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).

to their B_{12b}. On this basis we began to use density at 526 mμ as an optical assay. The purities given herein have been calculated on the basis of our final crystalline product having $E^{1\%}_{1\,cm.}$ 55.7 at 526; thus the crystals dried at 105° assay (by definition) 1000 mcg. per mg.

Isolation yields from crude product to crystals are stated in terms of absorption at 526 millimicrons. Thus, while the final crystalline product had an absorption spectrum identical with that of B_{12b} isolated by Fricke, *et al.*,[12] it is not assumed that all of the original biological or optical activity was due to B_{12b}.

Isolation from Fermentation Harvest to Crude Product

The process is designed to facilitate isolation of both neomycin and vitamin B_{12b} from the fermentation harvest. The fermented medium is acidified and heated to release the vitamin from the mycelium and to speed filtration. Adsorption on carbon at *p*H 3 separates B_{12b} from neomycin; the latter is left in the filtrate and is isolated by a procedure very similar to one developed in these laboratories[18] for the extraction of streptomycin.

Elution from the active carbon and concentration of the eluates *in vacuo* affords approximately tenfold purification from the fermentation mixture. The subsequent fractionations with 75–95% acetone and with methanol remove much inactive material, and the resin treatment accomplishes removal of small ionic compounds, and brown pigments. The effluent solution shows the pink color characteristic of the vitamin although it is still in the order of 0.5% pure.[19]

The fermented medium (1000 gal.) was adjusted to *p*H 3.0 with sulfuric acid, heated to 140°F., held 10 min., and filtered with 2% filter-aid. The clarified filtrate was stirred with 1% Darco G-60, and filtered with 0.7% filter-aid. The active cake was washed with 150 gal. of water, then eluted with 4 portions of 75% acetone at *p*H 9.3. The pooled eluates (190 gal.) were flash-evaporated *in vacuo* to an aqueous concentrate of 13 gal. for which microbiological assay showed 575 mg. of B_{12} activity, 3.8 kg. of solids, purity 0.16 mcg. per mg.

The aqueous concentrate was adjusted to *p*H 2.0 with sulfuric acid and stirred with 2.5 lb. of filter-aid during the addition of 19 volumes of acetone. The suspension was filtered and water was recycled through the active cake during adjustment to *p*H 7.0. The resulting aqueous extract (30 l.) was stirred with 1 lb. of filter-aid during the addition of 3 volumes of acetone. The inactive cake was washed with 75% acetone; the combined 75% acetone extracts were stirred with 2 lb. of filter-aid during addition of 4 volumes of acetone to reprecipitate the active material.

The active cake was extracted with 4 portions of methanol. The methanol extract (29 l.) was evaporated *in vacuo*, with addition of water to an aqueous concentrate of 8 l. for which microbiological assay showed 600 mg. of B_{12} activity, 290 g. of solids, purity 2.1 mcg. per mg. The solution was deionized over 22 l. of "Ionac C-240" in the hydrogen cycle and 19 l. of "Ionac A-300" in the hydroxyl cycle.[20] The yellowish-pink effluent (22 l.) was concentrated *in vacuo* and freeze-dried to afford 100 g. of dry powder. Microbiological assay showed 425 mg. of B_{12} activity, purity 4.2 mcg. per mg., optical assay showed 550 mg. (as B_{12b}), purity 5.5 mcg. per mg. This material is designated throughout this paper as the "crude product."

(18) Vander Brook, Wick, DeVries, Harris and Cartland, *J. Biol. Chem.*, **168**, 463 (1946).

(19) We are indebted to Mr. G. C. Colovos for initial observations on the value of resin treatment.

(20) Ion exchange resins supplied by the American Cyanamid Co.

Purification of the Crude Product

The methods outlined below, and various others, are effective when applied directly to the crude product. An effective series of steps is less simple to attain, for many accomplish the removal of similar types of impurities. We will describe five steps which work effectively in series and accomplish a two-hundred-fold purification from crude product to crystalline vitamin B_{12b}. They are, in order, extraction from water into phenol and precipitation with acetone, extraction from water into diethylacetic acid[21] and back into water by addition of petroleum ether, chromatography on carbon with 50% acetone, rechromatography on carbon with 75% methanol and crystallization from aqueous acetone.

The extraction with phenol was used earlier on liver by LaLand and Klem.[22] They used only water-immiscible solvents to precipitate from phenol whereas we have found acetone quite useful. Extraction with diethylacetic acid is effective when used as described herein or in counter-current distribution against water.

