Anal. Calcd. for $C_{28}H_{40}O_7N_2$: C, 65.10; H, 7.81; N, 5.42. Found: C, 65.05; H, 7.80; N, 5.01.

To 1.283 g. of this diazoketone in 22 ml. of methanol at 55–60°, a suspension of silver oxide in 8 ml. of methanol, which had been freshly prepared from 1.5 ml. of 10% aqueous silver nitrate, was added in small portions with shaking during the course of one hour; the reaction mixture was then refluxed for three hours, cooled and centrifuged. The supernatant was evaporated to dryness, and the residue dissolved in 3 ml. of benzene and passed over a short column containing 3 g. of aluminum oxide (Harshaw). Elution with 75 ml. of benzene containing 5% methanol served to remove colloidal silver from the crude reaction product which was then hydrolyzed by refluxing for one hour in 15 ml. of 10% potassium hydroxide in 90% methanol. The reaction mixture was poured into a dilute potassium carbonate solution and extracted with ether, thereby removing 24 mg. of neutral material. An ether extract of the acidified aqueous phase yielded 1.032 g. of a colorless oil, which on the addition of little acetone, gave 717 mg. of thick rectangular plates, m. p. 213–215.5°. Repeated crystallization from acetone raised the melting point to 219.5–220°.

Anal. Calcd. for $C_{25}H_{42}O_5$: C, 71.05; H, 10.02. Found: C, 71.02; H, 9.90.

Methyl Ester of 25-Homocholic Acid (III).—This was prepared by dissolving 25-homocholic acid in little methanol and treating with ethereal diazomethane. The reaction product was repeatedly crystallized from acetone to yield needles, m. p. 166–167° (occasionally a lower-melting form was obtained, m. p. 150–151°).[9]

Anal. Calcd. for $C_{26}H_{44}O_5$: C, 71.52; H, 10.16. Found: C, 71.39; H, 10.06.

Coprostanetetrol-3(α),7(α),12(α),25 (IV).—A solution of methylmagnesium iodide, prepared from 316 mg. of magnesium and 0.8 ml. of methyl iodide in 50 ml. of absolute ether, was added to 1.062 g. of the methyl ester of 25-homocholic acid in 25 ml. of absolute benzene. The mixture was heated so that the ether distilled in the course of one hour, and was then refluxed for two hours. The product was decomposed by the addition of 50 ml. of cold saturated ammonium chloride, 50 g. of crushed ice and 15 ml. of 20% sulfuric acid. The aqueous phase was extracted repeatedly with ether; the combined benzene and ether extracts were washed with dilute hydrochloric acid, then with water, and finally evaporated. The residue was hydrolyzed by refluxing for one hour in a mixture of 15 ml. of methanol and 5 ml. of 10% potassium hydroxide, diluted with water and extracted with ether to yield 352 mg. of neutral material. The aqueous phase gave, on acidification, 581 mg. of a crystalline product, m. p. 215°, consisting of 25-homocholic acid. The neutral fraction crystallized readily from acetone to yield 252 mg., m. p. 185.5–187°; repeated crystallization from acetone raised the melting point to 188–189°. This product is readily soluble in alcohol, moderately soluble in acetone, and rather difficultly soluble in ethyl acetate; it may also be crystallized from the latter solvent. Like scymnol, it gives an intense orange-red color when dissolved in concentrated sulfuric acid at room temperature; cholic acid, when treated similarly, gives a yellow color with marked green fluorescence. The three aforementioned compounds give a positive Hammersten test.

Anal. Calcd. for $C_{27}H_{48}O_4$: C, 74.26; H, 11.08. Found: C, 74.15; H, 11.09.

Tetraacetate of Coprostanetetrol-3(α),7(α),12(α),25.—118 mg. of the above product (IV), m. p. 188–189°, was dissolved in 1 ml. of anhydrous pyridine and 2 ml. of acetic anhydride added. The solution was heated for twenty-five hours on the steam-bath under anhydrous conditions, and then evaporated *in vacuo*. The residue was taken up in ether and washed successively with dilute hydrochloric acid, dilute sodium bicarbonate solution and water. Evaporation yielded 154 mg. of a product which on treatment with methanol gave a micro-crystalline product, m. p. 142°; recrystallization from the same solvent raised the melting point to 142.5°.

Anal. Calcd. for $C_{35}H_{56}O_8$: C, 69.50; H, 9.33. Found: C, 69.70; H, 9.15.

Acknowledgments.—The author is indebted to Mr. Irwin Schreibman and Miss Edith Goldberg for their technical assistance. Mr. James Rigas performed the microanalyses reported in this paper.

Summary

1. 25-Homocholic acid has been prepared from cholic acid by application of the Arndt–Eistert method.

2. Coprostanetetrol-3(α),7(α),12(α),25 has been prepared from 25-homocholic acid.

PHILADELPHIA, PA. RECEIVED FEBRUARY 1, 1947

[CONTRIBUTION FROM THE RESEARCH LABORATORIES, PARKE, DAVIS AND COMPANY]

Isolation and Characterization of Vitamin Bc from Liver and Yeast.[1] Occurrence of an Acid-labile Chick Antianemia Factor in Liver

BY J. J. PFIFFNER, S. B. BINKLEY, E. S. BLOOM AND B. L. O'DELL[2]

Introduction

The chemical nature of the antianemia substances present in liver and in yeast is a problem which has attracted attention for some time. In 1925 Whipple and Robscheit-Robbins[3] demonstrated that the feeding of beef liver exerted a favorable influence on severe secondary anemia of dogs and the following year Minot and Murphy[4] reported the effectiveness of whole liver therapy in pernicious anemia. In spite of numerous attempts[5] the so-called antipernicious anemia principle has not been obtained in pure form probably because of the fact that its concentration can be

(1) Angier, *et al.* (*Science*, **103**, 667 (1946)), demonstrated by degradation and synthesis the structure of the liver *L. casei factor* to be N[4-[(2-amino-4-hydroxy-6-pteridyl)-methyl]amino}benzoyl]-glutamic acid and named the compound pteroylglutamic acid. A sample of the synthetic compound was generously supplied us by the Lederle Laboratories. We found it to be identical with the compound which we isolated from liver and yeast and which we tentatively called vitamin Bc (*Science*, **97**, 404 (1943)) pending elucidation of its structure.

(2) Present addresses: S. B. Binkley, Bristol Laboratories, Syracuse, N. Y.; B. L. O'Dell, University of Missouri, Columbia, Mo.; E. S. Bloom, E. I. Dupont de Nemours & Co., Wilmington, Del.

(3) Whipple and Robscheit-Robbins, *Am. J. Physiol.*, **72**, 395 (1925); **72**, 419 (1925).

(4) Minot and Murphy, *J. Am. Med. Assoc.*, **87**, 470 (1926).

(5) For review of literature see Subbarow, Hastings and Elkin, in "Vitamins and Hormones," Academic Press, Inc., 1945, Vol. III, pp. 237–296.

measured only on pernicious anemia patients in relapse.

Wills[6] in 1931 demonstrated the curative effect of yeast and yeast extract as well as crude liver extract in macrocytic tropical anemia and showed that the effect was not due to the antipernicious anemia principle. She corroborated these findings on nutritionally deficient monkeys[7] but difficulties with the monkey assay prevented any significant progress in purifying the active substance.[8] The successful development of a nutritional anemia in chicks by Hogan and Parrott[9] provided a new assay tool for the study of antianemia active substances in liver and yeast. The present report deals with the isolation and characterization of a new chick antianemia compound from hog liver, horse liver and yeast. Evidence is also presented on the occurrence in liver of an as yet unidentified acid-labile chick antianemia factor which has high microbiological growth activity.

Part I

Isolation of Vitamin Bc from Liver.—Some years ago Hogan, Boucher and Kempster[10] developed a simplified ration for growing chicks. Various commercial liver extracts were used as a supplement of the vitamin B complex. The dietary conditions appeared adequate at the time but, on continued use, Hogan and Parrott[9] obtained quite variable results. They traced failures of the diet to variations in the liver extracts employed and observed that some of the chicks suffered from a severe anemia. With certain alterations in the dietary regimen they were able to develop a macrocytic hyperchromic anemia consistently in chicks and to demonstrate that the anemic state was due to the lack of an unrecognized component of the vitamin B complex. For convenience they designated the chick antianemia factor vitamin Bc. On the basis of these observations O'Dell and Hogan[11] developed a curative assay procedure and a concentration method which in their hands yielded products about 300 times the potency of fresh liver. Their method was based upon the adsorption of vitamin Bc on fuller's earth at a *p*H of 1 and elution with a dilute aqueous ammonia solution.

We undertook the isolation of this vitamin from hog liver in 1940 on the basis of the work of Hogan and Parrott.[9,12] We were unable to apply adsorption with fuller's earth at *p*H 1 effectively. Yields were poor, ranging from 6 to 25%, with a concentration of only 3 or 4 times. The low yield was apparently due to the sensitivity of the activity to the strongly acid conditions necessary for adsorption. In casting about for a more suitable adsorbent we examined the Amberlite ion exchange resins and found that Amberlite IR-4 adsorbed vitamin Bc almost quantitatively and released the vitamin activity to dilute aqueous ammonia solution in 60–80% yield. Superfiltrol was also found to be a suitable adsorbent liberating the factor to dilute ammonia solutions. The application of these two adsorption steps yielded fractions containing about 0.1–0.2% of the vitamin.

(6) Wills, *Brit. Med. J.*, **1**, 1059 (1931).

(7) Wills and Bilimoria, *Indian J. Med. Research*, **20**, 391 (1932).

(8) For review of the literature see Day, in "Vitamins and Hormones," Academic Press, Inc., 1944, Vol. II, pp. 71–105.

(9) Hogan and Parrott, *J. Biol. Chem.*, **132**, 507 (1940); **128**, Proc. xlvi (1939).

(10) Hogan, Boucher and Kempster, *J. Nutrition*, **10**, 535 (1935).

(11) O'Dell and Hogan, *J. Biol. Chem.*, **149**, 323 (1934).

(12) Professor Hogan of the University of Missouri kindly placed at our disposal the details of the curative assay procedure he was then using.

