with hydrobromic acid. The hydrogenation of apo- and isoapo-β-erythroidine yielded the same compound, octahydro-apo-β-erythroidine. Oxidation of apo-β-erythroidine gave formic acid.

Interpretation of these reactions and degradation products of β-erythroidine allows one to formulate tentative structures for these products.

RAHWAY, N. J. RECEIVED JULY 18, 1950

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF MERCK & CO., INC.]

Vitamin B_{12}. XIII. Additional Data on Vitamin B_{12a}

BY EDWARD A. KACZKA, ROBERT G. DENKEWALTER, ARNOLD HOLLAND AND KARL FOLKERS

In a recent communication,[1] crystalline vitamin B_{12a} was described as a reaction product of vitamin B_{12} and hydrogen over a platinum catalyst. The described physical properties of vitamin B_{12a} showed that it could be differentiated from vitamin B_{12} although these two compounds are obviously very closely related in composition and properties. The results of the first preliminary biological tests which were reported,[1] were not intended as a critical and final evaluation, but to show that vitamin B_{12a} possesses high vitamin B_{12} activity.

Vitamin B_{12a} has been examined further, and samples, including the one described,[1] have been found to be about 98% pure by solubility analyses. Vitamin B_{12a} separates from aqueous acetone in needle-like or bladed crystals which belong to the orthorhombic systems. The color of the crystals is somewhat darker red than those of vitamin B_{12}. On the micro-block, the crystals start to darken at about 200°, with more decomposition appearing to take place than with vitamin B_{12}, but do not melt below 300°. The refractive indices of two samples (dried *in vacuo* at room temperature) which resulted from two separate hydrogenation experiments are given in Table I. When the crystals of sample 2 were heated for two hours at 100° *in vacuo*, the refractive indices were found to be: α, 1.604 ± 0.004; β, 1.640 ± 0.004; γ, 1.654 ± 0.004. This heating of crystals of vitamin B_{12a} does not seem to alter very much the β and γ indices, but does seem to cause a progressive increase in the α index. The refractive indices may be used as a criterion for identity and reproducibility of vitamin B_{12a}.

TABLE I

REFRACTIVE INDICES OF VITAMIN B_{12a}

Sample	α	β	γ
1	1.580 ± 0.002	1.640 ± 0.002	1.657 ± 0.002
2	1.580 ± 0.002	1.640 ± 0.002	1.656 ± 0.002
Vitamin B_{12a} (from *S. griseus*)	1.584 ± 0.002	1.640 ± 0.002	1.657 ± 0.002

Amorphous and crystalline concentrates from culture broths of *Streptomyces griseus*, which showed vitamin B_{12} activity in microbiological assays, yielded a red crystalline compound after further fractional crystallization. The absorption spectrum of this red crystalline product is like that of vitamin B_{12a}, but differs from that of vitamin B_{12} as shown in Fig. 1. The slight variations in the absorption spectra of vitamin B_{12a} and the crystals from *S. griseus* in the regions 265–275 mμ, 300–320 mμ and 350–358 mμ are influenced by

(1) Kaczka, Wolf and Folkers, THIS JOURNAL, 71, 1514 (1949).

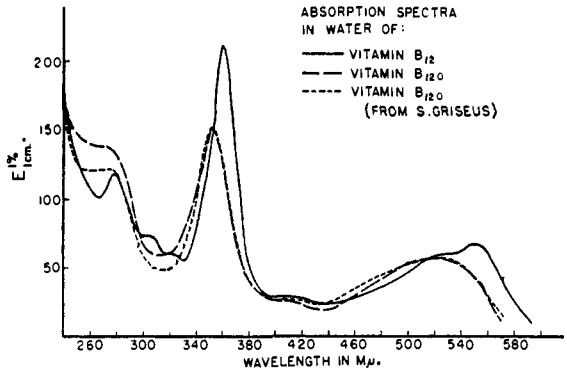

Fig. 1.

slight variations in the *p*H of the solutions and this influence is demonstrated in Fig. 2. During the early work, the *p*H variations of water solutions of samples of vitamin B_{12a} were not determined. It is evident that vitamin B_{12a} and the crystals from *S. griseus* cannot be differentiated by absorption spectrum (Fig. 1) or refractive indices (Table I).

