[CONTRIBUTION FROM THE RESEARCH DIVISION OF SHARP AND DOHME, INC.]

Biological Studies of Biocytin

BY LEMUEL D. WRIGHT, EMLEN L. CRESSON, KATHERINE V. LIEBERT AND HELEN R. SKEGGS

Biocytin from yeast extract and synthetic biocytin were examined concurrently for activity in promoting growth of a variety of microörganisms in basal media free of biotin, in reversing the antimetabolite homobiotin, in combining with avidin, and in stimulating the aspartic acid deaminase system of treated bacteria. Biological tests also were employed in studies involving paper chromatographic behavior of the compounds and in studies involving stability of the compounds to acid and enzymatic hydrolysis. All biological comparisons confirm the identity of isolated and synthesized biocytin. Paper chromatography and bioautographic techniques also were employed to furnish additional evidence that biocytin as isolated and synthesized is the biotin complex of yeast extract.

Many microörganisms with a nutritional requirement for biotin or biotin-like compounds utilize biocytin for purposes of growth in basal media free of compounds related to biotin in structure or function. Other similar fastidious microörganisms, including two whose requirement for biotin or biotin-like compounds was induced by mutation, do not utilize biocytin.[1,2] More recent studies, described in this paper, in which biocytin obtained by isolation from yeast extract[2,3] and by synthesis[4] have been found to have the same spectrum of microbiological activity, furnish additional evidence that biocytin from both sources is identical. It may be noted further that the spectrum of microbiological activity obtained with biocytin from both sources is the same as that previously reported for the biotin complex of yeast extract.[2] These data, further substantiated by the results obtained by paper chromatography, afford evidence for the identity of biocytin and the biotin complex of yeast extract.

Homobiotin has been reported to be an effective antimetabolite of biotin against *Lactobacillus casei*.[5] Synthetic biocytin and biocytin from yeast extract have been examined for activity in reversing this analog and were found to be equally active. At high levels of inhibition biotin is a more effective reversing agent than biocytin.

Previous studies have disclosed that while avidin combines readily with biotin, presumably through attachment to the urea ring,[6] combination of avidin can occur with compounds related to biotin in structure.[6,7,8] The present studies, involving avidin as encountered either in a commercial avidin concentrate or as present in fresh egg white, demonstrate that avidin readily combines with crystalline biocytin from either yeast extract or synthetic source with the same degree of affinity. Affinity ratios obtained in the present studies agree well with that previously reported[9] for isolated biocytin.

Lichstein and Umbreit have reported that biotin stimulates the aspartic acid (and reportedly serine and threonine) deaminase activity of bacteria that have been exposed for a time to *p*H 4 *M* phosphate buffer.[10] Lichstein has reported further that yeast extract is approximately 100 times more active than can be accounted for on the basis of its biotin content in stimulating the deaminase activity of treated cells.[11] These data led Lichstein to postulate the existence in yeast extract of a more highly active, presumably coenzyme, form of biotin that yields biotin on hydrolysis.[12] Biocytin (in the form of a concentrate) was examined by Lichstein, *et al.*,[13] who found that the material did not stimulate the aspartic acid deaminase activity of treated cells under his conditions. Isolated biocytin (crystalline) subsequently has been tested in these laboratories and found active in this system.[14] Activity observed with biocytin always was equal to or less than that of an equivalent of biotin. The present data confirm the latter findings and demonstrate that isolated and synthesized biocytin have essentially the same activity in the system.

The bioautographic technique[15] has been applied to a study of the extent to which isolated and synthesized biocytin partition on paper with a series of developing solvents. In 14 different solvents, isolated and synthesized biocytin have comparable *RF* values. The same procedures have been applied to furnish additional evidence that isolated biocytin[2,3] is identical with the biotin complex of yeast extract. In 10 solvent systems the biotin activity of yeast extract corresponds in *RF* value with that observed with isolated biocytin.

The biotin complex of yeast extract and isolated biocytin previously have been described as unavailable to *Lactobacillus arabinosus* as a source of biotin.[2,3] In the present experiments, isolated and synthesized biocytin have been compared with respect to the extent to which acid of various concentrations yields biotin as a product of hydrolysis. Isolated and synthesized biocytin are equally resistant to acid hydrolysis.

The biotin of yeast extract as well as isolated biocytin previously have been described as resistant to the action of a variety of commercial enzymes.[2]

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(2) L. D. Wright, E. L. Cresson, H. R. Skeggs, T. R. Wood, R. L. Peck, D. E. Wolf and K. Folkers, *ibid.*, **72**, 1048 (1950).