Carbon chromatography with 50% acetone followed by rechromatography over carbon with 75% methanol accomplishes more than either solvent system used twice. Smith and Cuthbertson[23] have chromatographed on carbon, eluting with aqueous ethanol or propanol. Fractional crystallization of the chromatographic eluates from aqueous acetone repeats the published work.

These procedures may be used advantageously at other stages of the purification scheme than those illustrated here. Carbon chromatography, using 25–50% acetone, will usually accomplish a 3–10-fold purification at any stage in the process. Phenol extraction is widely applicable; we have used it late in the process to minimize exposure of the personnel to the reagent. Ammonium sulfate–isopropyl alcohol distribution is a useful alternative to phenol extraction, applicable to the crude product. The aqueous extract is adjusted to 0.5–0.9 saturation with ammonium sulfate and extracted with isopropyl alcohol. The vitamin B_{12b} is recovered from the organic phase at approximately three-fold increase in purity.

Phenol Extraction.—The crude product was dissolved in 800 ml. of water, stirred with 2 g. of filter aid and centrifuged to remove a trace of white gelatinous precipitate. The clear dark red solution assayed 530 mg. of B_{12b}, 95.9 g. of solid, purity 5.53 mcg. per mg. The aqueous solution was stirred with 80 ml. of 75% phenol and centrifuged. The upper red-orange aqueous phase was separated and re-extracted with 8 ml. of 75% phenol. The spent orange-colored aqueous solution containing 23 mg. of B_{12b} and 68 g. of solids was discarded. The phenol extracts were stirred with 800 ml., then 320 ml. of acetone to precipitate a dark red powder which was dried *in vacuo*. The spent acetone was faintly yellow, containing negligible activity or solids, and was discarded. The active powder was dissolved in water to afford 400 ml. of dark red solution assaying 510 mg. of B_{12b}, 28 g. of solids, purity 18 mcg. per mg., yield 96%.

Diethylacetic Acid Extraction.—The aqueous solution above was extracted with four 400-ml. portions of diethylacetic acid. The spent aqueous solution, although dark

(21) Sold as 2-ethylbutyric acid by the Carbide and Carbon Chemicals Corporation.

(22) LaLand and Klem, *Acta. Med. Scand.*, **88**, 620 (1936); U. S. Patent 2,134,256.

(23) Smith and Cuthbertson, *Federation Proc.*, **9**, 230 (1950).

red, assayed 79 mg. of B_{12b}, 20 g. of solids, purity 3.9 mcg. per mg., and was discarded. The colors of the four extracts were dark red, dark red, red, and light red, respectively. They were washed separately with one 100-ml. portion of water each; the aqueous wash removed 54 mg. of B_{12b}, 2.4 g. of solid, purity 22 mcg. per mg., and was discarded.

The pooled washed organic extract (1600 ml.) was shaken with 200 ml. of water and 1200 ml. of petroleum ether.[24] The upper organic layer, plus a little tar, was re-extracted with 100 ml. of water. The aqueous extracts were washed with a little petroleum ether and evaporated *in vacuo* to an organic-solvent free solution. The spent solvents had negligible activity or solids and were discarded. The aqueous solution (250 ml.) assayed 370 mg. of B_{12b}, 4.6 g. of solid, purity 80 mcg. per mg., yield 73%.

Carbon Chromatography in 50% Acetone.—The column of adsorbent was prepared by stirring 100 g. of Darco G-60[25] and 34 g. of Celite 545[26] with 500 ml. of water, transferring the smooth mixture to a glass pipe of 5.0 sq. cm. cross-section, and packing by passage of water under pressure until the adsorbent compressed to a constant height of 71 cm. The liquid holdup was determined to be 313 ml.

Aqueous B_{12b} (250 ml., from the above extraction) was forced into the column under pressure, and was followed with a wash of 41 ml. of water. Aqueous acetone (50%) was then applied until the end of the chromatography. Flow rates did not exceed 2.8 ml. per min.; pressure requirements varied up to 15 lb./sq. in. The eluate was collected automatically in small fractions, which were combined into larger pools for assay. Fraction numbers represent volume eluted, in ml., beginning with the introduction of the starting material into the column. Fraction 511–855 was collected in one pool during a period of non-functioning of the fraction cutter. Past experience would indicate that the first activity was eluted at *ca.* 604 ml., where the 50% acetone began to exit. Assays on the partially concentrated eluates are shown in Table I.