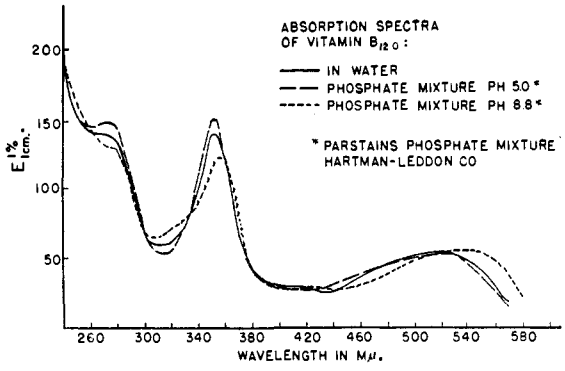

Fig. 2.

A sample (NP-92-58-4)[2] of vitamin B_{12b},[3] has been compared with vitamin B_{12a} obtained from the hydrogenation reaction with the following results: The two samples cannot be differentiated by absorption spectrum as illustrated by Fig. 3. The sample of vitamin B_{12a} used for this comparison was found to be 98.5% pure. When powdered samples of vitamin B_{12a} were dried at 100° for two hours *in vacuo* and then dissolved in water, the spectrum was found to be, on immediate

(2) Through the courtesy of Dr. T. H. Jukes of the Lederle Laboratories, Division of the American Cyanamid Company.

(3) Pierce, Page, Stokstad and Jukes, THIS JOURNAL, 71, 2953 (1949).

examination, as shown in Fig. 3; however, the absorption at 3150 Å. started to change within minutes, and after 24 hours was as shown in Fig. 3. Another sample of vitamin B_{12b} (Lederle NP-119-17-6)[4] was dried similarly and dissolved in water; the absorption was determined within two minutes, particularly at 3150 Å., and after time intervals of one and five hours. A decrease in the absorption of this solution of Vitamin B_{12b} was observed within this period similar to that seen for vitamin B_{12a}.

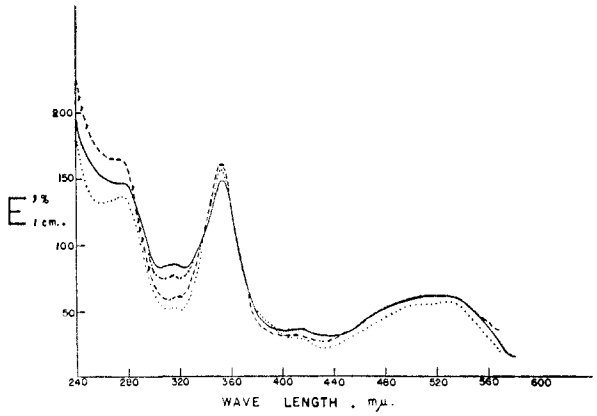

Fig. 3.—Spectra in water of vitamin B_{12a} after drying *in vacuo* at 100° for two hours: —, solution at t = *ca.* 1 min.; -·-, solution at t = *ca.* 15 min.; ---, solution at t = 24 hr.; · · ·, spectrum of vitamin B_{12b} (Lederle NP-92-58-4) after drying at 100° for two hours *in vacuo;* water solution at t = over 30 minutes.

A sample of vitamin B_{12a}, vitamin B_{12b} (NP-92-58-4), and a mixture of the two, were examined by paper-strip chromatography. The two samples and the mixture showed a single spot of comparable R_F values of about 0.14, and there was no evidence of a separation of the mixture, whereas mixtures of vitamins B_{12} and B_{12a}, and B_{12} and B_{12b} did separate. The solvent system used was 10% acetic acid–40% butanol–50% water.