At about this time Mills, Briggs, Elvehjem and Hart[13] demonstrated that the liver fraction of Hutchings, *et al.*,[14] which stimulated the growth of *L. casei*, contained a factor or factors essential for hemoglobin formation in growing chicks and remarked on the possible identity of the norite eluate factor and vitamin Bc of Hogan. We then assayed our vitamin Bc fractions and found them highly active in stimulating the growth of *L. casei*. Fractionation studies were continued using *L. casei* growth stimulating activity as a guide and steps which were found to be promising by this criterion were checked for efficiency in concentrating the chick antianemia activity.

In earlier studies on the *L. casei* growth factor Snell and Peterson[15] observed that it was not extracted from an aqueous solution with butyl alcohol at *p*H 7.5 or higher but was extracted at *p*H 4. Hutchings, Bohonos and Peterson[14] found charcoal a good adsorbent for the microbiological growth factor and suggested among other preparative steps the use of zinc precipitation which in their hands proved to be somewhat specific. The successive application of charcoal adsorption, differential butyl alcohol extraction, and zinc precipitation to our chick antianemia concentrates yielded fractions containing about 5 to 10% of the vitamin. Such concentrates were found to exhibit specific absorption in the ultraviolet. In further isolation work we employed three criteria, ultraviolet absorption characteristics, *L. casei* growth stimulating activity and chick antianemia activity in the order named. These properties ran parallel and the experiments led to the isolation from hog liver of a pure crystalline compound (Fig. 1) exhibiting both biological activities and possessing specific ultraviolet absorption. In a preliminary note[16] we retained the tentative designation vitamin Bc for the pure crystalline substance following the terminology of Hogan and Parrott.[9]

Because of the occasional scarcity of hog liver we had the opportunity of applying the isolation methods to horse liver. A crystalline compound was isolated from horse liver (Fig. 2) which was identical in all respects with the compound which we had previously isolated from hog liver.

In the course of the study many different fractionation procedures were tried as well as minor variations of individual steps. Procedures are presented by which the crystalline vitamin was obtained from either type of liver in yields of 1 to 4% of the quantity estimated to be present in the free form.[17]

Methods of Assay.[18]—In early experiments the curative chick assay as described by O'Dell and Hogan[11] was employed. In later work a prophylactic preventive assay technique was used as described by Campbell, Brown and Emmett.[19] The method of Mitchell and Snell[20] was employed in microbiological assays with *Lactobacillus casei* and *Streptococcus faecalis*. As a standard for chick assay a hot aqueous extract of hog liver which had been desiccated under reduced pressure at 65° was used. Microbiological potencies were evaluated against a partially purified hog liver extract and expressed as arbitrary units. When the crystalline vitamin was isolated these extracts

(13) Mills, Briggs, Elvehjem and Hart, *Proc. Soc. Exp. Biol. Med.*, **49**, 186 (1942).

(14) Hutchings, Bohonos and Peterson, *J. Biol. Chem.*, **141**, 521 (1941).

(15) Snell and Peterson, *J. Bact.*, **39**, 273 (1940).

(16) Pfiffner, Binkley, Bloom, Brown, Bird, Emmett, Hogan and O'Dell, *Science*, **97**, 404 (1943).

(17) Liver as it is removed from the animal contains essentially all the vitamin in a conjugated form. The ratio of the quantity of the free to the conjugated form varies with the efficiency of autolysis, intended or otherwise, in the storage and extraction procedure. The isolation and chemistry of a conjugated form of the vitamin will be discussed in a later paper.

(18) Assays were carried out in the vitamin and nutrition division. We wish to thank Dr. O. D. Bird for conducting the numerous microbiological assays and Mr. C. J. Campbell for the chick assays.

(19) Campbell, Brown and Emmett, *J. Biol. Chem.*, **152**, 483 (1944).

(20) Mitchell and Snell, *Univ. Texas Pub.*, No. 4137, 36 (1941).

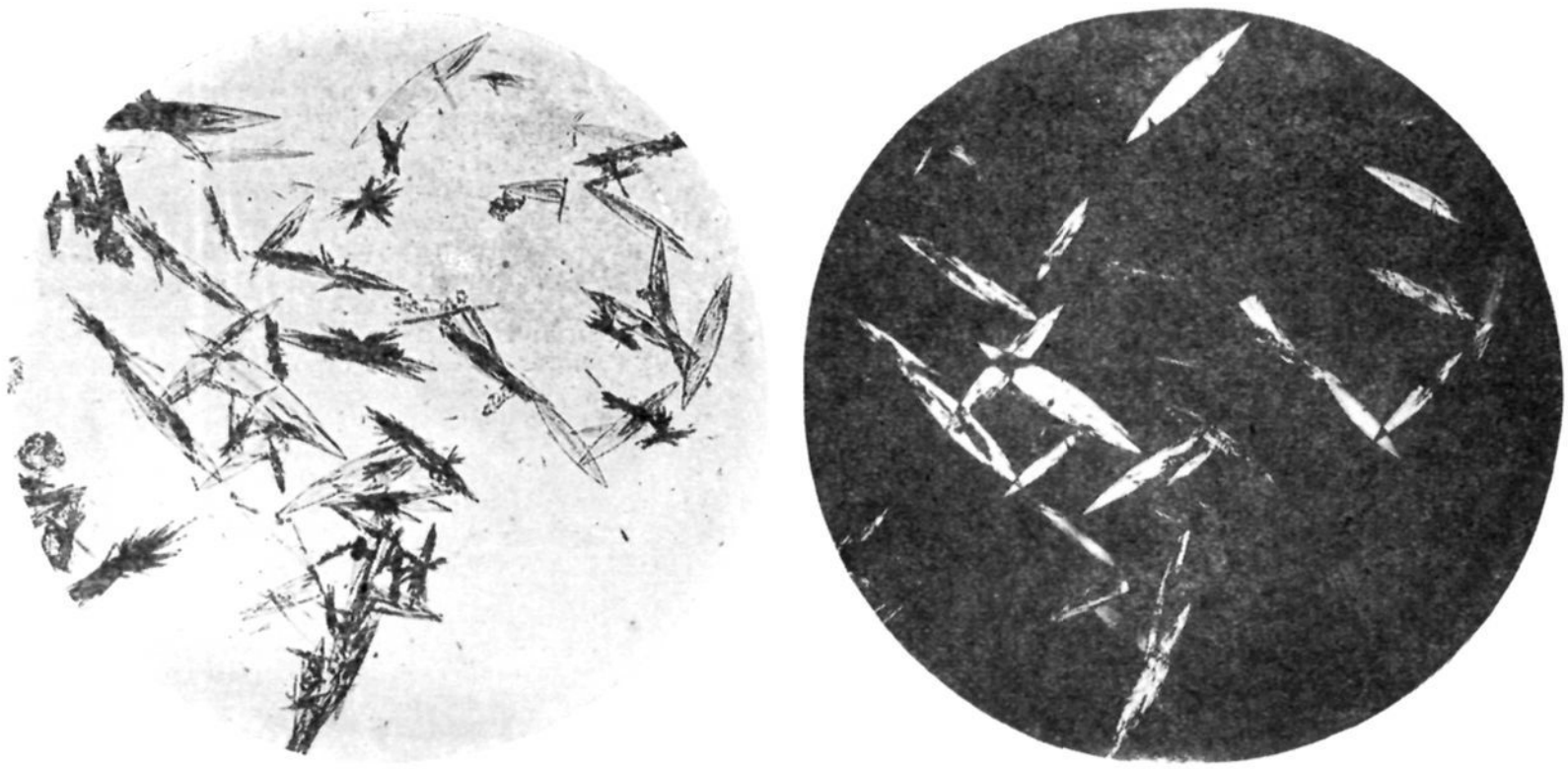


Fig. 1.—Vitamin Bc crystals from hog liver. Ordinary light on left. Polarized light on right (× 85).

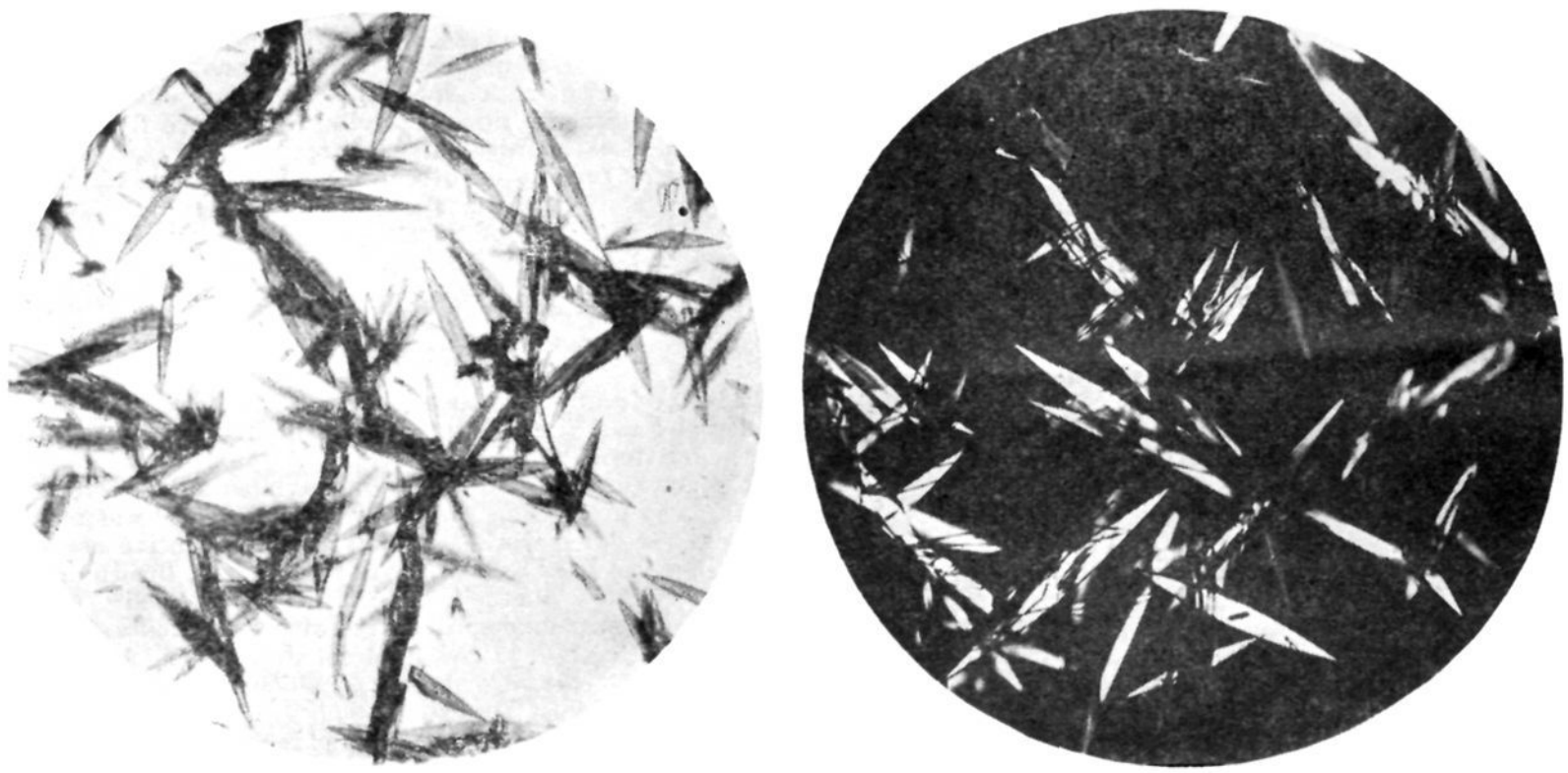


Fig. 2.—Vitamin Bc crystals from horse liver. Ordinary light on left. Polarized light on right (×85).