TABLE I

ASSAY DATA ON ELUATES FROM CARBON CHROMATOGRAMS

Fraction[a]	Mcg. B_{12b}	%	Mg.	Mcg./mg.
Chromatography in 50% acetone				
Starting material	370,000	100	4600	80
511–855	175,000	47	494	354
855–1227	20,000	5.4	98	204
1227–1528	8,700	2.4	76	114
1528–2595	65,700	17.7	150	438
2595–3120	20,600	5.6	48	430
3120–4084	24,500	6.6	84	292
4084–5645	15,200	4.1	81	188
5645–6215	2,600	0.7	27	96
Rechromatography of Fraction 511–1227				
Starting material	175,000	100	528	330
316–346	7,900	4.5	11	718
346–426	59,800	34.2	61	980
426–496	23,300	13.3	22	1060
496–603	16,200	9.2	17	952
603–800	11,200	6.4	15	747
800–1635	31,200	17.8	48	650
Rechromatography of Fraction 1528–4084				
Starting material	93,800	100	247	378
178–196	12,300	13.1	13	945
196–268	25,400	27.1	26	977
268–496	19,700	21.0	24	821
496–814	5,000	5.3	9	555

[a] Fraction numbers represent total volume eluted, in ml.

The fractions comprising the first peak (511–1227) were pooled and evaporated further to afford a concentrate of 250 ml. which assayed 182 mg. of B_{12b}, 550 mg. of solids, purity 330 mcg. per mg. The fractions comprising the second peak (1528–4084) were pooled and evaporated further to afford a concentrate of 55 ml. which assayed 103 mg. of B_{12b}, 272 mg. of solids, purity 378 mcg. per mg.

Rechromatography of Fraction 511–1227.—The column of adsorbent was prepared as above using 11 g. of Darco G-60 and 3.7 g. of Celite 545 in a glass tube of 1.0 sq. cm. cross-section. The liquid holdup was 37 ml.

Aqueous B_{12b} (240 ml., 96% aliquot of Fraction 511–1227) was forced into the column under pressure, and was followed with a wash of 25 ml. of water. Aqueous methanol (75%) was then applied until the end of the chromatography. Flow rates were 0.1–0.3 ml. per min., pressure 1–2 lb./sq. in. Assays on the partially concentrated eluates are shown in Table I. The concentrated fractions were placed in a water-bath at 30° and blown to dryness in tared tubes with a stream of nitrogen. Red amorphous residues were obtained.

Fraction 346–426 was crystallized as follows. The residue from the drying of a 93% aliquot, contained in *ca.* 3 ml. of water, was filtered, and diluted with acetone until a solution of 26 ml. was obtained which was hazy at 35°. The mixture was placed at room temperature in an acetone atmosphere over calcium sulfate. The resulting slow dehydration and absorption of acetone afforded 52.8 mg. of dark red needles of 0.5–1.0 mm. length, which lost 5.1% moisture on drying at 105° for 2 hr. *in vacuo*. The dried crystals assayed $E^{1\%}_{1\,cm.}$ 55.5 at 526 mμ, a recovery of 90% from the chromatographic eluate.

Rechromatography of Fraction 1528–4084.—The column of adsorbent was prepared as above using 6.0 g. of Darco G-60 and 2.0 g. of Celite 545 in a glass tube of 0.49 sq. cm. cross-section. The liquid holdup was 20 ml.

Aqueous B_{12b} (50 ml., 91% aliquot of Fraction 1528–4084) was forced into the column under pressure and was followed with a wash of 54 ml. of water. Aqueous methanol (75%) was then applied until the end of the chromatography, Flow rates were *ca.* 0.1 ml. per min., pressure 4 lb./sq. in. Assays on the partially concentrated eluates are shown in Table I. The concentrated fractions were placed in a water-bath at 30° and blown to dryness in tared tubes with a stream of nitrogen. Red amorphous residues were obtained.

Fraction 178–268 (91.4% aliquot) was crystallized from aqueous acetone in the manner previously described. Dark red needles (28.2 mg.) were obtained which were much smaller than the first ones, and which lost 9.9% moisture on drying at 105° for 2 hr. *in vacuo*. The dried crystals assayed $E^{1\%}_{1\,cm.}$ 55.7 at 526 mμ, a recovery of 73% from the chromatographic eluate.

