TABLE II

5-PHENYL-5-(p-SUBSTITUTED PHENYL-AZOPHENYL)-HYDANTOINS (HN—CO, OC—NH >C< C_6H_5, C_6H_4—N=N—R)

	R	M.p., °C.	Yield, %	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found	Nitrogen, % Calcd.	Nitrogen, % Found	Color
1	4-HO-C_6H_4	277–278 d.	77	67.73	67.59	4.33	4.38	15.05	14.88	Bright yell.
2	4-HO-$C_{10}H_6$	278.5 d.	71	71.08	71.01	4.30	4.17	13.26	13.07	Dark red
3	2-HO-$C_{10}H_6$	294.5 d.	87	71.08	70.96	4.30	4.28	13.26	13.16	Vermillion
4	$(CH_3)_2N$-C_6H_4	274.0–274.5 d.	78	69.15	69.08	5.30	5.29	17.53	17.59	Orange
5	2-NH_2-6-SO_3H-$C_{10}H_5$	[a]	71	59.87	59.61	3.82	3.88	13.97	13.76	Maroon

[a] When heated, this material began to darken about 290°, but had not melted at 360°; on a platinum foil, heating caused the compound to swell to about ten times its original volume and to evolve much gas before burning slowly.

hydrochloric acid. The mixture was shaken at room temperature under hydrogen at a pressure of about 1.5 atmospheres. After 13 hours, the consumption of hydrogen ceased; there had been utilized about twelve mole-equivalents of hydrogen, and white, crystalline material had separated from the reaction mixture. Although a fresh portion of catalyst was added and shaking under hydrogen pressure was continued for two hours, no more gas was utilized. From the suspended solid was obtained 4.1 g. of small needle-like crystals of melting point 294–295° dec.; the filtrate gave an additional 1.7 g. of product m.p. 288–290° dec.; total yield 6.16 g. (98%). After repeated recrystallization from alcohol, 5,5-di-(4-methylcyclohexyl)-hydantoin melted at 297–298° dec.

Anal. Calcd. for $C_{17}H_{28}N_2O_2$: C, 69.82; H, 9.65; N, 9.58. Found: C, 69.99; H, 9.76; N, 9.70.

Preparation of 5-(Amino- or Hydroxyaryl-)-p-azophenyl-5-phenylhydantoin.—Usually, 10 g. (0.0375 mole) of 5-(4-aminophenyl)-5-phenylhydantoin was added to 6.5 ml. (0.075 mole) of concentrated hydrochloric acid and the mixture was stirred until a gummy mass resulted; the latter could be dissolved by addition of 100 ml. of water (this hydantoin does not dissolve readily in dilute acid). Chipped ice was added until the temperature of the solution reached 0°, 2.85 g. (0.0413 mole) of sodium nitrite (dissolved in 10 ml. of water) was added beneath the surface of the solution by means of a pipet. While the cold, diazotized mixture[18] was being stirred for 30 minutes, a solution was made of the compound to be coupled. In those cases where the coupling agent was an amine, 2.25 g. (0.0375 mole) of urea was added to the suspension of the diazonium compound and the stirring was continued for 15 minutes (to destroy excess nitrous acid) before the alcoholic solution of the amine (0.0375 mole) was added. If the compound to be coupled was acidic, 0.0375 mole of such material was dissolved in 500 ml. of water containing 3.0 g. (0.075 mole) of sodium hydroxide. To the well-stirred appropriate alcoholic solution (of an amine), or alkaline solution (of a phenol or sulfonic acid), chilled to 0°, was added the solution of the diazotized aminophenylhydantoin. Stirring was continued for 30 minutes after the addition had been made.

(18) Chilling the solution caused formation of a flocculent precipitate, but addition of the nitrite solution produced separation of a much greater amount of flocculent material.

