the organism the weight of mycelium per flask was determined. The data, summarized in Table VI, demonstrate that the biotin activity of the AN factor for *Neurospora crassa*, like that of biotin itself or desthiobiotin, is negated readily by avidin.

WEST POINT, PENNA.

[CONTRIBUTION FROM THE RESEARCH LABORATORIES, SHARP AND DOHME DIVISION AND CHEMICAL DIVISION, MERCK & CO. INC.]

Biotin *l*-Sulfoxide. II. The Isolation of a Crystalline Factor with Biotin Activity from *Aspergillus niger* Culture Filtrates

BY LEMUEL D. WRIGHT, EMLEN L. CRESSON, JOHN VALIANT, DONALD E. WOLF AND KARL FOLKERS

RECEIVED DECEMBER 9, 1953

A compound with biotin activity for certain microbial species, tentatively termed AN factor, has been isolated in crystalline form from *Aspergillus niger* culture filtrates where growth had taken place in the presence of added pimelic acid. The isolation scheme involved adsorption on Norit A, elution with alcoholic ammonia, chromatography on Superfiltrol–Celite, ethanol precipitation of inactive material, chromatography on alumina, adsorption chromatography on Dowex-50 (H^+), ion exchange chromatography on Dowex-1 (OH^-), countercurrent distribution between butanol and water and, finally, crystallization from aqueous acetone.

A previously unrecognized derivative of biotin has been found to exist in certain mold culture filtrates, where growth had taken place in the presence of added pimelic acid.[1] The factor was tentatively termed the AN factor since it was first detected in culture filtrates of *Aspergillus niger*, although its distribution is by no means limited to this source. This paper describes procedures by which the AN factor was isolated from *Aspergillus niger* culture filtrates.

The AN factor was found to be adsorbed readily on an unusually small amount of Norit. Activity was eluted with alcoholic ammonia. Adsorption on and elution from Norit afforded a reasonable amount of concentration and was adopted as a first step. In batch-adsorptions it was observed that the activity contained in the Norit eluate was not well adsorbed by various earth adsorbents. It was found, however, that if the adsorptions were carried out chromatographically on a mixture of Superfiltrol and Celite, and the ratio of adsorbing mixture to dry matter of the Norit eluate was about 50, satisfactory separation of activity from inert material was achieved. The column was washed with water which served to carry through much inactive material prior to the factor.

Further concentration of the factor was obtained by adding ethanol to precipitate inert matter from the selected and concentrated eluates. On passing this ethanolic solution through alumina, concentration of the factor resulted because a large portion of the inert solids was retained while the factor was not adsorbed. The selected concentrated eluates were then applied to a strong sulfonic acid-type resin in the H^+ form. Much of the solid matter was retained by the resin. The AN factor was not held by the resin, but its passage was retarded so that the less strongly adsorbed impurities could be separated.

Paper electrophoretic studies had indicated that the AN factor is a monocarboxylic acid or its equivalent so that anion exchange chromatography was indicated. The activity of high potency AN factor concentrates was retained quantitatively by a column of strong anion exchange resin in the OH^- form. However, quantitative adsorption did not occur on a very large excess of resin with concentrates of low potency. Activity could be chromatographically eluted with very dilute acid to afford concentration of the factor.

Preliminary experiments showed that the AN factor does not have a partition coefficient for any of a number of water–solvent combinations favorable enough for batchwise purification by solvent extraction. Solubility of the factor in 1-butanol was sufficient, however, to make concentration by countercurrent distribution practical, provided a large number of transfers was employed. Material at this stage of concentration could be induced to give crystals by cooling a concentrated aqueous solution to which a small amount of acetone had been added.

In order to obtain sufficient source material to work out an isolation scheme and to operate the procedure enough times to acquire crystalline material essential for chemical and microbiological characterization, it was necessary to process over 20,000 liters of *Aspergillus niger* medium. Such a quantity was necessary since the factor is present to the extent of only 2 parts per million in the dry matter of the mold filtrate. While enrichment of the medium undoubtedly would have led to greater microbiological production of the factor, it was felt that the introduction of much extraneous material would complicate the isolation.