This sample of vitamin B_{12b} was found to be insoluble in 85% acetone solution previously saturated with vitamin B_{12a}. Vitamin B_{12b} is readily soluble in 85% acetone. These data indicate not only that the sample of vitamin B_{12b} was substantially pure, but that the major component was identical with vitamin B_{12a}. An infrared absorption band[8] at 2140 cm.$^{-1}$ exhibited by vitamin B_{12}, but which is absent in the spectrum of vitamin B_{12b}, is also absent in the spectrum of vitamin B_{12a}.

TABLE II

MICROBIOLOGICAL DATA

		L. lactis (Titrimetric)[5] units/μg.	*L. lactis* (Cup)[6] units/μg.	*L. leichmannii*[7] Aseptic addn. of sample, units/μg.	*L. leichmannii*[7] Sample auto claved with medium, units/μg.
1	Vitamin B_{12a}	7,700	11,000	9,500	2,100
2	Vitamin B_{12a} (from *S. griseus*)	6,600	10,800		2,350
3	Vitamin B_{12b} (Lederle NP-92-58-4)	6,800	11,000	10,000	1,900
4	Vitamin B_{12}	11,000	11,000	11,000	11,000

(4) Kindly sent by Dr. R. Stokstad.

(5) Caswell, Koditschik and Hendlin, *J. Biol. Chem.*, **180**, 125 (1949).

(6) Foster, Lally and Woodruff, *Science*, **110**, 507 (1949).

(7) Hendlin and Soars, *J. Biol. Chem.* in press.

Vitamin B_{12a}, the crystals from *S. griseus* and vitamin B_{12b} were assayed microbiologically using *Lactobacillus lactis* and *Lactobacillus leichmannii*. The results which are summarized in Table II show that those samples cannot be differentiated by these assays.

Vitamin B_{12} has shown "animal protein factor" activity[9] when fed to rats on a diet devoid of animal protein and containing 0.25% of thyroid powder. Vitamin B_{12a} was first assayed[1] at a level of 0.125 μg. in this test and, as seen in Table III, the response was equivalent to that elicited by 0.063 μg. of vitamin B_{12}; when one-half the dose of vitamin B_{12a} was fed, the gain in weight was also comparable to that elicited by an equal weight of vitamin B_{12}. Thus, vitamins B_{12} and B_{12a} have comparable activities in this test.

TABLE III

ACTIVITY OF VITAMIN B_{12a} IN RATS

Substance	Dose fed daily, μg.	No. of male rats	Wt. increment, g., 15 days
Controls (undosed)	...	10	27
Vitamin B_{12}	0.063	10	56
Vitamin B_{12}	.125	10	61
Vitamin B_{12a}	.063	10	60
Vitamin B_{12a}	.125	10	59

It was stated[1] that vitamin B_{12a} has 30 ± 15% of vitamin B_{12} activity in chicks. Additional tests with vitamin B_{12a}, as well as a preliminary comparison of vitamin B_{12a} and vitamin B_{12b} (NP-92-58-4), indicate that both the same order of vitamin B_{12} activity, approximately 50%, in the chick assay, under the conditions employed.

These comparisons of vitamin B_{12a} and the sample (NP-92-58-4) of vitamin B_{12b} have not revealed a difference between them, but have provided results which can be interpreted only on the basis that vitamins B_{12a} and B_{12b} are identical.

Dr. Randolph West made the first clinical test of vitamin B_{12a} on a pernicious anemia patient. He reported that it possessed activity like vitamin B_{12} and in one patient about 30% of a maximal hematological response was observed with a dose of 25 μg.[1] Dr. Tom Spies[10] has further examined the activity of vitamin B_{12a} and has found that it is effective in promoting clinical improvement and in producing positive hemopoietic responses in people with Addison's pernicious anemia, tropical sprue, non-tropical sprue, nutritional macrocytic anemia and in one case of megaloblastic anemia of infancy. Because of the extreme variability from patient to patient, comparative studies on the effect per unit of weight of vitamin B_{12} and vitamin B_{12a} will take some time to evaluate. However, it has been established that vitamin B_{12a}, like vitamin B_{12}, produces a rise in reticulocytes,

(8) Brockman, Pierce, Stokstad, Broquist and Jukes, THIS JOURNAL, **72**, 1042 (1950).