When the resulting azo compound did not contain a basic group, the product was readily caused to precipitate from solution by acidification with glacial acetic acid. However, if the amino group was present, addition of sodium acetate or ammonium chloride proved adequate to cause separation of the dyestuff. Digestion of the resultant suspension on a steam-bath for one hour usually caused the solid to coagulate and to filter more readily. Glacial acetic acid and ethyl acetate were used with some success as solvents for purification of the azo compounds; however, because of the large solubility of the dyes in pyridine, the best medium for crystallization was pyridine–water.

The structure of each of the azo derivatives is predicated upon the usual tendency exhibited in coupling by the respective phenol, amine or aminosulfonic acid. Physical and analytical data for these azo derivatives are listed in Table II.

AUSTIN, TEXAS

[CONTRIBUTION FROM THE DEPARTMENT OF MICROBIOLOGICAL CHEMISTRY, SHARP AND DOHME DIVISION, MERCK & CO., INC.]

Biotin *l*-Sulfoxide. I. The Occurrence of a Previously Unrecognized Form of Biotin in Certain Fermentation Sources

BY LEMUEL D. WRIGHT AND EMLEN L. CRESSON

RECEIVED DECEMBER 9, 1953

Microbiological evidence is presented for the existence of a previously unrecognized form of biotin in certain mold filtrates where growth had taken place in the presence of added pimelic acid. The growth-promoting material has been tentatively named AN factor because it was first encountered in culture filtrates of *Aspergillus niger*. AN factor has a characteristic spectrum of microbiological activity. It may be distinguished from biotin and desthiobiotin and less readily from biocytin by paper chromatography. AN factor is more labile than biotin to both acid and alkali. It migrates on paper electrophoresis as a monocarboxylic acid. The factor may be reduced with zinc and hydrochloric acid or aluminum and sodium hydroxide to biotin and with Raney nickel to desthiobiotin. It combines with avidin.

The addition of pimelic acid or higher homologs to an *Aspergillus niger* fermentation was found by Eakin and Eakin to be associated with an increase in the biosynthesis of "biotin" as determined microbiologically with *Saccharomyces cerevisiae*.[1] Concurrently the nutritive requirement of *Corynebacterium diphtheriae* for pimelic acid[2] was found by du Vigneaud, *et al.*,[3] to be satisfied equally well with much smaller amounts of biotin indicating that pimelic acid probably is a precursor of biotin for this organism. Subsequent microbiological studies of Tatum[4] have furnished evidence that a factor formed from pimelic acid by certain molds is des-

(1) R. E. Eakin and E. A. Eakin, *Science*, **96**, 187 (1942).

(2) J. H. Mueller, *ibid.*, **85**, 502 (1937).

(3) V. du Vigneaud, K. Dittmer, E. Hague and B. Long, *ibid.*, **96**, 186 (1942).

(4) E. L. Tatum, *J. Biol. Chem.*, **160**, 455 (1945).

thiobiotin. It is now commonly accepted that the biosynthesis of the vitamin proceeds through pimelic acid and desthiobiotin as intermediates.

While the above interpretation of the role of pimelic acid and desthiobiotin in the biosynthesis of biotin is an attractive one, it should be appreciated that the evidence in support of this hypothesis is largely circumstantial. With the discovery by Work[5] that α,ϵ-diaminopimelic acid occurs as a previously uncharacterized component of a number of bacteria and the demonstration by Davis[6] and by Dewey and Work[7] that α,ϵ-diaminopimelic acid, presumably as a result of a single decarboxylation, can function as a precursor of lysine, at least in *Escherichia coli*, the possibility immediately became apparent that pimelic acid functions as a precursor of the lysine moiety of biocytin, recently identified as ϵ-N-biotinyl-L-lysine,[8,9] rather than as a precursor of biotin or desthiobiotin.