A consideration of the isolation scheme which was finally developed for successfully obtaining crystalline AN factor reveals several features that are worthy of note: (a) Although a number of steps were essential in the isolation procedures so that the over-all yield was low, no single step was associated with marked or unaccountable loss of activity; (b) the isolation scheme did not involve drastic conditions such as extremes of *p*H, heat, or deliberate oxidizing conditions that might have been expected to alter the compound; and (c) the isolation scheme, involving the processing of many thousand liters of culture filtrate, was very economical of reagents.

The chemical and microbiological characteriza-

(1) L. D. Wright and E. L. Cresson, THIS JOURNAL, **76**, 4156 (1954).

tion of the AN factor is described in an accompanying paper.[2]

Experimental

Determination of AN Factor.—AN factor was determined microbiologically with *Neurospora crassa* as described in the previous paper and expressed in terms of *d*-biotin as the standard.[1]

Production of AN Factor by *Aspergillus niger* Fermentation.—The medium employed for the production of AN factor by *Aspergillus niger* had the following composition per liter: sucrose, 30 g.; sodium nitrate, 2 g.; potassium phosphate dibasic, 1 g.; magnesium sulfate (heptahydrate), 0.5 g.; potassium chloride, 0.5 g.; ferrous sulfate (heptahydrate), 0.01 g.; pimelic acid, 1 mg.; *p*H 4.0–4.5. The initial fermentation was carried out in a 200-liter industrial-type fermentor. After the procedures for fermentation and preliminary concentration of the factor were established, the fermentations were carried out in a 4,000-liter industrial-type fermentor. The medium was sterilized at 120° for 30 minutes prior to use. Inoculum for seeding the tanks was built up in 20-fold stages in accordance with prevailing fermentation procedures. All incubations were at 30°. Aeration was at a rate of 0.5 volume of sterile air per volume of medium per minute. The period of incubation was approximately 72 hours. Pertinent data on one production lot carried out on a 200-liter scale are summarized in Table I. After 65 hours of growth the mycelium was removed by Sharples centrifugation to give 200 l. of solution containing 1,740 g. of solids and 4.6 mg. of AN factor as biotin (2.6 μg./g.).

TABLE I

TYPICAL DATA ON THE PRODUCTION OF AN FACTOR BY *Aspergillus niger*

Sample	Duration of growth, hours	*p*H	Solids, g./200 l.	AN factor mg./200 l.	AN factor μg./g.
1	35	3.6	4540	3.0	0.66
2	41	3.2	3840	3.2	.83
3	47	2.7	3360	2.6	.78
4	52	2.5	2900	2.8	.97
5	59	2.5	2320	3.8	1.6
6	65	2.5	2120	4.4	2.1

Adsorption and Elution with Norit.—One kilogram of Norit A was added to 200 liters of clarified *Aspergillus niger* culture filtrate at the final *p*H of the fermentation and stirred for one hour. The Norit was collected with the aid of Super-Cel on a large ceramic filter. The Norit cake was suspended in 20 liters of elution mixture (prepared by mixing 10 liters of water, 10 liters of ethanol and 1 liter of 28% aqueous ammonia) and stirred mechanically for one hour. The Norit then was filtered off on a Büchner funnel and the filtrate concentrated *in vacuo* to a volume of about 1000 ml. This adsorption and elution step is effective in concentrating AN factor 5- to 10-fold to yield concentrates containing 10–20 μg. of AN factor as biotin/g. From the original 200 liters of culture filtrate containing 1,740 g. of solids which assayed 2.6 μg. as biotin/g., approximately 1 liter of concentrate was obtained. This concentrate contained 246 g. of solids which assayed 12.1 μg. as biotin/g. (87% recovery).

Larger batches of *Aspergillus niger* medium were processed essentially as described above except that the filtrations were carried out in an Oliver-type centrifugal filter. On occasion two separate elutions of the Norit cake were employed.

It was convenient to stockpile concentrated eluate by storage in the cold room. Aliquots were taken as required for subsequent steps in the isolation procedure.