were evaluated in terms of the pure substance. Biological activities of crude fractions can therefore be expressed in terms of the pure vitamin. Observations on ultraviolet absorption were made with a Beckman spectrophotometer.[21]

Extraction of Liver.—One thousand pounds of frozen hog liver was allowed to thaw at room temperature overnight and then ground in a meat chopper. The finely ground tissue was allowed to stand for ten hours to autolyze. It was extracted twice with hot water (85°), about 220 gal. being used in the first extraction and half that amount in the second. The extracts were filtered (filter press) and evaporated in a vacuum steam pan to a volume of approximately 60 gal. This solution contained 45 kg. of solids and approximately 1.5 g. of vitamin Bc activity. The vitamin content of extracts of autolyzed hog liver varied from 0.2 to 1.8 g. per 1000 lb. of tissue.

(21) We wish to thank Dr. J. M. Vandenbelt for the ultraviolet absorption measurements.

Amberlite Adsorption and Elution.—The solution was filtered slowly through 3.5 cu. ft. of Amberlite IR-4[22] in a 6-in. column. The rate of flow was adjusted to about 10 gal. per hour. The column was washed with 12.5 gal. of

(22) Amberlite IR-4 was obtained from the Resinous Products and Chemical Company, Philadelphia, Pa. We have found that Amberlite IR-1 also will adsorb the vitamin but only from more highly purified fractions. The indications are that these resins are acting as adsorbents rather than as true ion exchange agents since the vitamin can be eluted readily from either resin with diluted ammonia water while the conventional methods of regenerating the resins failed to release the vitamin in good yield. Amberlite IR-4 as received from the manufacturer was washed thoroughly with water before use. The same batch of resin was used repeatedly and lost none of its efficiency as an adsorbent for vitamin Bc by continued use. It was regenerated in the usual manner. For details on the use of the Amberlite resins see Myers, Eastes and Myers, *Ind. Eng. Chem.*, **33**, 697 (1941).

water. The percolate and washing contained only about 10 mg. of the vitamin. The resin adsorbate was transferred to a tank and eluted twice with 20-gal. portions of 5% aqueous ammonia solution. This elution was carried out at room temperature with thirty minutes of stirring. The combined eluates were evaporated to about 6 gal. in a steam-pan under reduced pressure. This solution which was at *p*H 5.5 contained about 1.0 g. of vitamin Bc activity and 3 kg. of solids.

Superfiltrol Adsorption and Elution.—The concentrate was diluted to 50 gal. with water, the *p*H adjusted to 3 with sulfuric acid, and 15 pounds of Superfiltrol[23] added to adsorb the vitamin. After stirring the mixture for one hour it was filtered. The filtrate contained about 15 mg. of vitamin Bc activity. The Superfiltrol adsorbate was washed with 20 gal. of 50% ethanol which removed 180 g. of solids and about 2 mg. of vitamin Bc activity. The adsorbate was then eluted twice with 30-gal. portions of hot 5% ammonia in 50% ethanol, the mixture being held at the boiling temperature for half an hour before filtering. The combined eluates were boiled down to about 6 liters under reduced pressure. This concentrate contained 480 g. of solids and 540 mg. of vitamin Bc activity.

Norite Adsorption and Elution.—The solution was diluted to 12 liters, sulfuric acid (10%) added to *p*H 3, and 200 g. of Norite SG-11 added. After stirring the mixture for one hour it was filtered and the adsorption was repeated on the filtrate with a similar quantity of Norite SG-11. The filtrate from the second adsorption contained about 4 mg. of vitamin Bc activity. The Norite adsorbates were washed with water and then eluted twice with 5-liter portions of 5% ammonia in 50% ethanol, refluxing the mixture for thirty minutes before filtering. The combined eluates were concentrated to a volume of 1.5 liters and contained 140 g. of solids and 270 mg. of vitamin Bc activity. Adsorption with Norite SG-11 was repeated from a volume of 6 liter at a *p*H of 3 using 100 g. of adsorbent, stirring for one hour and eluting twice with 1.5-liter portions of 5% ammonia in 50% ethanol. After the eluates were concentrated to a volume of 400 ml. the concentrate was found to contain 30 g. of solids and 150 mg. of vitamin activity.

Butyl Alcohol Extraction.—The solution which was at *p*H 5.6 was extracted continuously for forty-eight hours with butyl alcohol at a pressure of about 6 cm. of mercury. The butyl alcohol extractives weighed 7 g. and contained only 3 mg. of vitamin Bc activity. Hydrochloric acid (10%) was added to adjust the aqueous solution to *p*H 3 and butyl alcohol extraction was continued for forty-eight hours. The aqueous solution contained about 20 g. of solids and 10 mg. of vitamin Bc activity. The butyl alcohol extract contained 4 g. of solids and 85 mg. of the activity. It was concentrated to approximately 100 ml. and chilled for several days in the refrigerator.

Barium and Zinc Precipitations.—The solid (2.5 g. containing 78 mg. of vitamin activity) which separated from the butyl alcohol was collected and extracted on the steam-bath 4 times with 200-ml. portions of 90% methanol at the boiling temperature. The combined methanol extracts were cooled to room temperature, filtered and the barium salts precipitated by the addition of 0.2 *N* barium hydroxide in slight excess. The barium salts were filtered off, the excess alkali washed out with 90% methanol, taken up in water and the water insoluble barium salts discarded. The water soluble barium salts were precipitated in a volume of 1 liter at neutral reaction with excess zinc acetate. The zinc salts were collected (*ca.* 0.3 g. dry weight containing 70 mg. of activity), suspended in 40 ml. of water and decomposed by adding 1.5 g. of ammonium oxalate and heating on the steam-bath. After chilling overnight at 0–5° the precipitated zinc oxalate was removed at the centrifuge and washed with water. The water washings were added to the decanted supernatant solution making a volume of about 50 ml.

(23) Superfiltrol was obtained from the Filtrol Corporation, 315 West 5th Street, Los Angeles, California.

Acid Precipitation and Crystallization.—The *p*H was adjusted to 2.8 with hydrochloric acid (10%) and the crude vitamin precipitated almost immediately. On standing overnight in the refrigerator the precipitation was complete. Seven mg. of the vitamin in the supernatant solution failed to precipitate. The precipitate was collected at the centrifuge, washed well with water, leached out twice with 15-ml. portions of 0.1 *N* barium hydroxide and reprecipitated by addition of 10% hydrochloric acid to *p*H 2.8. The precipitate was collected and crystallized by dissolving in 80 ml. of hot water, clearing of small amounts of pigmented debris at the centrifuge and allowing to cool slowly. The product, 40 mg., separated in spherules. Another 20 mg. of crystalline material was recovered by working up the later side fractions.

The crystallinity of the product at this stage was often not apparent even under high magnification. However between crossed nicols the spherules exhibited parallel extinction typical of spherulites composed of radiating fibers or fine needles.

The compound as it first crystallized was often contaminated with traces of brown pigment material and on analysis contained up to 1% of ash. Further purification was effected by suspending the crystals in warm water and taking the vitamin into solution by the addition of hydrochloric acid (10%) to 0.1 *N* concentration. Insoluble contaminating pigment was separated at the centrifuge. Addition of an amount of sodium hydroxide equivalent to the hydrochloric acid used precipitated the vitamin in a crystalline or partially crystalline form. Thorough washing with cold water followed by one or more recrystallizations from hot water yielded the compound in the form of thin yellow platelets (Fig. 1), which were collected and dried in vacuum over calcium chloride.

Physical and Chemical Properties.—The color of the desiccator-dried compound varies from a pale lemon yellow to a light orange depending upon the size of the crystals and their state of aggregation. The individual crystals are pale yellow in color. They are pleochroic with the deeper color parallel to the elongation. The alpha index is 1.560 (±0.005) and is measured parallel to the elongation. The gamma index is 1.760 (±0.005) and is measured perpendicular to the elongation. The largest crystal observed was 0.170 × 0.030 × 0.002 mm. The two larger dimensions were measured by the use of a micrometer ocular. Because of the extreme thinness the crystals always lie flat on the microscope slide and the thickness was measured by determining the phasal difference in polarized light and calculating the thickness from double refraction. The minimum dimension can thus be measured by a method which gives a higher degree of accuracy than can be obtained in the other directions. The thickest crystal observed was 0.004 mm. in thickness. A unique feature of these crystals is the uniformity with which they crystallize from a free liquid giving platelets all of which are nearly the same thickness irrespective of the other dimensions of the crystal. It is this characteristic which makes the determination of the third index of refraction difficult.[24]

The compound has no melting point. It darkens and chars from about 250°. The compound is very slightly soluble in cold water (0.0016 mg. per ml. at *p*H 3, 25°) but soluble to about 0.1% in boiling water. It is slightly soluble in methanol and less so in ethanol and butanol. It is insoluble in acetone and chloroform but relatively soluble in acetic acid, phenol and pyridine. The acidic nature of the compound is evident from its ready solubility in water on addition of sodium, ammonium or barium hydroxide while its basic nature is revealed by its ready solubility in water on the addition of mineral acids. The ultraviolet absorption characteristics of the compound are presented in Fig. 3. Crude crystalline products in solution are highly fluorescent in ultraviolet light whereas analytically pure material in comparable concentration

(24) We wish to thank Professor C. B. Slawson of the University of Michigan for making the crystallographic observations and for his permission to include them in this paper.