In an effort to show that the addition of pimelic acid to an *Aspergillus niger* fermentation leads to the formation of increased amounts of biocytin rather than biotin or desthiobiotin the procedures of Eakin and Eakin were duplicated with the exception that biotin was determined by microbiological assay with *Neurospora crassa* rather than with *Saccharomyces cerevisiae*. *Neurospora crassa*, which was arbitrarily selected as the assay organism for the microbiological investigation, grows readily on a simple sucrose–inorganic salt medium provided a suitable biotin source is available. Biotin, desthiobiotin or biocytin are known to satisfy this requirement.[10,11] Pimelic acid is not utilized by this strain[12] or two other *Neurospora* species.[13] The conclusions of Eakin and Eakin were confirmed with respect to the effect of pimelic acid in augmenting the biosynthesis of material with biotin activity. When the culture filtrates were bioautographed the presence of what appeared to be biotin or possibly desthiobiotin could be demonstrated by R_f values and spectrum of microbiological activity against a number of organisms.[12] In addition, bioautography with *Neurospora crassa* showed the existence of much larger amounts of a biotin derivative with R_f values closely resembling biocytin. It differed from biocytin in its low order of activity as a source of biotin for *Lactobacillus casei* and *Saccharomyces cerevisiae*. This spectrum of microbiological activity could not be reconciled with any biotin derivative that had previously been isolated from natural material. Thus, while the hypothesis that pimelic acid in *Aspergillus niger* functions as a precursor of the lysine portion of biocytin could not be established, the data did afford evidence for the existence of a previously unrecognized component of the culture filtrate.

(5) E. Work, *Biochem. J.*, **49**, 17 (1951).

(6) B. D. Davis, *Nature*, **169**, 534 (1952).

(7) D. L. Dewey and E. Work, *ibid.*, **169**, 533 (1952).

(8) L. D. Wright, E. L. Cresson, H. R. Skeggs, R. L. Peck, D. E. Wolf, T. R. Wood, J. Valiant and K. Folkers, *Science*, **114**, 635 (1951).

(9) R. L. Peck, D. E. Wolf and K. Folkers, THIS JOURNAL, **74**, 1999 (1952).

(10) V. G. Lilly and L. H. Leonian, *Science*, **99**, 205 (1944).

(11) L. D. Wright, E. L. Cresson, K. V. Liebert and H. R. Skeggs, THIS JOURNAL, **74**, 2004 (1952).

(12) L. D. Wright and E. L. Cresson, unpublished data.

(13) W. J. Robbins and R. Ma, *Science*, **96**, 406 (1942).

The factor was found to be synthesized from pimelic acid by various penicillia as well as by *Aspergillus niger*. Limited studies indicated that more factor is synthesized when growth occurs on a crude medium as compared to a synthetic one. Aeration favors the microbiological production. One strain of *Actinomycetes sp.* examined apparently failed to synthesize the factor.

After it was found that the AN factor has a spectrum of microbiological activity different from that of biocytin, experiments were undertaken in an effort to demonstrate that biotin is one moiety of the factor. Acid hydrolysis does indeed produce a slight increase in the microbiological activity of the factor for lactobacilli and, when carried out on crude concentrates, biotin could be detected in the hydrolyzed mixtures by paper chromatography. High potency concentrates yielded a mixture of biotin and "biotin sulfoxide." At no concentration of acid did the biotin or "biotin sulfoxide" obtained as an hydrolytic product ever exceed 20% of the apparent biotin content determined by *Neurospora crassa*. These findings led to the tentative hypothesis that the AN factor is a conjugate of biotin with microbiological activity for *Neurospora crassa* greater than that expected from its "total" biotin content or biotin content after acid hydrolysis. AN factor is much more labile to alkali than is biotin with complete loss of microbiological activity for both *Neurospora crassa* and *Lactobacillus arabinosus*.

Paper electrophoretic studies were employed to determine the acid strength of the AN factor. Biotin, N-biotinylaspartic acid and biocytin, representatives of a monocarboxylic acid, a dicarboxylic acid and a neutral compound, respectively, were available for comparative studies. Under standardized conditions AN factor migrates toward the anode at the same rate as biotin whose rate of migration is intermediate between that of biocytin and biotinylaspartic acid. AN factor was, therefore, concluded to be a monocarboxylic acid.

Reduction of microgram quantities of high potency AN factor concentrates with zinc and hydrochloric acid or aluminum and sodium hydroxide yielded biotin. Raney nickel reduction experiments gave desthiobiotin. The identity of these reduction products was established by microbiological assay using appropriate organisms and/or bioautographic procedures. The recovery of biotin was poor in the studies, possibly because of adsorption on the metals used.