Chromatography on Superfiltrol-Celite.—An amount of eluate from the preceding adsorption and elution procedure containing about 2.2 kg. of solids was neutralized to *p*H 7.0 and diluted to a concentration of 10% solids. The solution was mixed with a little Super-Cel and clarified by filtration. The clarified filtrate was poured onto a large chromatographic column (1.5 feet in diameter, 6 feet in height) charged with 200 pounds of Superfiltrol-Celite (1:2) mixture.

When the adsorbent had taken up the above-mentioned clarified filtrate, the column was developed with water and 20-liter cuts were collected. After about 6 such eluate fractions containing a sizable proportion of the inert-dry matter applied to the column were collected, it was convenient to collect eluates of larger volume. About 600 liters of water was required to obtain complete elution in 96 hours. The fractions were assayed for AN factor and total solids; those fractions containing most of the factor were combined and concentrated *in vacuo* in a Mojonnier vacuum concentrator[3] to a volume of about 10 liters. This chromatographic step is effective in concentrating AN factor 5- to 8-fold to yield concentrates containing 50–100 μg. of AN factor as biotin/g. Data for a typical column are summarized in Table II. Fractions 7–12 combined and evaporated *in vacuo* to give a solution containing approximately 357 g. of solids and 29.15 mg. of AN factor as biotin (82 μg./g.).

TABLE II

TYPICAL DATA ON THE CONCENTRATION OF AN FACTOR BY CHROMATOGRAPHY ON SUPERFILTROL-CELITE

Fraction	Volume, liters	Solids, g.	AN factor content (as biotin) Mg.	AN factor content (as biotin) μg./g.
Starting sample	10	2368	40	17
1	20	329.0	0	0
2	20	705.0	0	0
3	20	851.6	0	0
4	20	296.0	0.38	1
5	20	97.8	1.16	12
6	20	65.8	1.40	21
7	60	125.4	6.60	53
8	60	76.2	8.40	110
9	90	86.4	9.90	116
10	70	50.4	3.35	66
11	30	18.6	0.90	48
12	80	41.6	1.44	35

Precipitation of Inert Matter with Ethanol.—The concentrated Superfiltrol-Celite eluates obtained from the preceding chromatography with a volume of about 10 liters were treated with an equal volume of ethanol. The precipitate that formed was filtered off with suction and the clear filtrate concentrated *in vacuo* to about 1 liter. This solution then was treated with an equal volume of ethanol. The precipitate was again filtered off and discarded. The precipitation with ethanol is effective in concentrating the AN factor about 5-fold to yield concentrates containing 500–800 μg. of AN factor as biotin/g. In a typical experiment 10,920 ml. of the Superfiltrol-Celite eluate which contained 158.4 g. of solids and assayed 179 μg. of AN factor activity as biotin/g. yielded 2,000 ml. of a second ethanolic solution which contained 40.4 g. of solids assaying 634 μg. of AN factor activity as biotin/g. (90.5% recovery).

Chromatography on Alumina.—The 50% ethanolic solution obtained in the previous step was applied to a glass chromatographic column (6 inches in diameter, 4 feet in height) charged with 20 pounds of highly activated alumina.

When the alumina had taken up the 50% ethanolic solution, the column was developed with additional 50% ethanol. One-liter eluate fractions were collected. Satisfactory recovery of the factor required the collection of about 10 such fractions. The fractions were assayed for AN factor and dry matter; those fractions containing most of the AN factor were combined and concentrated *in vacuo* to a volume of about 300 ml. This chromatographic step is effective in concentrating AN factor about 8- to 10-fold to yield concentrates containing about 2,000 μg. of AN factor as biotin/g. Data for a typical column are summarized in Table III. Fractions 4–7 combined and evaporated *in vacuo* to give a solution containing approximately 11.65 g. of solids and 23.61 mg. of AN factor as biotin (2,020 μg./g.).

Chromatography on Dowex-50 (H^+).—The concentrated alumina eluates obtained from the preceding chromatog-

(2) L. D. Wright, E. L. Cresson, J. Valiant, D. E. Wolf and K. Folkers, THIS JOURNAL, **76**, 4163 (1954).