TABLE I

SUMMARY OF CHICK AND MICROBIOLOGICAL (*L. casei*) ASSAY RESULTS (HOG LIVER FRACTIONATION)

		Potency per gram		
			Microbiological	
		Chick anemia units[a]	Units[c]	Crystalline equivalent, mcg.
1	Aqueous liver extractives	2.5	325	16.3
2	Fraction not adsorbed on Amberlite IR-4	<0.5	<8	<0.4
3	Elute of Amberlite IR-4 adsorbate	27	2,320	126
4	Fraction not adsorbed on Superfiltrol	<4.5	<40	<2
5	Elute of Superfiltrol adsorbate	53	8,500	425
6	Fraction not adsorbed on Norite	<7	<300	<15
7	Elute of Norite adsorbate	177	28,600	1,430
8	Butyl alcohol extractives from acidified elutes of Norite adsorbate	5,250	400,000	20,000
9	Precipitate from acidified ammonium oxalate solution of zinc precipitate	>10,000[b]	6,880,000	344,000
10	Amorphous fraction before crystallization	> 5,000	10,000,000	500,000
11	Crystalline product	>25,000[b]	20,000,000	1,000,000

[a] Assayed by curative method. [b] Assayed by preventive method. [c] One microbiological unit is 100 times the amount which in 10 ml. of medium produces approximately half maximum growth of *L. casei*.

(0.002%) has only a trace of blue green fluorescence at *p*H 11 and none at *p*H 1. The results of elementary analysis on ash-free specimens are recorded in Table III.

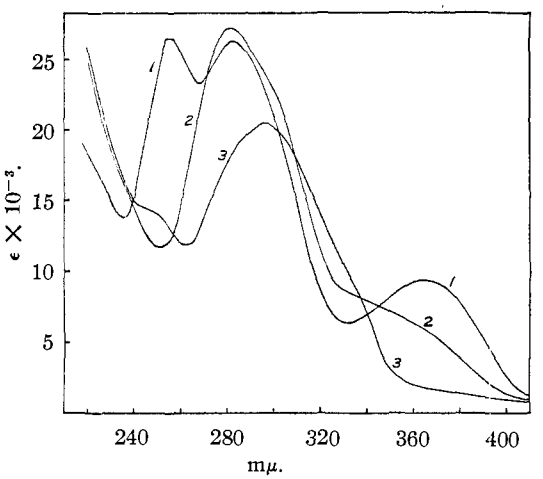


Fig. 3.—Molecular extinction curves of vitamin Bc at several *p*H levels: 1, *p*H, 11; 2, *p*H 3; 3, *p*H 1.

Biological Activity.—The data in Table I show the assay results by the chick and microbiological methods on progressive types of fractions encountered in the isolation procedure. There is general correlation between the biological activity as determined by the two methods of assay. The effects of the pure compound on growth and hematopoiesis in the chick have been reported by Campbell, Brown and Emmett.[19]

Isolation of Vitamin Bc Methyl Ester from Hog Liver.—It was found that by esterification of the crude zinc salts and isolation of the vitamin as its methyl ester a simpler procedure than that used for the isolation of the unesterified compound was feasible. Frequently it was possible to delete several of the adsorption steps. The following is an example of a procedure used to isolate the methyl ester from the crude zinc salt.

Fifteen hundred pounds of hog liver was processed to the zinc salt as described for the isolation of the free acid except that the superfiltrol adsorption and one Norite adsorption were eliminated. The zinc salt, containing 72 mg. of vitamin Bc activity, was dissolved in about 800 ml. of water by the addition of an excess of ammonium oxalate. The oxalate was removed with barium chloride and sufficient barium hydroxide to maintain a *p*H of about 8. A second zinc precipitation was made by adding excess zinc acetate, adjusting to *p*H 6.7–7.0, and heating on the steam-bath for about half an hour at 60°. After standing overnight the zinc salt was collected and dried in a vacuum desiccator. This crude salt, 6 g. containing 51 mg. of vitamin activity, was dissolved by shaking with 325 ml. of 2% hydrogen chloride in absolute methanol and allowed to stand twenty-four hours at room temperature. The solution was concentrated under reduced pressure to a volume of 10 to 15 ml., 100 ml. of water was added and the *p*H was adjusted to about 3 with sodium hydroxide (10%). After shaking to redissolve the precipitated zinc hydroxide the ester was collected at the centrifuge and extracted with 15- to 20-ml. portions of hot 50% methanol. Upon cooling, this solution deposited 84 mg. of a yellow solid which contained 45 mg. of vitamin Bc activity. The methyl ester hydrochloride was prepared by dissolving the crude ester in 100 ml. of 1.5% hydrogen chloride in absolute methanol, allowing to stand twenty-four hours, concentrating *in vacuo* to a volume of 5 to 10 ml. and precipitating the hydrochloride by addition of 30–40 ml. of dry acetone. The white crystalline hydrochloride was collected, decomposed with 10 ml. of water and the solution adjusted to *p*H 3.0. The resulting ester was collected and twice recrystallized from hot 50% methanol. The product, 28 mg., separated in the form of small platelets and clusters of platelets.

Properties of Vitamin Bc Methyl Ester from Hog Liver.—The methyl ester is less soluble in water and slightly more soluble in organic solvents, especially in the simpler aliphatic alcohols, than the free acid.

Esterification does not affect the specific ultraviolet absorption characteristics of the compound. Analytical results are recorded in Table III.

The ester is less than 10% as active as the free acid in stimulating the growth of *L. casei* and *S. fecalis*. This residual growth activity may be due to partial hydrolysis during incubation.

Isolation of Vitamin Bc and Vitamin Bc Methyl Ester from Horse Liver.—Although, as in the case of hog liver, the content of vitamin Bc activity in horse liver is highly variable, it was found to be a much richer source of the vitamin than hog liver. This greater potency aided not only in supplying more vitamin Bc activity in the source material, but also permitted further simplification of the isolation procedure. For the isolation of the methyl ester from horse liver it was possible to dispense with the butanol extraction and precipitation of the barium salts from methanol.

In fresh horse liver nearly all of the vitamin is in the conjugated form, but it was found that it could be effectively released by allowing the chopped liver to autolyze in an equal weight of water at 45° for five hours. The autolysate was then processed in a manner similar to that used for hog liver. The process for the preparation of the

methyl ester from horse liver included the following successive steps: Amberlite IR-4 and Norite adsorption and elution, neutral and acid butanol extraction, preparation of the barium and zinc salts followed by esterification and recrystallization. Using a procedure embodying these steps on an aqueous extract from 1000 lb. of autolyzed horse liver, containing 4.6 g. of vitamin Bc activity, there was isolated 162 mg. of crystalline methyl ester which represents a yield of 3.5% of original vitamin Bc activity.

The products isolated from horse liver were identical with the compounds isolated from hog liver in crystal form (Fig. 2), solubility properties, biological activities on the chick and bacteria, specific ultraviolet absorption properties and elementary composition (Table III).

Part II

Evidence for the Occurrence in Liver of an Acid-Labile Factor Having Antianemic Activity for the Chick and Growth Activity for *L. casei* and *S. faecalis*

Prior to isolation of a pure physiologically active compound and a study of its stability properties the low yields were thought to be due to the lability of the vitamin. Once the pure compound was isolated, however, and it was found to be relatively stable to mineral acid evidence accumulated pointing to the presence in both hog liver and horse liver, particularly in the latter, of a compound(s) possessing the same biological properties as vitamin Bc but which is acid-labile. This acid-labile factor has not been isolated but concentrates were prepared from horse liver which contained about 5% of the factor expressed in terms of vitamin Bc as determined by assay with bacteria (*L. casei* and *S. faecalis*).

Stability of Vitamin Bc to Mineral Acids.—The percentage of *L. casei* activity remaining after allowing crystalline vitamin Bc to stand at room temperature in various concentrations of aqueous hydrochloric acid is shown below.

	2 days, %	10 days, %	22 days, %
0.1 *N* HCl	86.5	100	85
2.0 *N* HCl	100	86.5	80
9.6 *N* HCl	100	...	75

That the vitamin is also stable to methanol–hydrogen chloride was first evidenced by the fact that relatively pure zinc salts of the vitamin could be esterified and the ester hydrolyzed without significant loss of activity. The following results were obtained when crystalline vitamin Bc and a crude concentrate, to which the pure vitamin (20 mcg.) was added, were treated with 1.5% methanol–hydrogen chloride for various periods of time, the esterification mixtures subsequently hydrolyzed with dilute alkali and assayed with *L. casei*.

	Vitamin activity (mcg.)			
	Vitamin Bc	(A) Crude conc.	(B) Crude conc. +20 mcg. Bc	Difference (B − A)
Untreated sample	100	46	66	20
MeOH-HCl 5 min.	110			
MeOH-HCl 24 hours	100	8.0	31	23
MeOH-HCl 92 hours	80			
MeOH-HCl 1 week		10	30	20

It is apparent that vitamin Bc is stable to methanol–hydrogen chloride for several days either in the pure state or when added to a crude concentrate. The recoveries are within the error of the microbiological assay method.

Lability of Chick Antianemia Activity and Microbiological Growth Activity of Liver Extracts to Mineral Acids.—Crude horse liver extracts were dried, treated with 1.5% methanol–hydrogen chloride and subsequently hydrolyzed with 0.1 *N* sodium hydroxide. All the samples were assayed with *L. casei* before and after treatment; one sample was assayed with the chick. The results were as follows:

Liver extract	Microbiological Before mcg./g.	Microbiological After mcg./g.	Activity remaining %
X1376	165	40	24
X1377	150	48	32
X1554A6	195	63	32
X1554A	74	21	28
	chick.		
X1554A	88	27	31

Approximately 65–75% of the microbiological and chick antianemia activities were destroyed by this acid treatment. This explains in part the low yields obtained in isolating the crystalline acid-stable vitamin.