Avidin was found to render AN factor microbiologically unavailable to *Neurospora crassa*. Preliminary studies indicate that a larger quantity of avidin is required to negate AN factor than is necessary for a microbiologically equivalent amount of biotin.[14]

Experimental

Microbiological Procedures.—Microbiological assays with *Neurospora crassa* were carried out with a *p*-aminobenzoic acid-dependent mutant.[15] The basal medium had the following composition per liter: sucrose, 20 g.; ammonium tartrate, 5 g.; ammonium nitrate, 1 g.; potassium dihydrogen phos-

(14) L. D. Wright and H. R. Skeggs, *Arch. Biochem.*, **12**, 27 (1947).

(15) E. L. Tatum and G. W. Beadle, *Proc. Natl. Acad. Sci. U.S.*, **28**, 234 (1942).

phate, 1 g.; magnesium sulfate (heptahydrate), 0.5 g.; sodium chloride, 0.1 g.; calcium chloride, 0.086 g.; trace elements,[16] 1 ml.; *p*-aminobenzoic acid, 10 mg. Assays were carried out in 125-ml. erlenmeyer flasks with 25 ml. of medium per flask. The standard curve involved 0.0, 0.0005, 0.0010, 0.0015, 0.0020, 0.0030 and 0.0050 μg. amounts of biotin. The extent of growth was measured by weighing the pressed, dried mycelium. Other details were according to customary procedures used with *Neurospora crassa*.[17] Microbiological assays with *Lactobacillus arabinosus* (ATCC 8014) were carried out as previously described in detail.[18] Assays with *Leuconostoc dextranicum*[19] were done similarly except that 1 μg. of folic acid/tube was added to the basal medium. Assays with *Lactobacillus casei* (ATCC 7469) involved the addition of folic acid and a 72-hour incubation period. Microbiological assays with yeast were carried out essentially as described for the determination of pyridoxine except that pyridoxine was added to the basal medium (10 μg/100 ml.) and biotin was omitted.[20] Commercially available (Fleischmann) dehydrated yeast was used as the assay organism.

Bioautography with *Neurospora crassa* involving paper chromatograms and paper electrophoretograms was accomplished by cutting the papers into sections which were eluted in erlenmeyer flasks with 25 ml. of basal medium. After removal of the papers following a 1-hour elution period, the flasks were plugged, autoclaved, seeded with *Neurospora crassa*, incubated, and the extent of growth determined in the usual manner. It was convenient to cut paper chromatograms into 21 sections such that each section represented 0.05 of an R_f unit. When the mycelium weight was plotted as a function of the paper section number a smooth curve connecting the points permitted location of the R_f value by interpolation to within 0.02 unit. Paper electrophoretograms were cut into 0.5-cm. sections permitting, by the same technique, the location of a microbiologically active component to within 0.1 cm. Bioautography was carried out with lactic acid bacteria and yeast by accepted techniques.[21] 2,3,5-Triphenyltetrazolium chloride addition to the basal medium helped to locate accurately areas of microbiological activity.[22]

Microbiological Production.—*Aspergillus niger* was grown in 500-ml. amounts in 1000-ml. erlenmeyer flasks on a basal medium with the following composition per liter: sucrose, 30 g.; sodium nitrate, 2 g.; dipotassium phosphate, 1 g.; magnesium sulfate (heptahydrate), 0.5 g.; potassium chloride, 0.5 g.; ferrous sulfate (heptahydrate), 0.01 g.; pimelic acid where used, 1 mg.; *p*H 4.0–4.5. Flasks were incubated at 30° for 5–7 days. Where incubation with shaking was carried out this was done at the rate of about 200 rotations per minute. The data, summarized in Table I, demonstrate that the production of the factor by *Aspergillus niger* is increased by addition of pimelic acid to the medium