Some Properties of Crystalline Vitamins B_{12b} and B_{12}

The preceding sections have described the isolation and crystallization of two chromatographic fractions of vitamin B_{12b}. We do not fully understand why the chromatography in 50% acetone should have separated them since the final products were identical in all microbiological and physical properties. The crystalline material obtained represents a 19% yield from the crude pink product, where optical assay is first applicable. Adjacent fractions at high purity could no doubt have been crystallized to improve the yield.

Ultraviolet and Visible Spectra.[27]—In Fig. 1 are shown the absorption curves for vitamins B_{12} and B_{12b} in neutral aqueous solution; the data are presented in Table II. Our vitamin B_{12b} curve is identical with that of Fricke, *et al.*[12] The vitamin B_{12} was extracted in these laboratories from streptomycin fermentations. Its absorption curve is identical with that of material purchased from Merck & Company, Inc., although ours appears to be about 94% pure.[14] The literature contains an

(24) "Skellysolve B," b. p. 60–70°, supplied by the Skelly Oil Company.

(25) Activated carbon supplied by the Darco Corporation.

(26) Diatomaceous filter aid supplied by Johns-Manville.

(27) Since submission of our manuscript for publication, Pierce *et al.*, have published absorption data on their purest B_{12b} which substantiate those reported here: Pierce, Page, Stokstad and Jukes, THIS JOURNAL, 72, 2615 (1950).

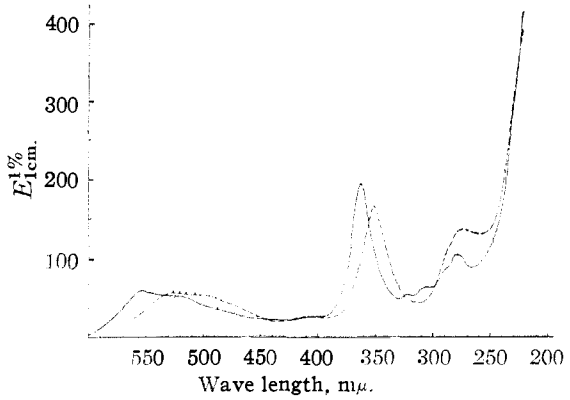

Fig. 1.—Ultraviolet absorption spectra in neutralaqueous solution of: —, vitamin B_{12}; – – –, vitamin B_{12b}.

absorption curve of B_{12} which is apparently about 87% pure.[13] Whereas vitamin B_{12} is relatively unaffected, high *p*H causes bathochromic shift of the B_{12b} spectrum.

TABLE II

ULTRAVIOLET AND VISIBLE ABSORPTION SPECTRA

Vitamin B_{12}		Vitamin B_{12b}	
Mμ	$E^{1\%}_{1\ cm.}$	Mμ	$E^{1\%}_{1\ cm.}$
552	59	550	34
430	21	527	56
362	194	500	53
330	48	430	18
323	54	410	25
319	52	400	24
308	63	351	165
300	61	310	43
279	104	274	136
267	88	260	131
240	158	240	180
220	412	220	389

similar to a published curve of Barer, Cole and Thompson[28] on a single crystal obtained from Lester Smith.

The 2136 cm.$^{-1}$ band appearing in B_{12}, and not in B_{12b} is in a spectral region where few groups absorb. The carbodiimide band (—N=C=N—) gives a very strong absorption in this region and probably the absorption of even one such group would be detected in a molecule of this size. Other 1,2-dienoids which absorb near 2100 cm.$^{-1}$ are C=C=O and N=C=S.[29] We are indebted to Mrs. J. L. Johnson for the infrared data and interpretation.

Magnetic Susceptibility.—Both vitamins B_{12b} and B_{12} are diamagnetic, indicative of an octahedral arrangement around covalent cobalt(III). Our samples were run through the kindness of Dr. M. Calvin and his associates.[30]

Microbiological Assay.—In Table III are presented the results of three microbiological assays in which vitamin B_{12b} is run against vitamin B_{12} as standard. It will be seen that B_{12b} is about 140% as active as B_{12} on *L. leichmannii* assay with aseptic addition of sample. On *L. lactis* Dorner assay, B_{12b} is 100% as active as B_{12} with aseptic addition of sample, and 62% as active when the sample is autoclaved with the assay medium.[16] Vitamin B_{12b} is 95% as active as B_{12} on the Sulfanilamide–*E. coli* plate assay.[31]

TABLE III

MICROBIOLOGICAL ASSAY OF CRYSTALLINE VITAMIN B_{12b} *vs.* B_{12}

L. lactis Dorner		*L. leichmannii*	Sulfanilamide–*E. coli*
1000[a]	680[b]	1490[a]	1010
1000[a]	570[b]	1370[a]	900

Results expressed as micrograms of vitamin B_{12} activity per mg. of vitamin B_{12b}. [a] Aseptic addition of samples. [b] Samples autoclaved with medium.

