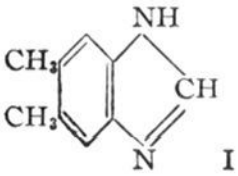
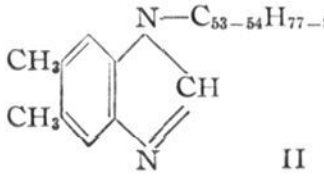
VITAMIN B_{12}. VI. 5,6-DIMETHYLBENZIMIDAZOLE, A DEGRADATION PRODUCT OF VITAMIN B_{12}

Sir:

Degradation of vitamin B_{12} by acid hydrolysis has given a new basic compound which has been identified by its reactions and by synthesis as 5,6-dimethylbenzimidazole (I).



I



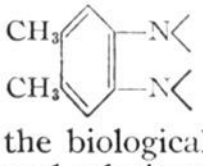
II

The crystalline product melted at 205–206°. *Anal.* Calcd. for $C_9H_{10}N_2$: C, 73.94; H, 6.90; N, 19.17. Found: C, 74.36; H, 6.47; N, 19.21. Potentiometric titration showed an equivalent weight of 144 ± 5; calcd., 146. In 95% ethanol solution in the presence of 0.01 *N* hydrochloric acid, the absorption spectrum of the compound was characterized by maxima at 2745 Å. (E_M7500) and at 2840 Å. (E_M8100). In similar solution in the presence of 0.01 *N* sodium hydroxide, maxima were observed at 2470 Å. (E_M3900), 2775 Å. (E_M 4900), 2810 Å. (E_M5250) and 2880 Å. (E_M5700). The compound was optically inactive. It gave a crystalline picrate, melting point 273–275°. *Anal.* Calcd. for $C_{15}H_{13}N_5O_7$: N, 18.66. Found: N, 18.76.

Treatment of the degradation product with benzoyl chloride in aqueous alkali according to the method of Bamberger and Berlé[1] for the cleavage of benzimidazoles to dibenzamidobenzenes afforded a compound, melting point 262–263°, which was identical with a synthetic sample of the new 4,5-dibenzamido-1,2-dimethylbenzene, melting point 262–262.5° (*Anal.* Calcd. for $C_{22}H_{20}N_2O_2$: C, 76.72; H, 5.85; N, 8.14. Found: C, 76.70; H, 6.01; N, 8.25), prepared by benzoylation of 4,5-diamino-1,2-dimethylbenzene.

The assigned structure of the degradation product was confirmed by the synthesis of 5,6-dimethylbenzimidazole by condensation of 4,5-diamino-1,2-dimethylbenzene with formic acid. The resulting compound had melting point and mixed melting point 204–205°. Its absorption spectrum was identical with that of the natural product, within experimental error. A provisional formula[2] for vitamin B_{12} is represented in II, which is based on the assumption that the dimethylimidazole is terminal and linked to a nitrogen.

It is noted that the 1,2-diamino-4,5-dimethylbenzene moiety (III) appears in 5,6-dimethylbenzimidazole and vitamin B_{12}, and also in riboflavin.



III

Elucidation of the biological implications of this chemical structural relationship will undoubtedly prove of interest.

When a sample of riboflavin was hydrolyzed under the same conditions, a similar isolation technique failed to yield any 5,6-dimethylbenzimidazole.

The authors wish to thank Miss Janice Mayfield for technical assistance, Dr. N. R. Trenner and Mr. R. P. Buhs for the potentiometric titration, and Mr. R. Boos and his associates for the microanalyses.

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(1) Bamberger and Berlé, *Ann.*, **273**, 346 (1893).
(2) Brink, *et al.*, THIS JOURNAL, **71**, 1854 (1949).