TABLE I

ACTIVITY OF *Aspergillus niger* CULTURE FILTRATE AS A SOURCE OF BIOTIN FOR VARIOUS MICROÖRGANISMS

Organism	Material assayed: Unshaken cultures, Unsuppl. medium, μg./ml.	Unshaken cultures, Pimelic acid suppl. medium, μg./ml.	Shaken cultures, Unsuppl. medium, μg./ml.	Shaken cultures, Pimelic acid suppl. medium, μg./ml.
Neurospora crassa	0.0016	0.012	0.013	0.040
Lactobacillus casei	.00013	.00011	.0024	.010
Lactobacillus arabinosus	.00008	.00018	.0010	.0035
Leuconostoc dextranicum	.00034	.0066	.0042	.0055

and by continuous shaking of the cultures during growth. The relative inactivity of the factor as a source of biotin for lactobacilli as compared to *Neurospora crassa* also is clearly shown. A strain of penicillia grown with shaking on the medium described above produced 0.05 μg./ml. of biotin activity by *Neurospora crassa*. *Penicillium chrysogenum* in a crude medium such as used in penicillin production produced 0.37 μg./ml.

Paper Chromatography.—The bioautographic data are summarized in Table II. For comparison, 0.01-μg. amounts of biotin and desthiobiotin were subjected to paper chromatography with AN factor concentrates of approximately equal activity as determined by *Neurospora crassa* assay. Comparison of the behavior, using a number of solvents, serves to establish that the AN factor is not biotin or desthiobiotin. In all solvents used R_f values were remarkably similar to corresponding values for biocytin.[11] The relative inactivity of the AN factor for several biocytin-utilizing organisms, as described above, served, therefore, as a better means of differentiating the factor from biocytin than did paper chromatography. AN factor could not be detected on bioautography with yeast as the indicating organism when present in amounts considered adequate on the basis of activity with *Neurospora crassa*. Extensive paper chromatographic studies of the AN factor are considered in a separate paper of this series.[23] In all the paper chromatographic work described in this paper and a subsequent one[23] the ascending procedure[24] was employed. The development time was usually 16–18 hours. Development was at room temperature which was not excessive during the period of this study. Under these conditions of paper chromatography the solvent usually travelled about 30 cm. After development the papers were air-dried in a hood.

TABLE II

R_f VALUES OF BIOTIN, DESTHIOBIOTIN AND AN FACTOR

Solvent	R_f values of: Biotin[a]	Desthiobiotin[b]	AN factor[c,d]
Benzyl alcohol (satd. with H_2O)	0.44	0.58	0.13
Pyridine (70%), water (30%)	.86	.91	.83
Isobutyric acid, (satd. with H_2O)	.87	.94	.70
Isoamyl alcohol (satd. with H_2O)	.00	.00	.00
1-Butanol (40%), water (50%) and acetic acid (10%)	.78	.89	.38
Isoamyl alcohol–5% KH_2PO_4	.79	.72	.83
1-Butanol (satd. with 10% urea)	.14	.36	.00

[a] Determined with *Lactobacillus arabinosus*. [b] Determined with *Leuconostoc dextranicum*. [c] Determined with *Neurospora crassa*. [d] In the form of a low potency concentrate.

Stability to Acid and Alkali.—One ml. aliquots of a solution of a low potency AN factor concentrate, free of other biotin sources according to paper chromatography, were pipetted into separate tubes. One-ml. quantities of sulfuric acid were added to the tubes to give a range of concentration from 0 to 9 *N*. The tubes were autoclaved at 120° for 1 hour. After neutralization and dilution, the solution contained in each tube was assayed with *Neurospora crassa* and with *Lactobacillus arabinosus*. The data, summarized in Table III, indicate marked instability of the factor to acid as demonstrated by *Neurospora crassa* assay and small but significant increase in microbiological activity for *Lactobacillus arabinosus*. When the acid hydrolysis was carried out on crude concentrates of the AN factor and a portion of the 3 *N* acid hydrolysate bioautographed, unchanged AN factor, biotin, and a small amount of "biotin sulfoxide" were found present. The solvent system was 1-butanol–water–acetic acid and the R_f values were 0.47, 0.81 and 0.57, respectively, with *Neurospora crassa*. "Biotin sulfoxide" always is encountered when biotin is chromatographed on unwashed Whatman 1 paper with 1-butanol–water–acetic acid as the solvent.[25] When a similar experiment was carried out on a high potency concentrate, "biotin

(16) N. H. Horowitz and G. W. Beadle, *J. Biol. Chem.*, **150**, 325 (1943).