Properties of the Acid-Labile Factor.—In addition to its sensitivity to mineral acid, the acid-labile factor differs from vitamin Bc in other respects. Whereas zinc precipitates relatively pure vitamin Bc almost quantitatively from aqueous solution, 30 to 50% of the vitamin activity in the purified horse liver concentrate remained in the zinc filtrate. Assays of the zinc filtrates on *S. faecalis* gave values one and one-half to two times as high as when assayed on *L. casei*, but after treatment of either the filtrate or the zinc salt with methanolic hydrogen chloride the two organisms gave the same assay value. A summary of observations on zinc filtrates from horse liver is presented in Table II which shows the difference in assay on the two microörganisms and the amount of the activity which remains in the filtrate. Although nearly one-half of the activity present in the barium salt remained in the zinc filtrate, a large part of the acid-labile factor

TABLE II

DISTRIBUTION OF MICROBIOLOGICAL ACTIVITY WITH ZINC PRECIPITATION

Prep. no.	Barium salt *L. casei*, mg.	Zinc filtrate *L. casei*, mg.	Zinc filtrate *S. faecalis*, mg.	Zinc salt *L. casei*, mg.	Zinc salt *S. faecalis*, mg.	Hydrolyzed ester of zinc salt *L. casei*, mg.	Hydrolyzed ester of zinc salt *S. faecalis*, mg.
B146	41	25.6		22.6	39.0	8.6	
B152	677	200	400	444	770	242	246
B172	128	65	131	38	91	31.6	
B180	2080	1020	1560	730	1410	392	364

was also precipitated by the zinc as evidenced by the fact that esterification of the zinc salt with methanol–hydrogen chloride and subsequent hydrolysis of the ester resulted in a marked loss of activity. Thus the zinc salt of the acid-labile factor is more soluble than the zinc salt of vitamin Bc, but the acid-labile factor is partially precipitated by zinc under the conditions used.

In order to study more thoroughly the stability of the acid-labile factor a sample of the zinc filtrate (B152 in Table II) was dried and dissolved in 1.5% methanol–hydrogen chloride. At intervals samples were removed and hydrolyzed with 0.1 *N* sodium hydroxide for assay.

	Vitamin Bc activity			
	L. casei		*S. faecalis*	
	mcg.	%[a]	mcg.	%[a]
Original	890		1760	
Dried sample	800	90	1570	89
0.1 *N* NaOH (30 min.)	860	96	2100	119
Methanolic HCl				
(a) 5 minutes	480	54	505	29
(b) 44 hours	190	21	190	11
(c) One week	155	17	155	9
(d) Two weeks	145	16	135	8

[a] Percentage of activity remaining after treatment.

The activity was not impaired by drying or by treating with dilute alkali but within five minutes after the addition of methanol–hydrogen chloride about 50% of the *L. casei* activity was destroyed and after forty-four hours it had dropped to 21% of the original assay at which level it remained nearly constant for two weeks. The *S. faecalis* assay value very quickly dropped to that of *L. casei*.

Comparative assays with the chick and *L. casei* of zinc filtrates before and after treatment with methanol–hydrogen chloride yielded the following results:

	Sample 1		Sample 2	
Assay	Before, mg.	After,[a] mg.	Before, mg.	After,[a] mg.
L. casei	18.0	5.5	16.6	2.6
Chick	10.8	3.5	8.6	3.0

[a] 1.5% methanol–hydrogen chloride for forty-eight hours at room temperature. Hydrolyzed in 0.1 *N* sodium hydroxide by warming in the steam-bath for thirty minutes.

These observations demonstrate that the acid-labile factor concentrated in the zinc filtrate is an antianemia agent for the chick.

That the destruction of activity in methanol–hydrogen chloride is a function of the acid rather than the methanol and the anhydrous condition is evidenced by the fact that activity is also lost in aqueous acid solution. Samples of the zinc filtrate were allowed to stand at room temperature for seventy-two hours at *p*H 7.0, 3.0 and 1.0, respectively, and subsequently assayed.

	Vitamin Bc activity	
	L. casei, mcg.	*S. faecalis*, mcg.
Original sample	105	201
*p*H 7.0	101	144
*p*H 3.0	45	85
*p*H 1.0	29	40

The destruction in dilute aqueous acid is not as rapid as with 1.5% methanol–hydrogen chloride, but at *p*H 1.0 the activity is readily destroyed.

Discussion.—Cooperman, *et al.*,[25] have presented evidence for the presence of a heat-labile substance in liver and raw milk which stimulates the growth of *S. faecalis*. It was observed that its occurrence correlates closely with that of the heat-labile monkey antianemia factor reported by Cooperman.[26]

Stokes and Larsen[27] have observed that the SLR factor[28] can be converted to folic acid by microörganisms but that autoclaving in water results in the destruction of most of the activity. This destruction could be prevented by sodium thioglycollate or ascorbic acid. The acid-labile factor is not identical with either of these heat-labile factors since autoclaving for thirty minutes at 15 lb. pressure did not destroy the activity.

The acid-labile factor appears to be closely related to vitamin Bc chemically since during the concentration procedure it follows along very closely with vitamin Bc and since none of the fractionation procedures tried have led to a concentrate which is completely destroyed by acid. However, it is possible that the acid-labile factor is converted by acid to vitamin Bc or another stable compound which is less active. Chemically it differs from vitamin Bc chiefly in its acid lability and the greater solubility of its zinc salts.

Part III

Isolation of Vitamin Bc from Yeast.—Yeast and yeast extracts are rich in chick antianemia activity but have little microbiological growth activity. Using the chick assay technique[11] as a guide concentrates of this chick antianemia factor were prepared, virtually free of microbiological growth activity. When such concentrates were digested with desiccated hog kidney the microbiological growth activity was enormously increased and was then found to parallel the chick antianemia activity when both were expressed in terms of crystalline vitamin Bc. Application of the methods used on hog liver and horse liver when applied to such digestion mixtures yielded a crystalline compound (Fig. 4) identical in all respects with crystalline vitamin Bc from liver. The compound was also isolated as its methyl ester from the digestion mixtures by the previously described methods.

Materials.—The starting material was a plasmolyzed extract of brewers' yeast, usually Fleischman Type 3 (Standard Brands, Inc.). This extract assayed 50 mcg. per g. on chicks and between 1 and 2 mcg. per g. microbiologically.

(25) Cooperman, *et al.*, *J. Biol. Chem.*, **163**, 769 (1946).
(26) Cooperman, *et al.*, *Science*, **102**, 645 (1945).
(27) Stokes and Larsen, *J. Bact.*, **50**, 219 (1945).
(28) Keresztesy, Rickes and Stokes, *Science*, **97**, 405 (1943).

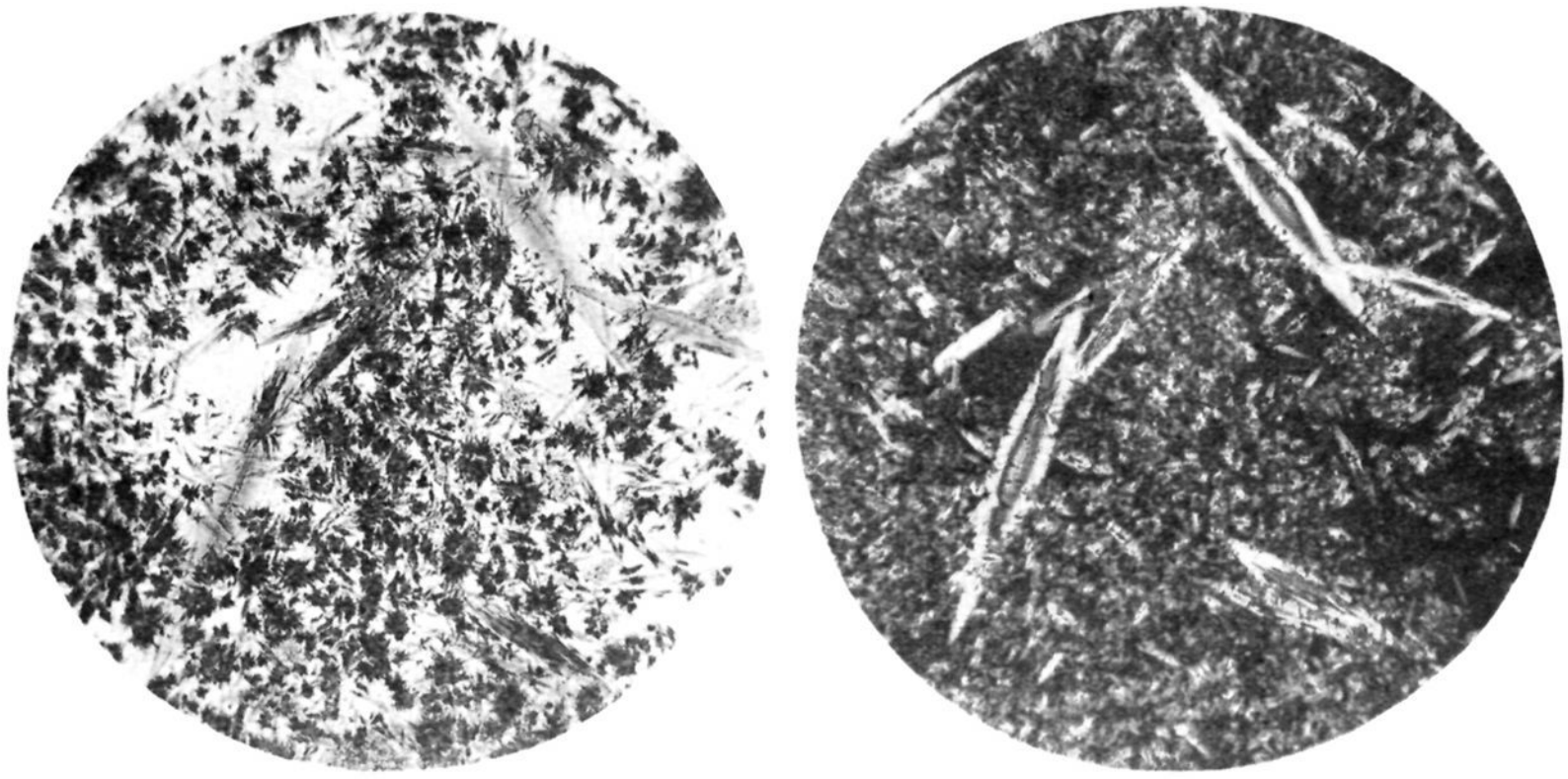


Fig. 4.—Vitamin Bc crystals from yeast digest. Ordinary light on left. Polarized light on right (× 85).