(3) Model LTFL low temperature vacuum concentrator, Mojonnier Brothers Company, 4601 West Ohio Street, Chicago 44, Ill.

TABLE III

TYPICAL DATA ON THE CONCENTRATION OF AN FACTOR BY CHROMATOGRAPHY ON ALUMINA

Fraction	Volume, liters	Solids, g.	AN factor content (as biotin) Mg.	μg./g.
Starting sample	2	100.4	26.9	258
1	1	1.37	0.06	41
2	1	2.46	.63	256
3	1	2.41	.90	374
4	1	2.72	.91	335
5	1	3.94	8.20	2080
6	1	2.63	11.80	4490
7	1	2.36	2.70	1150
8	1	1.90	0.50	263
9	1	1.45	0.11	76

raphy, in a volume of about 300 ml., were neutralized to *p*H 7.0 and clarified by centrifugation. The clarified centrifugate was applied to a glass chromatographic column (3 inches in diameter, 3 feet in height) charged with 2.5 kg. of Dowex-50, 20–60 mesh in the H^+ form (prepared by washing with a large volume of 1 *N* hydrochloric acid then with water until the washings were essentially neutral).

When the resin had taken up the aqueous solution, the column was developed with distilled water. One-liter eluate fractions were collected. Satisfactory recovery of the factor required the collection of about 15 such fractions. The fractions were assayed for AN factor and dry matter; those fractions containing most of the AN factor were combined and concentrated *in vacuo* to a volume of about 30 ml. This chromatographic step is effective in concentrating AN factor about 10- to 15-fold to yield concentrates containing about 50,000 μg. of AN factor as biotin/g. Data for a typical column are summarized in Table IV. Fractions 4–13 combined and evaporated *in vacuo* to give a solution containing approximately 0.46 g. of solids and 23.10 mg. of AN factor as biotin (50,500 μg./g.).

TABLE IV

TYPICAL DATA ON THE CONCENTRATION OF AN FACTOR BY CHROMATOGRAPHY ON DOWEX-50 (H^+)

Fraction	Volume, ml.	Solids, g.	AN factor content (as biotin) Mg.	μg.
Starting sample	300	7.73	30.15	3,900
1	1000	0.12	0	0
2	1000	2.35	0	0
3	1000	0.62	0.60	970
4	1000	.21	2.40	11,400
5	1000	.09	4.60	51,100
6	1000	.06	5.70	95,000
7	1000	.04	4.10	102,500
6	1000	.01	2.80	280,000
9	1000	< .01	1.50	>150,000
10	1000	< .01	0.80	>80,000
11	1000	< .01	.48	>48,000
12	1000	< .01	.36	>36,000
13	1000	< .01	.36	>36,000

Ion Exchange Chromatography on Dowex-1 (OH^-).—The concentrated Dowex-50 eluates from the preceding chromatography in a volume of about 30 ml. were neutralized to *p*H 7.0 and clarified by centrifugation. The clarified centrifugate was applied to a column of 50 g. of Dowex-1 (OH^-), 20–60 mesh, contained in a 100-ml. buret. The resin was prepared in the OH^- form by washing *liberally* with 10% sodium hydroxide solution and then with distilled water until the washings were neutral.

When the resin had taken up the aqueous solution, the column was washed with distilled water until the washings were neutral. Five-hundredths normal hydrochloric acid was applied to the column and 100-ml. eluate fractions were collected. Satisfactory recovery of the factor required the collection of about 20 such eluate fractions after the *p*H of the eluates fell below 7.0. The fractions were assayed for AN factor and those fractions containing most of the AN factor were concentrated *in vacuo* to a small volume and then lyophilized to dryness. This chromatographic step is effective in concentrating AN factor about 2–3-fold to yield concentrates containing about 100,000–150,000 μg. of AN factor as biotin/g. Data for a typical column are summarized in Table V. Fractions 2–18 combined and evaporated *in vacuo* to give a solution that was then lyophilized to give 0.162 g. of solids and 19.17 mg. of AN factor as biotin (118,000 μg./g.).