(17) E. E. Snell, "Vitamin Methods," Vol. I, Academic Press, Inc., New York, N. Y., 1950.

(18) L. D. Wright, *Biological Symposia*, **12**, 290 (1947).

(19) V. Whitside-Carlson, W. R. Starnes, C. L. Rosano and W. W. Carlson, *Proc. Soc. Exptl. Biol. Med.*, **77**, 344 (1951).

(20) L. Atkin, A. S. Schultz, W. L. Williams and C. N. Frey, *Ind. Eng. Chem., Anal. Ed.*, **15**, 141 (1943).

(21) W. A. Winsten and E. Eigen, *J. Biol. Chem.*, **177**, 989 (1949).

(22) J. E. Ford and E. S. Holdsworth, *Biochem. J.*, **53**, xxii (1953).

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

(24) R. J. Williams and H. Kirby, *Science*, **107**, 481 (1948).

(25) This phenomenon first observed by B. D. Davis (personal communication of Nov. 22, 1949).

sulfoxide" (R_f 0.59 in the same solvent system) rather than biotin was the predominant product.

TABLE III

EFFECT OF AUTOCLAVING WITH ACID ON THE MICROBIOLOGICAL ACTIVITY OF AN FACTOR

Acid concn., N	AN factor content by assay *Neurospora crassa* μg./ml.	*Lactobacillus arabinosus* μg./ml.
0	4.8	0.20
0.6	4.4	.24
1.2	3.9	.31
3.0	3.1	.52
6.0	1.8	.48
9.0	0.86	.12

One-ml. aliquots of a solution of a low potency AN factor concentrate, free of other biotin sources according to paper chromatography, were pipetted into separate tubes. The alkaline hydrolysis was carried out by adding 1-ml. aliquots of sodium hydroxide solution to the tubes to give a range of concentration from 0 to 4.5 *N*. The tubes were autoclaved at 120° for 1 hour. After neutralization and dilution, the solutions were assayed with *Neurospora crassa* and with *Lactobacillus arabinosus*. The results obtained, demonstrating marked lability of the AN factor to alkali by either method of assay, are summarized in Table IV.

TABLE IV

EFFECT OF AUTOCLAVING WITH ALKALI ON THE MICROBIOLOGICAL ACTIVITY OF AN FACTOR

Alkali concn., N	AN factor destroyed *Neurospora crassa* assay, %	*Lactobacillus arabinosus* assay, %
0	0	0
0.3	46	45
0.6	52	59
1.5	70	78
3.0	91	96
4.5	97	95

Paper Electrophoresis.—An apparatus essentially as described by Gordon, *et al.*,[26] was used. The *p*H 5.9 buffer used had the following composition: 50 ml. of 0.2 *M* potassium acid phthalate and 43 ml. of 0.2 *M* sodium hydroxide diluted to 400 ml. Migration was for 3 hours at 400 volts. Areas of activity were located by cutting the paper into 0.5-cm. sections which were evaluated with *Neurospora crassa* as described for paper chromatography. The data of Table V show that the AN factor migrates toward the anode at the same rate as biotin. This rate is between that of biocytin and biotinylaspartic acid. The migration that is observed with biocytin is attributable largely, if not entirely, to electroendosmosis.