Concentration of the Conjugated Vitamin.—Six thousand grams of yeast extract was dissolved in 40 liters of warm water. The solution was adjusted to *p*H 3 by the addition of 1:1 sulfuric acid and 500 g. of supercel was stirred in. The mixture was filtered and the filter cake washed with 2 liters of water. To the clear filtrate and washings was added 3000 g. of Norite SG-11. After stirring for one hour the adsorbate was filtered off, washed with 2 liters of water and 2 liters of 50% ethanol. The filtrate and washings were discarded. They contained less than 5% of the activity in the starting material as measured by the antianemic response in the chick while microbiologically the filtrate had no activity. The charcoal adsorbate was eluted twice with 20-liter portions of hot 5% ammonia in 50% ethanol. The eluates were concentrated to about 1.5 liters by distillation under reduced pressure. The solution, containing 650 g. of solids and 60% of the chick activity, was diluted to 5 liters, adjusted to *p*H 2.7 and treated with 500 g. of Norite. The adsorbate was filtered off, washed with 2 liters of 50% ethanol and then eluted twice with 5 liters of 5% ammonia in 50% ethanol. The eluates were concentrated by distillation under reduced pressure to a volume of approximately 450 ml. The concentrated eluates contained 114 g. of solids and about 100 mg. of vitamin activity. The solution which was at *p*H 5.5–6 was extracted with butyl alcohol for forty-eight hours under reduced pressure. It was then acidified to *p*H 3 with hydrochloric acid (10%) and the butyl alcohol extraction repeated. About 90 mg. of vitamin Bc (chick activity) was accounted for in the aqueous solution and about 1.5 mg. by microbiological assay on *L. casei* and *S. faecalis*. The solution contained 82 g. of solids. At this stage the yield of vitamin activity as assayed on the chick was 30%.

Enzyme Digestion and Isolation of Vitamin Bc.—The solution was diluted to 5 liters, 550 g. of finely ground fresh hog kidney[29] was added, the *p*H adjusted to 4.2, and the mixture incubated at 45° for two days under toluene.

The enzyme digest was filtered, adjusted to *p*H 3 and treated with 50 g. of Norite to adsorb the vitamin. The filtrate contained only 0.1 mg. of vitamin and was discarded. The adsorbate was washed with 200 ml. of 50% ethanol and then eluted 3 times with 700 ml. of 5% ammonia in 50% ethanol. The eluates were concentrated by distillation under reduced pressure to a volume of 500 ml. This solution contained 7 g. of solids and 70 mg. of vitamin activity as determined by both chick and microbiological assay.

Further fractionation was carried out by the methods described including butanol extraction and purification through the barium and zinc salts, yielding by final recrystallization from water, 5.1 mg. of a yellow crystalline product. The crystal habit is indicated in Fig. 4. The over-all yield was 1.7%.

Physical and Chemical Properties.—The solubility properties of the compound were similar to those of vitamin Bc isolated from liver. The crystal form is the same as that of the liver product. The individual crystals had the same alpha and gamma indices as those of the crystalline vitamin isolated from hog and horse liver.[24] The ultraviolet absorption curves are identical with the curves of the crystalline compound isolated from liver. The compound decomposed but did not melt below 360°. Analytical results are recorded in Table III.

Biological Activity.—The antianemia and growth effects in the chick of the compound isolated from yeast have been reported by Campbell, Brown and Emmett[30] and by Campbell, McCabe, Brown and Emmett.[31] No differences were detected in the effects of the compound isolated from yeast and that isolated from liver. Likewise the compound isolated from yeast was found by Dr. Bird of this Laboratory to have the same growth effects on *Lactobacillus casei* and *Streptococcus faecalis* as vitamin Bc from liver. Similar findings were obtained by Krueger and Peterson.[32]

Isolation of Vitamin Bc Methyl Ester from Yeast Digest.—The vitamin Bc methyl ester was isolated in a manner similar to that described for the preparation of the methyl ester from hog liver. The following is an example of the procedure used for the isolation of vitamin Bc methyl ester from the zinc salts prepared from the enzyme hydrolysate of 7.2 kg. of yeast extract. The zinc salts, containing about 40 mg. of vitamin Bc activity, were esterified by dissolving in 100 ml. of 1.2% methanol-

(29) Hog kidney contains a small amount of vitamin activity. Control experiments demonstrated that 550 g. of fresh hog kidney when digested under the above conditions contributed only 0.41 mg. of vitamin Bc activity to the reaction mixture. We wish to thank Dr. O. D. Bird for carrying out the enzyme digestion.

(30) Campbell, Brown and Emmett, *J. Biol. Chem.*, **154**, 721 (1944).

(31) Campbell, McCabe, Brown and Emmett, *Am. J. Physiol.*, **144**, 348 (1946).

(32) Krueger and Peterson, *J. Biol. Chem.*, **158**, 145 (1945).

TABLE III

ANALYSES OF CRYSTALLINE VITAMIN Bc

Sample	Source	Drying conditions[b] Press., mm.	Temp., °C.	Period of drying, hr.	Volatile loss, %	C, %		H, %		N, %	OMe, %	Method of preparation
1[a]	Hog liver	14	100[c]	2	2.2	50.50	50.63	4.78	4.78	19.91(D)		Isolated directly as acid
2[a]	Hog liver	0.05	145	7	8.6	51.61		4.42		18.91(K)		Isolated directly as acid
3[a]	Horse liver	.001	140	4	11.7	52.44	52.46	4.28	4.49	19.83, 19.64(D) 22.37, 22.29(K)		Isolated directly as acid
4	(a) Yeast	Desiccator dried ($CaCl_2$)		.6 (mo.)		47.0	47.1	4.9	5.0			By regeneration of pure methyl ester
	(b)	.001	80	2	8.9	52.9		4.7				
	(c)	.001	140	4	9.2	52.3		4.3				
5[a]	(a) Yeast	.001	140	5	8.6	52.44		4.37		22.04, 21.95(D)		Isolated directly as acid[i]
	(b)	.001	140	6	9.8	52.51		4.43				
	(c)	.001	140	4	8.6	51.52	51.53	4.30	3.90			
	(d)	.001	175	4	9.3	53.06		4.05				
6	Yeast	.001	140	4	8.5	52.11		4.37		20.26, 20.20(D)	0.0	By regeneration from Sample 13
7	Yeast	.001	140	6	9.1	52.60		4.69				Isolated directly as acid
8	Yeast	.05	145	7	9.6	52.54	52.54	4.39	4.40	22.12(D) 22.07, 22.01(K)		By regeneration of pure methyl ester
Average[d]					9.3	52.27		4.39		22.12		
$C_{19}H_{19}O_6N_7$						51.70		4.34		22.22		
$C_{21}H_{20}O_6N_8$						52.50		4.20		23.23		
ANALYSES OF CRYSTALLINE VITAMIN Bc DIMETHYL ESTER												
9	Hog liver	.05	145	7	4.6	54.12		5.11		21.08(K)		By esterification of crude zinc salts
10	Horse liver	.05	145	7	4.7	53.34		4.96		21.20(K)		
11[e]	Horse liver	.05	145	7	6.9	54.25		5.08		13.23	13.26	By esterification of crude zinc salts
12	Horse liver	.05	145	7	3.6	53.88		5.17				
13[h]	Yeast	.001	140[b]	2		53.43		5.26				By esterification of Sample 5
14[a]	Yeast	.001	140	3	4.8	54.05	54.13	5.14	5.14	19.86, 20.17(D)	12.91 13.17	By esterification of crude zinc salts
15	Yeast	.001	175	4	2.8	54.02	53.92	5.06	5.02			By esterification of crude zinc salts
16	Yeast	.05	145	7	5.7	54.38	54.27	5.14	5.20	21.20(D) 21.13, 21.02(K)	13.14 13.26	By esterification of crude zinc salts
Average					5.2	53.89		5.12		21.10[g]	13.16	
$C_{19}H_{17}O_4N_7(OCH_3)_2$						53.73		4.94		20.89	13.22	
ANALYSES OF SYNTHETIC PTEROYLGLUTAMIC ACID (LIVER *L. casei* FACTOR)												
17[j]		.05	145	8	7.4	51.71	51.62	4.62	4.68	22.38(K)		
		.001	145	4	9.0	52.43	52.34	4.58	4.62			

[a] Some of the analytical findings on these samples have been previously recorded. [b] Dried to constant weight in a drying pistol using a Hayman pig unless otherwise specified. [c] No drying pig used. [d] The C and H figures on sample 1 and the results of analysis (a) on sample 4 and (d) on sample 5 are excluded from the average. The results of nitrogen analyses on samples 1, 2 and 6 and the Dumas results on sample 6 are excluded from the average. [e] Recrystallized from dioxane–water mixture 1:3 .[f] Not dried to constant weight. [g] The Dumas results on sample 14 are excluded. [h] A sample dried under the same conditions except that the temperature was 110° gave C, 52.71; H, 5.13. [i] This sample yielded a negative test for methoxyl and methylimide groups by the micro-Zeisel method and for methyl groups attached to carbon by the Kuhn–Roth method. Determination of primary amino groups by Van Slyke's micro-method yielded 3.16% amino N (reaction time six hours) and 3.14% amino N (reaction time fifteen hours); theory for $C_{19}H_{17}O_6N_8 \cdot NH_2$ is 3.18%. [j] Sample supplied by Lederle Laboratories. Values corrected for 0.19% ash.

hydrogen chloride and allowing to stand overnight. The solution was concentrated *in vacuo* to a volume of 5 ml. and 45 ml. of water was added to the brown sirupy solution. It was then adjusted to *p*H 3.0 by the addition of 6 *N* sodium hydroxide with cooling. The resulting precipitate was collected at the centrifuge and extracted with 15-, 10- and 5-ml. portions of hot 50% methanol. Upon cooling the solution deposited 34 mg. of crude ester (dried in vacuum desiccator over calcium chloride) which was dissolved in 50 ml. of 1.2% methanol–hydrogen chloride. After a few hours this solution was evaporated under reduced pressure to a small volume (2–3 ml.) to which was added 10 ml. of dry acetone. After standing in the refrigerator a short time the crystalline hydrochloride precipitated. It was collected at the centrifuge, washed with acetone and decomposed by stirring with 10 ml. of water. The resulting yellow ester was collected and washed with water; yield 18.8 mg. The ester was recrystallized from 60 ml. of hot 50% methanol and allowed to cool. The solution deposited 16 mg. of crystalline vitamin Bc methyl ester.