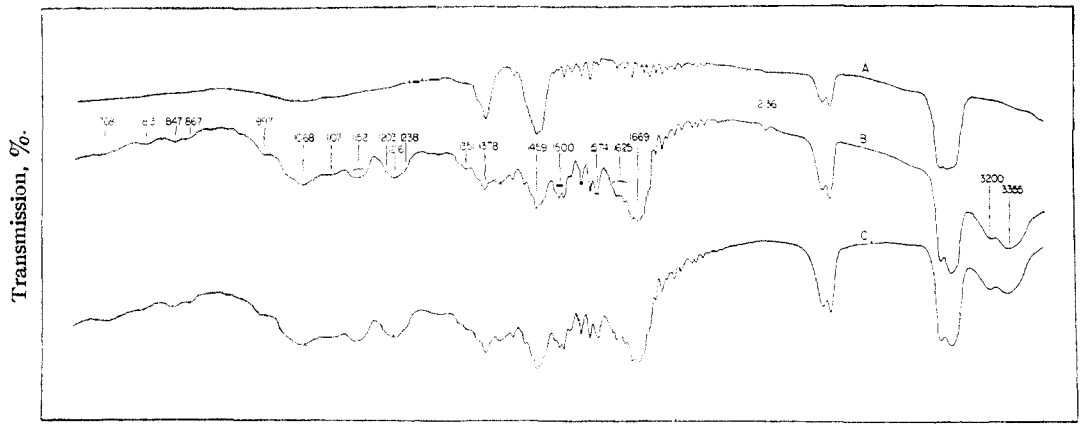

Fig. 2.—Infrared absorption spectra in Nujol mull of: A, Nujol mull and atmospheric absorption; B, vitamin B_{12}; C, vitamin B_{12b}.

Infrared Spectra.—In Fig. 2 are shown the infrared absorption curves for vitamins B_{12} and B_{12b} in Nujol mull. The curves are identical for B_{12} and B_{12b} with the single exception of the absence of the 2136 cm.$^{-1}$ band in B_{12b}. The spectrum of our B_{12} is identical with that found for B_{12} purchased from Merck & Company, Inc., and is

(28) Barer, Cole and Thompson, *Nature*, **163**, 198 (1949).

(29) ADDED IN PROOF: The 2136 cm.$^{-1}$ band has since been ascribed to cyanide absorption.[18b]

(30) Wallman, Cunningham and Calvin, personal communication.

(31) An "inhibition analysis" assay with *E. coli* as test organism, described by Shive, *Ann. N. Y. Acad. Sci.*, in press, adapted to a plate method by variations of inoculum, sulfanilamide and agar concentrations by R. E. Crandall and J. J. Stefaniak, Eli Lilly and Company, unpublished work.

These assay results are in line with the observations of others ([3b,c,12]) on the instability of B_{12b} in the presence of certain reducing agents and assay media constituents such as thiamin and ascorbic acid. The apparent differences in microbiological assay attributed to vitamins B_{12a} and B_{12b}[7] may well be the result of assay methods.

Acknowledgments.—The production and isolation of vitamin B_{12b} is obviously the result of group effort by many individuals. We are grateful to Dr. G. F. Cartland for active direction of the program, to Dr. C. E. Meyer and Messrs. R. A. Delor and H. H. Buskirk for countless microbiological assays, to Dr. George Pish for ultraviolet data, and to Messrs. L. Scholten, W. A. Struck and N. A. Drake for other physical and optical measurements.

Summary

An isolation process for vitamin B_{12b} is described by which it may be obtained as a co-product of neomycin produced by fermentation. The steps include heating the acidified fermentation medium to free the vitamin from the mycelium, adsorbing on carbon, eluting with aqueous acetone, evaporating to a small volume, fractionating between 75 and 95% acetone, extracting into methanol, transferring to water, and deionizing to afford a pink amorphous product of 0.5% purity. The crude product is then extracted from water into phenol, precipitated with acetone, extracted from water into diethylacetic acid, precipitated by petroleum ether, chromatographed on carbon with 50% aqueous acetone, then with 75% aqueous methanol and finally crystallized as dark red needles from aqueous acetone.