TABLE V

PAPER ELECTROPHORESIS OF BIOTIN AND DERIVATIVES

Factor(s) migrated	Areas of microbiol. activity and distance from origin, cm.
Biotin	13.0
Biotin + AN factor	12.8
Biocytin	8.3
Biocytin + AN factor	8.3, 13.7
Biotinylaspartic acid	16.0
Biotin + biotinylaspartic acid	13.1, 16.0

Reduction Experiments.—Amounts of high potency AN factor concentrate[27] containing 2 μg. of activity by microbiological assay with *Neurospora crassa* in 10 ml. of water were treated in one experiment with 200 mg. of zinc dust and 2 ml. of 1 *N* hydrochloric acid and in a second experiment with 200 mg. of granular aluminum and 2 ml. of 2 *N* sodium hydroxide solution. After 2 hours the reductions were stopped by neutralization of the acid and alkali, respectively, the solutions were diluted to a convenient volume, and assayed for biotin activity with *Lactobacillus arabinosus*. The data obtained indicated a 7- to 9-fold increase in biotin activity for this organism. Although the reaction mixtures were not chromatographed, the requirement of *Lactobacillus arabinosus* for biotin is sufficiently specific that the results may be taken as presumptive evidence that biotin is a product of the reduction of the AN factor with nascent hydrogen.

An amount of high potency AN factor concentrate[27] containing 10 μg. of activity by microbiological assay with *Neurospora crassa* was refluxed in 100 ml. of ethanol with 1 g. of Raney nickel. The ethanol was decanted and the Raney nickel eluted by refluxing a number of times alternately with ethanol and with methanol. The supernatant solution together with the nickel eluates was concentrated *in vacuo* to dryness. Bioautography involving paper chromatography in 1-butanol–water–acetic acid (4:5:1) and location of activity with *Neurospora crassa* showed the untreated concentrate to contain only one microbiologically active component with R_f of 0.47. This corresponds to the previously determined R_f value of the AN factor. After reduction, only one microbiologically active component was present with an R_f of 0.88. In this system biotin has an R_f of about 0.83 while desthiobiotin has an R_f of about 0.89. Differential microbiological assay of the reduced material with *Lactobacillus arabinosus* which cannot utilize desthiobiotin to satisfy its biotin requirement and *Leuconostoc dextranicum* for which desthiobiotin is essentially equivalent to biotin confirmed the R_f value as corresponding to desthiobiotin.

An equivalent amount of biotin was similarly reduced. Before reduction bioautography showed the existence of two microbiologically active components with R_f values of 0.83 and 0.58 corresponding to biotin and "biotin sulfoxide," respectively. After reduction only one microbiologically active component was present with R_f of 0.87.

Affinity of Avidin for the AN Factor.—A microbiological assay with *Neurospora crassa* was set up containing, in addition to the regular biotin standards, four flasks of biotin, four of desthiobiotin and four of the AN factor in amounts previously determined to give just maximal growth of the organism. After the usual addition of medium, autoclaving and seeding, increasing levels of a sterile egg white dilution as a source of avidin were added to each series of four flasks. The egg white was purposely diluted so that 0.5 ml. would just combine with 0.005 μg. of biotin. After growth of

TABLE VI

AFFINITY OF AVIDIN FOR BIOTIN, DESTHIOBIOTIN AND AN FACTOR

Biotin or derivative	Biotin equiv., mμg.	Egg white dilution, ml.	Weight of *Neurospora crassa* mycelium, mg.
Biotin	0		1.4
	0.5		21.7
	1.0		22.6
	1.5		30.0
	2.0		33.8
	3.0		39.5
	5.0		43.5
	5.0	0	41.3
	5.0	0.5	0.0
	5.0	1.0	0.0
	5.0	2.0	0.0
Desthiobiotin	5.0	0	44.3
	5.0	0.5	0.5
	5.0	1.0	0.1
	5.0	2.0	0.0
AN factor	5.0	0	43.0
	5.0	0.5	0.8
	5.0	1.0	0.8
	5.0	2.0	0.4

(26) A. H. Gordon, J. Gross, D. O'Connor and R. Pitt-Rivers, *Nature*, **169**, 19 (1952).

(27) L. D. Wright, E. L. Cresson, J. Valiant, D. E. Wolf and K. Folkers, THIS JOURNAL, **76**, 4160 (1954).

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).