(9) Emerson, *Proc. Soc. Exp. Biol. and Med.*, **7B**, 392 (1949).

(10) Personal communication.

red blood cells, white blood cells, platelets and hemoglobin, and promotes a return of the bone marrow to normal.

Experimental

Vitamin B_{12a} from the Reaction of Vitamin B_{12} with Hydrogen in Presence of a Platinum Catalyst.—A solution containing 349 mg. of vitamin B_{12} in 70 ml. of water was shaken with approximately 300 mg. of platinum oxide catalyst and hydrogen gas under substantially atmospheric pressure at *ca.* 25° for 40 minutes. During the reaction, the color of the solution changed from red to brown. The solution was then diluted with acetone to a volume of *ca.* 400 ml. and, after a short time, the catalyst settled. After removal of the catalyst by centrifugation, the solution was further diluted with acetone to a volume of *ca.* 900 ml. and after a short time vitamin B_{12a} began to crystallize in the form of dark red slender needles. After allowing the solution to stand overnight, 200 mg. of vitamin B_{12a} was obtained. Recrystallization of this product was accomplished by dissolving the compound in *ca.* 30 ml. of water and diluting the solution with *ca.* 300 ml. of acetone; yield 150 mg. A solubility analysis showed that the product had a purity of 98.5%.

Acknowledgment.—We are indebted to Dr. Charles Rosenblum for measurements on the crystallographic properties of vitamin B_{12a}, Mr. Fred Bacher for solubility determinations, Dr. N. R. Trenner for infrared absorption spectrum determination, Dr. David Hendlin and Miss M. Soars for microbiological assays and to Drs. Gladys Emerson and Walther Ott for animal assays.

Summary

A new crystalline product, designated vitamin B_{12a}, has been produced from vitamin B_{12} by utilizing a catalytic hydrogenation technique and has been characterized by several criteria.

Using the described criteria, it is concluded that vitamin B_{12a}, the red crystalline product isolated from concentrates from *S. griseus*, and vitamin B_{12b} are identical.

Vitamin B_{12a} has a biological activity like that of vitamin B_{12} in assays using *L. lactis*, *L. leichmannii*, rats, chicks and humans.

RAHWAY, NEW JERSEY RECEIVED JULY 31, 1950

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN COMPANY]

The Isolation of Vitamin B_{12b} from Neomycin Fermentations

BY WILLIAM G. JACKSON, GEORGE B. WHITFIELD, WILLIAM H. DEVRIES, HARRISON A. NELSON AND JOHN S. EVANS

The isolation from liver of the anti-pernicious anemia factor, vitamin B_{12}, has been reported by workers in this country[1a] and in England[2]; its isolation from streptomycin fermentations has been announced.[1b,3a] The "animal protein factor" is apparently identical with, or owes its principal activity to vitamin B_{12} or B_{12b}.[4,5] Vitamin B_{12} has been converted to B_{12a} by hydrogenation,[6] and to B_{12b} by hydrogenation[7] and by "prolonged mild acid hydrolysis."[3b] Vitamin B_{12b} has been shown[8] to be the full equivalent of B_{12} against Addisonian pernicious anemia. We wish to report the isolation of vitamin B_{12b} from neomycin fermentations, and to provide the working details of an extraction process by which one may start with a neomycin fermentation[9] and obtain the vitamin in pure crystalline form.

Vitamin B_{12b} has been isolated from liver,[10] from aureomycin fermentations[11] and from streptomycin fermentations.[12,3a] It was differentiated from B_{12} by the location of its absorption peaks at 352 and 525 mμ.[11] The corresponding B_{12} peaks are at 361 and 548 mμ.[13,14] The relation of vitamin B_{12a} to B_{12b} has not been completely clarified.[15a,b] Both were obtained from B_{12} by supposedly similar hydrogenation conditions and their absorption spectra are nearly identical, save in the 315 mμ region where B_{12a} has a density $E^{1\%}_{1\,cm.}$ 80,[6] *vs.* $E^{1\%}_{1\,cm.}$ 43 for B_{12b}.