Properties.—The methyl ester isolated from yeast digests possessed solubility properties entirely similar to those of the vitamin methyl ester isolated from liver. Specific ultraviolet absorption properties of the methyl ester preparations isolated from liver and from enzyme digests of yeast concentrates were identical. Analytical results are recorded in Table III.

Part IV. Characterization

In keeping with its pterine nature vitamin Bc presented a number of difficulties in its molecular characterization somewhat comparable to those encountered by Wieland, Schöpf and their co-workers[33] in their extended investigations on the pterines, particularly xanothopterine. Although Angier, *et al.*,[1] have proved the structure of the

(33) Wieland and Purrmann, *Ann.*, **544**, 163 (1940), and earlier publications. See Schöpf (*Naturwissenschaften*, **28**, 478 (1940)) for a complete bibliography.

vitamin by degradation and synthesis it appears desirable to record the results of our study on the characterization of the compound since ample evidence is at hand to indicate that a number of biologically active compounds related to the vitamin remain to be isolated and characterized. The results which we have obtained also demonstrate the identity of the compound isolated from yeast digest with that isolated from liver.

Analysis.—The compound either in crystalline or amorphous form when dried in a vacuum desiccator under reduced pressure held from 5 to 11% of moisture which was lost only on drying in high vacuum at elevated temperatures. Some preparations could be brought to analytical dryness at 80° while other preparations required a drying temperature of 145°. The moisture loss could not be correlated with water of crystallization or with water of constitution. The possibility of the desiccator dried product being a hydrate was ruled out by optical crystallographic study of material dried at 145° for five hours in high vacuum. Drying under these conditions had no effect on the alpha and gamma optic indices. The thoroughly dried compound is quite hygroscopic, absorbing moisture from the atmosphere until it again contains 5 to 10%. Since drying to constant weight at 145° in high vacuum was found not to alter the physical or biological properties of the compound, these conditions were adopted in drying the compound and its stable derivatives for analysis. The derivatives of the vitamin also held relatively high percentages of moisture even on prolonged desiccator drying. The analytical results obtained on the acid isolated from hog liver, horse liver and yeast digest are summarized in Table III. It will be noted that the carbon values tend to be high. This might be explained by loss of water of constitution but against this stands the observation that crystallographic study failed to reveal any changes in crystal properties due to drying. Low nitrogen values were frequently encountered with the acid and its derivatives. The Dumas method yielded results often 1 to 2% too low while the ordinary micro-Kjeldahl procedure gave values as much as 3% too low. Satisfactory nitrogen values by the micro-Kjeldahl method were obtained only after employing a modified digestion procedure[34] although with this modified technique an occasional low value was obtained, *e. g.*, sample 2, Table III. Only values secured with this modified technique are included in Table III.

Molecular Weight.—The estimation of the molecular weight of vitamin Bc by cryoscopic methods was impractical because of the extreme insolubility of the compound in water and organic solvents generally, including camphor, urethan, formamide, phenol and the lactam of *cis*-hexahydro-*p*-aminobenzoic acid. However, the dimethyl ester was found to be sufficiently soluble in the two latter solvents to permit determinations to be made. Values obtained on the dry (145° to constant weight in high vacuum) dimethyl ester by the micro-Rast procedure using as solvent the lactam of *cis*-hexahydro-*p*-aminobenzoic acid[35] were 370, 377; theory 469.4. Slight discoloration of the melt indicated possible decomposition. The molecular weight of the dimethyl ester determined by the method of freezing point depression in phenol gave values of 463, 407 and 394.

Preparation of Vitamin Bc Dimethyl Ester.—A 40.8-mg. sample of vitamin Bc was dissolved in 100 ml. of 1.2% methanol-hydrogen chloride and the solution was allowed to stand at room temperature for twenty-four hours. The esterification mixture was concentrated *in vacuo* to a volume of 2-3 ml. At this volume the hydrochloride of the ester started to crystallize and to complete its separation 10 ml. of anhydrous acetone was added. After cooling in ice for a few hours the white solid was collected at the centrifuge and washed with 10 ml. of dry acetone. The hydrochloride was stirred with 8 ml. of water whereupon the yellow dimethyl ester was formed. It was collected at the centrifuge, washed well with water and dried in a vacuum desiccator over calcium chloride; yield, 35 mg. The product separated in the form of orange-yellow spherulites upon recrystallization from 150 ml. of 50% methanol. Analytical results are recorded in Table III, sample 13.

The acid was regenerated from the ester by allowing the latter to stand for forty-five minutes at room temperature in a 10% excess of 0.1 *N* sodium hydroxide. On acidification to *p*H 3 the acid precipitated and was recrystallized three times from water (sample 6, Table III).

Preparation of Vitamin Bc Diethyl Ester.—A 50-mg. sample of desiccator dried crystalline vitamin was dissolved in 100 ml. of 2% ethanol-hydrogen chloride to give a clear yellow solution. After standing thirty-six hours at room temperature the solution was concentrated under reduced pressure to a volume of about 10 ml. Thirty ml. of dry acetone was added to precipitate the ester hydrochloride. After standing in the refrigerator overnight, the white crystalline precipitate was collected at the centrifuge and washed twice with 10-ml. portions of dry acetone. The hydrochloride was decomposed by the addition of 10 ml. of water and the ester was collected at the centrifuge. It was washed with two 10-ml. portions of water. The resulting yellow colored product was extracted repeatedly with 25-ml. portions of boiling 50% methanol until nearly all was in solution. The hot solution was then filtered and allowed to cool slowly, depositing 41 mg. of crystalline ester. This was recrystallized from 50% methanol, then from 25% dioxane and finally from 50% methanol. The final product was collected at the centrifuge and dried in a vacuum desiccator over calcium chloride. It has no melting point. For analysis it was dried to constant weight at 145° in high vacuum (volatile loss 3.9%).

Anal. Calcd. for $C_{19}H_{17}O_4N_7(OC_2H_5)_2$: C, 55.52; H, 5.47; N, 19.71. Found: C, 56.10, 56.20; H, 5.90, 5.60; N (Kjeldahl), 19.90 (all values corrected for 0.3% ash).

Preparation of Vitamin Bc Dimethyl Ester Dihydrochloride.—To 46.6 mg. of vitamin Bc dimethyl ester was added 6 ml. of about 5% methanol-hydrogen chloride. The mixture was warmed to effect complete solution of the ester. To the warm solution was added 44 ml. of dry acetone, and the white hydrochloride separated immediately. After standing at room temperature for two hours, the mixture was cooled in ice for two hours. The product was collected at the centrifuge, washed with six 5-ml. por-

(34) We are greatly indebted to Dr. A. Elek of the Rockefeller Institute who as a personal favor supplied us with advance information on improved methodology (still under investigation) of the microdetermination of nitrogen.

(35) Wendt, *Ber.*, **75**, 425 (1942).

tions of dry acetone, and dried *in vacuo* over calcium chloride and sulfuric acid. The white crystalline solid weighed 40.3 mg. It does not melt.

For analysis the compound was dried in high vacuum at 60°, to constant weight (volatile loss, preparation 1, 1.20%; preparation 2, 0.50%).

Anal. Calcd. for $C_{19}H_{17}O_4N_7(OCH_3)_2 \cdot 2HCl$: C, 46.50; H, 4.65; N, 18.08; Cl, 13.07. Found: Prep. 1, C, 47.30, 47.40; H, 4.85, 5.91; N, 19.00 (Dumas), 19.15 (Kjeldahl); Cl, 12.84. Prep. 2, C, 46.80, 46.86; H, 4.62, 4.68; N, 17.87 (Dumas), 17.95 (Kjeldahl); Cl, 12.29.

Preparation of the Silver Salt of Vitamin Bc.—To a solution of 35.5 mg. of desiccator dried vitamin Bc isolated from yeast in 75 ml. of hot water was added a solution of 400 mg. of silver nitrate in 20 ml. of hot water. A yellow microcrystalline precipitate formed immediately. The mixture was boiled gently for a few minutes and allowed to cool to room temperature. The product was collected at the centrifuge, washed with water until the washings were free of nitrate ion, and dried *in vacuo* over calcium chloride. The yellow microcrystalline powder weighed 42.1 mg. For analysis it was dried in high vacuum at 145° to constant weight (volatile loss 6.80%).

Anal. Calcd. for $C_{19}H_{17}O_6N_7Ag_2$: C, 34.83; H, 2.62; N, 14.97; Ag, 32.93. Found: C, 35.70, 35.82; H, 2.66, 2.74; N, 14.79 (Dumas), 14.83 (Kjeldahl); Ag, 30.88, 30.77.

Preparation of the Disodium Salt of Vitamin Bc.—To 76.8 mg. of desiccator dried vitamin Bc there was added 3.14 ml. of 0.10 *N* sodium hydroxide, and the volume made up to about 4 ml. Upon the addition of 4 ml. of absolute methanol there occurred an immediate precipitation, which redissolved with warming. The hot solution was allowed to cool slowly in a bath of hot water and then to stand overnight at 4°. The yellow solid was collected at the centrifuge, washed with about 1 ml. of cold 50% methanol and dried. The properties of this material (9.5 mg.) precluded its being the sodium salt. The 50% methanol solution was evaporated in a stream of nitrogen to a volume of about 0.9 ml. The precipitate which formed upon the addition of 1 ml. of absolute methanol was redissolved by warming, and the solution was allowed to cool slowly. The product was collected at the centrifuge and washed successively with 0.5- to 1-ml. portions of 50% methanol, 75% methanol, 90% methanol, absolute methanol, 50% methanol–50% ether, and absolute ether. After drying *in vacuo* over calcium chloride, the yellow-orange microcrystalline powder weighed 46.7 mg. For analysis the product was dried in high vacuum at 145° to constant weight (volatile loss 13.6%). Lead chromate was used in the C and H analyses.

