, infrared).

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A CRYSTALLINE DECARBOXYLASE WITHOUT BIOTIN

Sir:

During an investigation of the mechanism of decarboxylation[1] we have crystallized the decarboxylase for acetoacetic acid from *Clostridium acetobutylicum*. The microörganism (American Type Culture Collection No. 862) was grown and harvested and the crude enzyme prepared by a modification of the procedure developed by Davies[2] and Seeley.[3] The partially purified enzyme was comparable in activity to the best samples obtained by Davies. Approximately 25 mg. of this preparation in 3 ml. of solution was dialyzed against 0.05 *M* phosphate buffer at *p*H 5.9 and then placed on a column of diethylaminoethyl cellulose.[4] On gradient elution to 0.05 *M* phosphate (*p*H 5.9)–0.1 *M* ammonium sulfate, the enzyme was spread over several fractions; these were combined and precipitated with 60% saturated ammonium sulfate. The precipitate was suspended in 1 ml. of 0.05 *M* phosphate (*p*H 5.9) and, on standing at room temperature, microscopic hexagonal plates of the enzyme crystallized out. The enzyme can be recrystallized from a solution in 0.05 *M* phosphate (*p*H 5.9) by adding ammonium sulfate to about 50% saturation. The crystalline enzyme is 2 to 3 times as active as Davies' best preparation. In a Spinco model E ultracentrifuge, the protein proved monodisperse, and showed a sedimentation constant (extrapolated to zero concentration) of about 15. Its molecular weight is then presumably of the order of 300,000.

Samples of the enzyme were hydrolyzed with 6 *N* sulfuric acid or 4.5 *N* potassium hydroxide solution at 120° for two hours, and the hydrolysate assayed microbiologically[5] for biotin, using *Saccharomyces cerevisiae* (American Type Culture Collection No. 7754). Control experiments showed that the vitamin, as measured by this assay, was not extensively destroyed under the experimental conditions employed; the method gave results approximating those in the literature for biotin-containing tissues.[6] The assay with hydrolyzed enzyme gave results which did not differ significantly from "blanks." Assuming that the concentration of the enzyme could be determined from the absorption in the 260–280 mμ region and that the enzyme was hydrolyzed under the above conditions, the data show that the enzyme contains less than 0.01 molecule of biotin per 300,000 molecular weight units. This vitamin has been implicated, and is presumably present, in another beta ketoacid decarboxylase,[7] and in the carboxylating enzyme in fatty acid synthesis.[8] Biotin may prove essential for the fixation of carbon dioxide by certain enzymes, but it is not present in the pure crystalline decarboxylase for acetoacetic acid.

The authors wish to thank the National Institutes of Health for financial support, and Mr. J. Kucera for his assistance in the determination of the sedimentation constant.

(1) For previous studies of decarboxylation, see F. H. Westheimer and W. A. Jones, THIS JOURNAL, **63**, 3283 (1941); R. Steinberger and F. H. Westheimer, *ibid.*, **71**, 4158 (1949); **73**, 429 (1951); S. Seltzer, G. A. Hamilton and F. H. Westheimer, *ibid.*, in press.

(2) R. Davies, *Biochem. J.*, **37**, 230 (1943).

(3) H. W. Seeley, in "Methods in Enzymology" (S. Colowick and N. Kaplan, Eds.) Academic Press, Inc., New York, N. Y., 1955, Vol. 1, p. 624.

(4) E. A. Peterson and H. A. Sober, THIS JOURNAL, **78**, 751 (1956); H. A. Sober, F. J. Gutter, M. M. Wyckoff and E. A. Peterson, *ibid.*, **78**, 756 (1956).

(5) E. E. Snell, in "Vitamin Methods" (P. Gyorgy, Ed.), Academic Press, Inc., New York, N. Y., 1950, Vol. I, p. 425; H. C. Lichstein, *J. Biol. Chem.*, **212**, 217 (1955).

(6) R. C. Thompson, R. E. Eakin and R. J. Williams, *Science*, **94**, 589 (1941).

(7) H. C. Lichstein, *Arch. Biochem. Biophys.*, **71**, 276 (1957).

(8) S. J. Wakil, E. B. Titchener and D. M. Gibson, *Biochim. Biophys. Acta*, **29**, 225 (1958).

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A NEW AND GENERAL METHOD FOR THE ISOLATION OF ANTI-PROTEIN ANTIBODIES

Sir:

Although a few general methods for the isolation of pure antibodies (Ab) directed against protein antigens (Ag) have been described,[1] the need exists for a convenient, reproducible and gentle method capable of good yields. We have developed such a method with which we have so far isolated pure

(1) H. C. Isliker, *Advances Protein Chem.*, **12**, 388 (1957).