TABLE V

TYPICAL DATA ON THE CONCENTRATION OF AN FACTOR BY CHROMATOGRAPHY ON DOWEX-1 (OH^-)

Fraction	Volume, ml.	AN factor content (as biotin), mg.	Fraction	Volume, ml.	AN factor content (as biotin), mg.
Starting sample	30	16.80	10	100	0.37
Water			11	100	.29
1	1400	0.00	12	100	.26
0.05 *N* HCl			13	100	.23
2	100	0.92	14	100	.17
3	100	3.03	15	100	.14
4	100	7.20	16	100	.11
5	100	2.55	17	100	.09
6	100	1.77	18	100	.08
7	100	1.25			
8	100	0.92			
9	100	.61			

It was convenient to stockpile concentrates in this lyophilized state where the factor appeared to be stable. During the processing of the *Aspergillus niger* culture filtrate as described in the preceding procedures, approximately 300 mg. of solids were accumulated containing by microbiological assay 30 mg. of AN factor as biotin (100,000 μg./g.).

Countercurrent Distribution and Crystallization of AN Factor.—Three hundred milligrams of AN factor concentrate containing about 100,000 μg. of AN factor as biotin/g. were subjected to a 200-plate countercurrent distribution. The solvent system was 1-butanol–water–acetic acid in the proportions of 5:5:0.1. Each phase had a volume of 10 ml. per tube. After the distribution, aliquots of the aqueous phase of every tenth tube from 0 through 100 and of the 1-butanol phase of every tenth tube from 110–200

TABLE VI

TYPICAL DATA ON THE CONCENTRATION OF AN FACTOR BY COUNTERCURRENT DISTRIBUTION BETWEEN 1-BUTANOL AND WATER

Tube	AN factor content (as biotin), mg.[a]	Tube	AN factor content (as biotin), mg.[a]
Starting sample	37.40[b]	100	0
0 (water-soluble end)	0	110	0
		120	0
10	0	130	0
20	0.034	140	0.224
30	1.140[c]	150	0.460[d]
40	1.460	160	0.100
50	0.096	170	0
60	0.036	180	0
70	0	190	0.136
80	0	200 (1-butanol-soluble end)	0
90	0		

[a] Since only every tenth tube was assayed for AN factor multiplication of the sum of the factor found in those tubes assayed by 10 gives a rough approximation of the total recovery. When this is done 36.86 mg. of activity or 99% of that distributed is accounted for. [b] This activity was present in 304 mg. of total solids (123,000 μg./g.). [c] Identified by paper chromatography as AN factor. [d] Identified by paper chromatography as biotin.

were assayed microbiologically for AN factor. The data obtained are summarized in Table VI. The contents of plates 35–42 were combined and concentrated *in vacuo* to dryness. The residuum which weighed 26 mg. contained 400,000 μg. of AN factor as biotin/g. This material was dissolved in 0.5 ml. of water and the solution clouded with acetone. The colorless crystalline material which formed on standing melted at 236–238° and contained by microbiological assay with *Neurospora crassa* about 750,000 μg. of AN factor as biotin/g.

WEST POINT, PENNA.

[CONTRIBUTION FROM THE RESEARCH LABORATORIES, SHARP AND DOHME DIVISION AND CHEMICAL DIVISION, MERCK & CO., INC.]

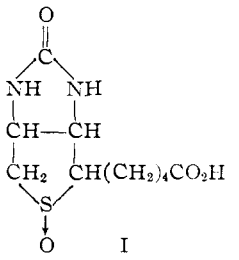
Biotin *l*-Sulfoxide. III. The Characterization of Biotin *l*-Sulfoxide from a Microbiological Source

BY LEMUEL D. WRIGHT, EMLEN L. CRESSON, JOHN VALIANT, DONALD E. WOLF AND KARL FOLKERS

RECEIVED DECEMBER 9, 1953

Crystalline AN factor isolated from *Aspergillus niger* culture filtrate, where growth had taken place in the presence of added pimelic acid, has been identified as biotin *l*-sulfoxide. Characterization involved a number of chemical and microbiological comparisons of the isolated material with an authentic sample of the sulfoxide.