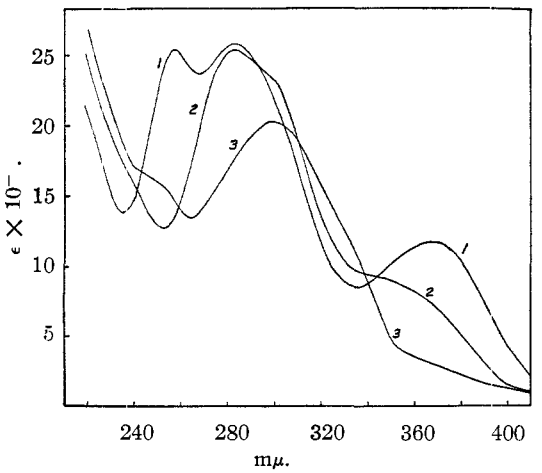


Fig. 5.—Molecular extinction curves of vitamin Bc diamide at several *p*H levels: **1**, *p*H 11; 2, *p*H 3; 3, *p*H 1.

Anal. Calcd. for $C_{19}H_{17}O_6N_7Na_2$: C, 47.01; H, 3.53; N, 20.20; Na, 9.48. Found: C, 47.55, 47.66; H, 3.80, 3.82; N, 20.33 (Dumas), 20.26 (Kjeldahl); Na, 8.17.

Preparation of Vitamin Bc Diamide.—Vitamin Bc methyl ester (39.7 mg., desiccator dried) was dissolved in 40 ml. of ammonia water (28%) and allowed to stand three days at room temperature in a completely filled, glass stoppered vessel. The solution was distilled to dryness under reduced pressure. The residue was dissolved in 20 ml. of hot water, filtered, concentrated to 10 cc. and allowed to cool. The product separated as a gel. On adding 2 drops of 0.1 *N* hydrochloric acid the product separated as a yellow flocculent precipitate. It was collected at the centrifuge, washed several times with cold water until the product tended to go colloidal and dried under reduced pressure over calcium chloride. The orange amorphous powder weighed 26.1 mg. It has no melting point. For analysis the product was dried at 145° to constant weight in high vacuum (volatile loss 7.30%).

Anal. Calcd. for $C_{19}H_{21}O_4N_9$: C, 51.93; H, 4.82; N, 28.69. Found: C, 51.84; H, 4.49; N, 27.69 (Dumas), 28.44 (Kjeldahl); corrected for ash 1.94.

The specific ultraviolet absorption characteristics of the diamide are indicated in Fig. 5. They are similar to those of the parent compound with the exception of a higher band at 365 mμ. The diamide is not utilized as a growth factor by either *L. casei* or *S. faecalis*.

Discussion.—Crystalline vitamin Bc did not lend itself to ready molecular characterization by the usual methods. However, sufficient analytical information was obtained to show that the compound was a dibasic acid containing two basic groups one of which was a primary amino group. Although most of the analytical data are in agreement with the known molecular formula $C_{19}H_{19}O_6N_7$ it will be noted that some of the results are more in keeping with a $C_{21}H_{20}O_6N_8$ formulation. The latter formula was ruled out by results of degradation studies.[35] The analytical results obtained on the products isolated from liver and from yeast digest taken in conjunction with other properties demonstrate their identity. This conclusion is at variance with that of Stokstad.[37] He believed that his product from yeast (yeast *L. casei* factor) was different from his liver compound (liver *L. casei* factor) which he felt was identical with vitamin Bc. The analytical values (Table III) and other published physical and biological properties of synthetic pteroylglutamic acid[38] taken in conjunction with our own degradation results[36] leave no doubt of the identity of crystalline vitamin Bc and crystalline liver *L. casei* factor.[37]

There remains the question of the identity of pteroylglutamic acid with the microbiological growth factor in spinach which Mitchell, Snell and Williams[39] called folic acid. Their better preparations[40] have many properties in common with pteroylglutamic acid, making it appear

(36) Wittle, *et al.*, THIS JOURNAL, in proof.

(37) Stokstad, *J. Biol. Chem.*, **149**, 573 (1943).

(38) Angier, Boothe, Hutchings, Mowat, Semb, Stokstad, Subba-Row, Waller, Cosulich, Fahrenbach, Hultquist, Kuh, Northey, Seeger, Sickels and Smith, *Science*, **102**, 227 (1945).

(39) Mitchell, Snell and Williams, THIS JOURNAL, **63**, 2284 (1941).

(40) Mitchell and Williams, *ibid.*, **66**, 271 (1944).

likely that this compound accounts for some of the biological activity in green plants. Our own limited experience with green plant sources indicates the presence therein of at least two microbiological growth factors both of which are active as antianemia agents in the chick.

Acknowledgment.—The authors wish to thank Mr. A. W. Spang for his microanalytical services and Mr. D. G. Calkins for assistance in much of the isolation work.

Summary

Methods are described for the isolation of crystalline vitamin Bc as the free acid and its dimethyl ester from hog liver.

The identical acid and ester were isolated from horse liver.

A method is described for the partial concentration of the chick antianemia activity in yeast extract. Following enzymatic digestion of such concentrates the identical acid and ester were isolated.

Some salts and derivatives of the vitamin are described.

Vitamin Bc was found to be identical with synthetic pteroylglutamic acid.

Evidence is presented for the occurrence in liver of a compound having biological properties similar to those of pteroylglutamic acid but differing from it chiefly in being very acid labile.

DETROIT, MICHIGAN RECEIVED FEBRUARY 17, 1947

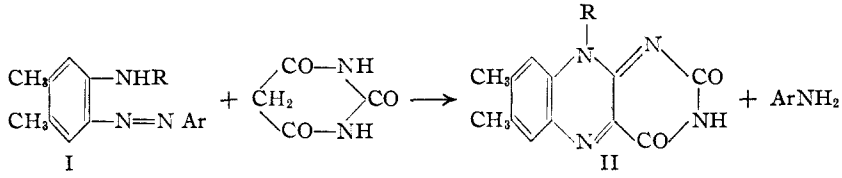
[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF MERCK AND CO., INC.]

The Reaction between o-Aminoazo Compounds and Barbituric Acid. A New Synthesis of Riboflavin

BY MAX TISHLER, KARL PFISTER, 3RD, R. D. BABSON, KURT LADENBURG[1] AND ANN J. FLEMING

Two methods have been reported previously for the syntheses of compounds having the isoalloxazine structure. These methods, designed chiefly for the synthesis of riboflavin, involve the reaction of *o*-phenylenediamines with alloxan[2] or with halogenated barbituric acids.[3]

A new synthesis of riboflavin[3a] is presented now consisting of the reaction between an appropriate *o*-aminoazo compound, I. and barbituric acid.[4]



I II

(a) R = D-ribityl
(b) R = tetraacetyl-D-ribityl

This synthesis is more direct and simpler than the older methods particularly since barbituric acid is more readily accessible than alloxan or the halogenated barbituric acids. Moreover, the *o*-phenylenediamine required in the old procedures is difficult to prepare and is unstable in contrast to the corresponding *o*-aminoazo compounds.

The *o*-aminoazo compounds have been previously utilized for the preparation of phenazines[5] and benzimidazoles.[6] Crippa in a series of papers described the preparation of quinoxaline derivatives from *o*-aminoazo compounds and methyl ketones or ethyl acetoacetate.[7]

The reaction between barbituric acid and *o*-aminoazo compounds of the type I is catalyzed by weak organic acids, such as acetic acid. The optimum yields are obtained when a mixture of the reactants in glacial acetic acid or in acetic acid diluted with dioxane is refluxed until the azo compound is consumed. The presence of mineral acids not only inhibits the reaction but also rapidly destroys the azo compound. The *o*-aminoazo compound is also destroyed by refluxing a solution in acetic acid but the rate of decomposition is much less. If the azo compound in acetic acid is heated for several hours and then the barbituric acid is added, the yield of riboflavin is considerably decreased. The isolation of the by-product *p*-nitroaniline from the reaction of the *o*-aminoazo compound (Ib, Ar = p-$NO_2C_6H_4$) with barbituric acid suggests that riboflavin is formed in accordance with equation I → II.

With the intention of investigating the rela-

(1) Present address: Technical Enterprises Inc., 31 South Street, New York, New York.

(2) (a) Kuhn, Reinemund and Weygand, *Ber.*, **67**, 1460 (1934); (b) Kuhn and Weygand, *ibid.*, **67**, 1939 (1934); (c) Karrer, Solomon, Schöpp and Schlittler, *Helv. Chim. Acta*, **17**, 1165 (1934).

(3) Tishler, Wellman and Ladenburg, THIS JOURNAL, **67**, 2165 (1945).

(3a) *Cf.* Tishler and Carlson, U. S. Patent 2,350,376 (1944); *C. A.*, **38**, 4963 (1944).

(4) Recently Bergel, Cohen and Haworth, British Patent 550,836 (1943); U. S. Patent 2,374,661 (1945), reported the preparation of riboflavin from 1-(D-ribitylamino)-2-phenylazo-4,5-dimethylbenzene (I, Ar = C_6H_5) and alloxantin in the presence of palladized charcoal. This reaction is an extension of the Kuhn and Karrer methods.[2] In the Bergel, *et al.*, procedure the azo group is undoubtedly reduced to the *o*-phenylenediamine by the alloxantin as the latter is dehydrogenated to alloxan.

(5) Witt, *Ber.*, **18**, 1119 (1885); **19**, 441 (1886); Krollpfeiffer, Müllhausen and Wolf, *Ann.*, **508**, 39 (1934).

(6) O. Fischer, *J. prakt. Chem.*, **107**, 16–49 (1924).

(7) G. B. Crippa, *Gazz. chim. ital.*, **59**, 330 (1929); **60**, 301 (1930); **62**, 394 (1932); **63**, 251 (1933); **66**, 649 (1936).