Crystalline vitamins B_{12} and B_{12b} are compared on three microbiological assays, and their ultraviolet, visible and infrared absorption curves are presented.

KALAMAZOO, MICHIGAN RECEIVED JUNE 14, 1950

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF CHAS. PFIZER AND CO., INC.]

Netropsin, a New Antibiotic Produced by a Streptomyces

BY A. C. FINLAY, F. A. HOCHSTEIN, B. A. SOBIN AND F. X. MURPHY

A new antibiotic has been obtained from culture filtrates of a hitherto undescribed Actinomycete, *Streptomyces netropsis*, which was isolated from a soil sample. The antibiotic has been assigned the name Netropsin.[1] Its toxicity appears to be high for parenteral administration; it may, however, have other therapeutic applications.

Netropsin has been prepared as its crystalline sulfate, hydrochloride and picrate. The free base is unstable and has not been isolated as yet. The analysis and molecular weight of netropsin salts indicate it to be a tetraacidic base corresponding to the formula $C_{32}H_{48}N_{18}O_4$. This formula assignment is based primarily upon the analysis of the sulfate; the hydrochloride and picrate are less easily purified and their absolute purity is in doubt. It is relatively stable in acid solution, having a half life of about two hours in 1 *N* sulfuric acid at 100°. When netropsin hydrochloride is dissolved in an equivalent quantity of 0.2 *N* sodium hydroxide, inactivation is complete in less than two hours at 25°.

The ultraviolet absorption spectrum of an aqueous solution of the sulfate at *p*H 5.5 exhibits two peaks, $E^{1\%}_{1\,cm.}$ 429 at 236 mμ, $E^{1\%}_{1\,cm.}$ 436 at 296 mμ (see Fig. 1). This spectrum is virtually unchanged at *p*H 10. The infrared absorption spectrum of a Nujol mull of netropsin sulfate exhibits no sharp peaks. Broad maxima, characteristic of large molecules, occur at the following estimated wave lengths: 3320, 1670, 1550, 1410, 1265, 1210, 1145, 1096, 1057, 961 and 810 $cm.^{-1}$. In Fluorolube-S mulls, additional bands occur at 3100, 1468 and 1434 $cm.^{-1}$. The hydrochloride shows no optical activity in water, methanol or dimethylformamide solutions. The titration of netropsin hydrochloride with sodium hydroxide in aqueous solution indicates all basic groups to be of similar strength.

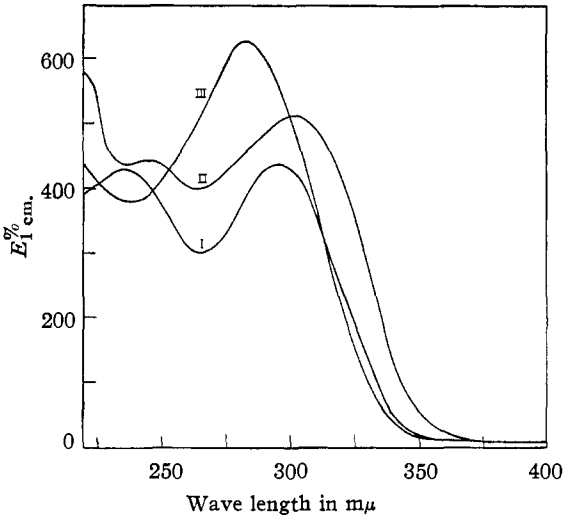

Fig. 1.—Absorption spectra: I, Netropsin sulfate in water, *p*H 5.5; II, degradation product I in 0.1 *N* NaOH; III, degradation product I in 0.1 HCl.

Netropsin gives a positive Sakaguchi test and a positive Ehrlich test. Ninhydrin, Tollens, fuchsin-aldehyde, murexide, ferric chloride, aminoantipyrine (for phenol) and 2,4-dinitrophenylhydrazine tests are all negative. Primary amino nitrogen, as determined by the method of Van Slyke, is absent. It absorbs hydrogen slowly in aqueous solution over Adams platinum catalyst to yield a biologically inactive product. Two crystalline basic hydrolysis products of netropsin have been isolated. The ma-

(1) Netropsin is the trade name of Charles Pfizer and Co. for the antibiotic produced from the fermentation of *Streptomyces netropsis*.