The AN factor[1,2] as encountered in culture filtrates or partially purified concentrates was found to have a characteristic spectrum of microbiological activity, to have the same acid strength as biotin, and to have the cyclic urea ring intact as evidenced by avidin combinability.[3] Under appropriate conditions the factor could be reduced to biotin or to desthiobiotin. Acid hydrolysis yielded small amounts of biotin or "biotin sulfoxide" as determined by microbiological assay. Alkaline hydrolysis led to complete inactivation of the factor. These properties were such as to suggest that the AN factor is quite similar to biotin, differing only in the state of oxidation of the sulfur atom. Three such derivatives of biotin have been described in the literature, only one of which has previously been isolated from natural material. Biotin sulfone has been prepared and studied microbiologically.[4] This compound was found in the present study to be an antimetabolite of biotin against *Neurospora crassa* and for this reason could be dismissed from consideration as the AN factor. Two isomeric forms of biotin sulfoxide (I) have been described.[5]



I

The two sulfoxides designated as the *dextro-* and *levo*-forms[6] were prepared by the oxidation of biotin with an equivalent of hydrogen peroxide. The *dextro*-form which is obtained in the larger amount in the oxidation is as active as biotin for *Lactobacillus arabinosus* and *Saccharomyces cerevisiae*. This isomer was also isolated from milk.[5] The *levo*-form of biotin sulfoxide is reported to be only about 5% as active as biotin for *Lactobacillus arabinosus* and less than one-thousandth as active as biotin for *Saccharomyces cerevisiae*.[5] It has not been isolated previously from natural sources.

In the present investigation attention was directed to the sulfoxides of biotin since in some bioautographic studies of biotin, using *Neurospora crassa* as the assay organism, the presence of two sulfoxides of biotin could be observed. These presumably originate by oxidation of biotin during the filter paper chromatography. One of these, occurring in the smaller amount, had an R_f value in 1-butanol–acetic acid–water identical with that of the AN factor. Through the courtesy of Dr. D. B. Melville, samples of the *dextro-* and *levo*-isomers of biotin sulfoxide have been obtained for chemical and microbiological examination. With the availability of the AN factor in crystalline form, it has been possible to make a number of chemical and microbiological comparisons with the two biotin sulfoxides. These comparisons, described in this paper, demonstrate conclusively that the AN factor and biotin *l*-sulfoxide are identical.

The crystalline AN factor and authentic biotin *l*-sulfoxide were found to have the same melting point which was not depressed when the two materials were mixed. The infrared absorption spectrum of the isolated substance corresponds with that of the known compound. The two compounds have the same spectrum of microbiological activity against a number of organisms. R_f values for the AN factor and biotin *l*-sulfoxide in a number of solvents are the same. The two compounds are equally labile to acid and alkaline hydrolysis. Although a comparison of crystalline AN factor with the AN factor-activity as it occurs in culture filtrates was of necessity less direct and dependent entirely upon the results of microbiological evaluations, the factor in culture filtrates has a spectrum of microbiological activity corresponding with that of the isolated substance. R_f values of the factor in culture filtrate agreed by one dimensional paper

(1) L. D. Wright and E. L. Cresson, THIS JOURNAL, **76**, 4156 (1954).

(2) L. D. Wright, E. L. Cresson, J. Valiant, D. E. Wolf and K. Folkers, *ibid.*, **76**, 4160 (1954).

(3) V. du Vigneaud, K. Dittmer, K. Hofmann and D. B. Melville, *Proc. Soc. Expil. Biol. Med.*, **50**, 374 (1942).

(4) K. Dittmer and V. du Vigneaud, *Science*, **100**, 129 (1944).

(5) D. B. Melville, D. S. Genghof and J. M. Lee, *Federation Proc.*, **9**, 204 (1950); D. B. Melville, *J. Biol. Chem.*, **208**, 495 (1954); D. B. Melville, D. S. Genghof and J. M. Lee, *ibid.*, **208**, 503 (1954).

(6) This nomenclature refers to the optical rotation of the sulfoxides and is not necessarily related to their spatial configuration.