ELECTRON MICROGRAPHS OF CRYSTALLINE EDESTIN

Sir:

Electron micrographs have been obtained showing molecular arrays in crystalline edestin. The protein was recrystallized six times from 5% sodium chloride by cooling slowly from 60 to 4°. Crystals were formed on the specimen screen with collodion film by applying a drop of the protein in 2.5% sodium chloride at 60° and cooling to room temperature. Salt was removed by washing with water or 75% alcohol. Since the crystals are relatively opaque in the electron microscope, a shadow-transfer technique was developed to render the surface structure visible. The specimen was shadowed with uranium or nickel coated normally with an evaporated film of silicon oxide, washed with acetone to remove the collodion and 0.05 *N* hydrochloric acid to remove the protein. In outline, such crystals are mostly equilateral triangles as shown in Fig. 1(a). The molecules on the triangular faces are arranged in a hexagonal pattern as shown in Fig. 1(b). It is concluded from the analysis that the triangular faces are the (111) planes of a face-centered cubic lattice. Measurements of the perpendicular distance between rows of molecules parallel to the triangle edges range from about 68 to 72 Å. with an average of 69.7 Å. Magnifications are judged to be accurate to within ±2%. From this measurement it is calculated that a = 114 Å. for the unit cell. The conclusion regarding the three-dimensional symmetry of the crystals was suggested by

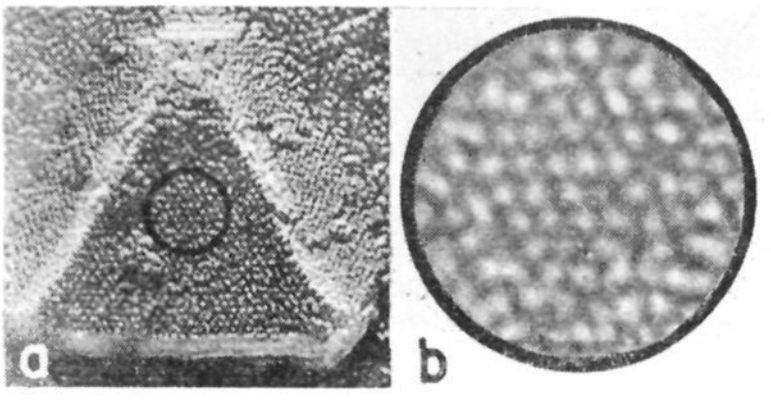

Fig. 1.—(a) Electron micrograph showing surface structure of edestin crystal, × 95,000; (b) circumscribed area of (a) showing molecules in (111) plane, × 396,000.

two pieces of information in addition to the geometrical array in the triangular faces. First, adjoining planes are frequently visible in which the molecular arrangement is like that of an orthographic projection of (200) faces. Secondly, where molecules are lying on the triangular surfaces or where steps occur, the shadow lengths indicate that the molecules are approximately as thick as their diameter in the triangular faces. Apparently the molecules are approximately spherical, about 80 Å. diameter in the dry crystal.

With an assumed density of 1.30 for the crystal,[1] the calculated molecular weight is 290,000 agreeing well with values deduced by other methods.[1] Although the unit cell of edestin has not been measured by X-ray methods, the structure of a similar globulin from tobacco seed has been reported as face-centered cubic with $a = 123$ Å. in the dry crystal.[2] Although this value is a little larger than that obtained for edestin, the agreement appears satisfactory insofar as there may be actual differences between the two proteins or differences in hydration as examined.

(1) See E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids, and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943.
(2) D. Crowfoot and I. Fankuchen, *Nature*, **141**, 522 (1938).