Methods of Assay.—Fermentation harvests and brown extracts were assayed microbiologically against crystalline B_{12} in a *Lactobacillus lactis* Dorner turbidimetric assay, adapted from that of Shorb.[16] The medium is a modification of that used by Guirard, *et al.*[17]

Extracts having a purity of 5 mcg. per mg. or better usually had a pink or yellowish-pink color and showed optical peaks at 526 mμ and at 352 mμ, strongly suggestive of the B_{12b} spectrum and with intensity at 526 mμ corresponding roughly to the microbiological potency. At this time Dr. Fricke of the Abbott Laboratories kindly supplied us with the complete quantitative ultraviolet and visible spectrum of crystalline B_{12b} isolated from streptomycin fermentations, and comparison showed our semi-purified extracts to be similar if not identical

(1) (a) Rickes, Brink, Koniuszy, Wood and Folkers, *Science*, **107**, 396 (1948); (b) *ibid.*, **108**, 634 (1948).

(2) Smith and Parker, *ibid.*, **43**, viii (proceedings) (1948).

(3) (a) Smith, Fantes and Ball, Abstracts of Papers, A. C. S. Meeting, Spring 1950, p. 10A; (b) Brockman, Pierce, Stokstad, Broquist and Jukes, *ibid.*, p. 11A; (c) Folkers, *ibid.*, p. 11A.

(4) Stokstad, Page, Pierce, Franklin, Jukes, Heinle, Epstein and Welch, *J. Lab. Clin. Med.*, **33**, 860 (1948).

(5) An excellent review article has just appeared summarizing the history of the anemia problem, animal protein factor, folic acid and the B_{12} vitamins: Smith, *J. Pharm. Pharmacol.*, **2**, 409 (1950).

(6) Kaczka, Wolf and Folkers, THIS JOURNAL, **71**, 1514 (1949).

(7) Brockman, Pierce, Stokstad, Broquist and Jukes, *ibid.*, **72**, 1042 (1950).

(8) Lichtman, Watson, Ginsberg, Pierce, Stokstad and Jukes, *Proc. Soc. Exptl. Biol. Med*, **72**, 643 (1949).

(9) Fermentation data are covered in a separate paper by Nelson, Calhoun and Colingsworth, presented at the September, 1950, A. C. S. meeting, Chicago, Ill.

(10) Stokstad, Jukes, Pierce, Page and Franklin, *J. Biol. Chem.*, **180**, 647 (1949).

(11) Pierce, Page, Stokstad and Jukes, THIS JOURNAL, **71**, 2952 (1949).

(12) Fricke, Lanius, DeRose, Lapidus and Frost, *Federation Proc.*, **9**, 173 (1950).

(13) Ellis, Petrow and Snook, *J. Pharm. Pharmacol.*, **1**, 60 (1949).

(14) U. S. Pharmacopoedia XIII, Third Sheet Supplement, p. 8.

(15) (a) Stokstad, Jukes, Brockman, Pierce and Broquist, *Federation Proc.*, **9**, 122 (1950). (b) ADDED IN PROOF: The identity of B_{12a} and B_{12b} has now been claimed (Brink, Kuehl and Folkers, *Science*, **112**, 354 (1950)) and the name "hydroxo-cobalamin" has been proposed (Kaczka, Wolf, Kuehl and Folkers, *ibid.*, p. 354). Vitamin B_{12}, by this nomenclature, is "cyano-cobalamin."

(16) Shorb, *J. Biol. Chem.*, **169**, 455 (1947); *J. Bact.*, **53**, 669 (1947).

(17) Guirard, Snell and Williams, *Arch. Biochem.*, **9**, 361 (1946).