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RECEIVED JUNE 16, 1949

CRYSTALLIZATION OF VITAMIN B_{12b}

Sir:

The existence of more than one pink clinically-active pigment in liver extract was noted by Smith.[1] In the present study, a crystalline fraction was separated by chromatography having absorption spectrum maxima different from those at 278, 361 and 550 mμ which have been reported for vitamin B_{12}.[2,3] The biological activity of an impure preparation of this new fraction, obtained from liver extract, for chicks and in the microbiological assay was described elsewhere.[4] A similar fraction was obtained from cultures of *Streptomyces aureofaciens*[5] by adsorption with charcoal followed by elution and chromatography upon silicic acid columns.[6] Two characteristic pink bands were thus separated and were eluted. Fractional precipitation of the first of these with acetone yielded small rod-like red crystals which contained cobalt and phosphorus and which showed absorption spectrum maxima at 273, 351 and 525 mμ. The secondary "peaks" at 307 and 325 mμ, which are shown by vitamin B_{12}, were absent. The crystals were biologically active in the chick assay[4] and in the assay with *L. leichmannii* 313.[7] Since the term "vitamin B_{12a}" has recently been applied to a biologically active compound related to vitamin B_{12},[8] the term "vitamin B_{12b}" is suggested for the preparation described in the present investigation.

The second pink fraction had an absorption spectrum which was characteristic of vitamin B_{12}.[2,3] It was concentrated to yield needle-like crystals which appeared similar to those of vitamin B_{12}.

(1) Smith, *Nature*, **161**, 638 (1948).
(2) Ellis, Petrow and Snook, *J. Pharm. and Pharmacol.*, **1**, 60 (1949).
(3) Brink, Wolf, Kaczka, Rickes, Koniuszy, Wood and Folkers, THIS JOURNAL, **71**, 1854 (1949).
(4) Stokstad, Jukes, Pierce, Page and Franklin, *J. Biol. Chem.*, in the press (Sept. 1949).
(5) Duggar, *Annals N. Y. Acad. Sci.*, **51**, 175 (1948).
(6) Smith and Parker, *Biochem. J.*, **43**, viii (1948).
(7) Hoffman, Stokstad, Franklin and Jukes, *J. Biol. Chem.*, **176**, 1465 (1948).
(8) Kaczka, Wolf and Folkers, THIS JOURNAL, **71**, 1514 (1949).

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RECEIVED JULY 22, 1949

PROTEIN SYNTHESIS BY CHYMOTRYPSIN

Sir:

Bergmann and Fruton[1] showed that chymotrypsin can synthesize anilides. It has now been found that an insoluble protein forms when very small quantities of chymotrypsin are added to Witte peptone. The clear peptone solution gradually changes into a solid gel. The reaction appears to have an optimum *p*H very close to 7.00. Crude lima bean trypsin inhibitor and crystalline lima bean trypsin inhibitor cause temporary inhibition.[2] The synthetic protein is soluble in hot water. It is precipitated by trichloroacetic acid and by a saturated ammonium sulfate solution which has been adjusted to *p*H 7.00. It gives the usual protein color reactions and a pink biuret test.

TABLE I

Sample	Chymotrypsin	After 16 hours	After 40 hours	Insoluble protein N, mg.
1	50 gamma	Viscous solution	Solid gel	3.60
2	1 mg.	Solid gel	Heavy precipitate	9.84
3	None	Clear solution	Clear solution	None

Each sample contained 0.66 g. of Witte peptone in 2 cc. of distilled water. The chymotrypsin was dissolved in 0.25 cc. of distilled water. Sample 3 contained 0.25 cc. of distilled water instead of the enzyme solution. The *p*H was 7.00. The temperature was 37°. A generous quantity of salt-free crystalline chymotrypsin was very kindly furnished by Dr. M. Kunitz of the Rockefeller Institute for Medical Research. Identical results were obtained with a commercial preparation of crystalline chymotrypsin Worthington Biochemical Laboratory—4 × crystallized, ap. 50% $MgSO_4$). Three different Witte peptone preparations gave identical results. A few commercial peptones (not Witte peptone) were unsuitable for the protein synthesis.

(1) N. Bergmann and J. S. Fruton, *Ann. N. Y. Acad. Sci.*, **45**, 409 (1944).
(2) H. Tauber, B. B. Kershaw and R. D. Wright, *J. Biol. Chem.*, in press.