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(13) H. C. Lichstein, J. F. Christman and W. L. Boyd, *ibid.*, **59**, 113 (1950).

(14) L. D. Wright, E. L. Cresson and H. R. Skeggs, *Proc. Soc. Exper. Biol. & Med.*, **74**, 334 (1950).

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

The release of biotin as determined by microbiological assay with *Lactobacillus arabinosus*[16] has been used as a measure of the extent to which biocytin is resistant to the more common enzymes. Isolated and synthesized biocytin were found equally resistant to the action of any one of six commercial enzymes.

Experimental

Studies of Microbiological Activity.—Previously described strains, media, and procedures[1] were used in studies of the extent to which isolated or synthesized biocytin can function *in lieu* of biotin as a growth factor for a variety of fastidious microörganisms. All determinations of microbiological activity involving lactic acid bacteria were obtained after 18–24 hours of incubation. With *Lactobacillus arabinosus* and *Lactobacillus casei* determinations also were made after 72 hours of incubation. Tests inoculated with *Neurospora crassa* and *Penicillium chrysogenum* were incubated for 4 days. Where isolated and synthesized biocytin were active they promoted the same rate of growth as determined by visual observation. Isolated and synthetic biocytin were found inactive in satisfying the biotin requirements of *Lactobacillus arabinosus* 17-5 (8014), *Leuconostoc mesenteroides* P-60 (8042) and *Penicillium chrysogenum* 62078. Isolated and synthetic biocytin were equally active in satisfying the biotin requirements of *Lactobacillus acidophilus* 05, *Lactobacillus acidophilus* (4357), *Lactobacillus acidophilus* K, *Lactobacillus brevis* (8287), *Lactobacillus casei* (7469), *Lactobacillus delbrückii* LD5 (9595), *Lactobacillus plantarum* (4943), *Neurospora crassa* (9278), *Propionibacterium shermanii* (8262) and *Streptococcus fecalis* R (8043).

Studies of Homobiotin Reversal.—*dl*-Homobiotin used in these studies kindly was supplied by Hoffmann-La Roche, Inc., Nutley, New Jersey. Inhibition ratios determined with *Lactobacillus casei* after 18–24 hours of growth, summarized in Table I, indicate the ratio of antimetabolite (homobiotin) to metabolite (biotin, isolated or synthesized biocytin) required to depress growth to one-half that obtained in the absence of antimetabolite.

TABLE I

REVERSAL OF DL-HOMOBIOTIN INHIBITION OF *Lactobacillus casei* WITH BIOTIN, ISOLATED OR SYNTHESIZED BIOCYTIN

Level of biotin, isolated or synthesized biocytin, γ/10 ml.	Inhibition ratios		
	Biotin	Isolated biocytin	Synthesized biocytin
0.0025	320	200	200
.01	350	200	200
.05	>10,000	250	250

Studies of Avidin Combinability.—Determinations of the relative affinity of avidin for isolated or synthesized biocytin compared to biotin were carried out with *Lactobacillus arabinosus* essentially as described previously.[7,8,9] A commercial avidin concentrate containing 50 "units" of activity per g. and sterile egg white were studied as sources of avidin. The affinity ratios are expressed as the ratio of analog (isolated or synthesized biocytin) to biotin at which, when avidin and biotin are used in stoichiometric equivalents, one-half the biotin remains free and available for growth of the test organism. With egg white as a source of avidin affinity ratios of 6.8 and 7.2 for isolated and synthesized biocytin, respectively, were obtained. With avidin concentrate an affinity ratio of 3.2 was obtained with both isolated and synthesized biocytin.

Studies of Aspartic Acid Deaminase Stimulation.—Determinations of activity in stimulating the aspartic acid deaminase system of bacteria previously exposed to *p*H 4 *M* phosphate buffer were carried out as described in detail in previous papers.[14,17,18] *Proteus vulgaris* was the organism used. The deamination reaction was carried out at *p*H 7.0. The results obtained are summarized in Table II.

Studies of Paper Chromatographic Behavior.—0.002-γ amounts of isolated and synthesized biocytin, each in 0.02 ml., were applied to sheets of Whatman #1 filter paper 2.5 cm. from one edge. Each paper then was developed in a selected solvent according to the ascending technique of Williams and Kirby[19] After the solvent fronts had moved 25–30 cm., the papers were removed from their respective solvents and air-dried. *RF* values were determined by the bioautographic procedure.[15] The basal medium was essentially that of Landy and Dicken[20] solidified with 1.5% agar. *Lactobacillus casei* was the microörganism employed. Results are summarized in Tables III and IV.

TABLE II

ISOLATED AND SYNTHESIZED BIOCYTIN IN ACTIVATION OF THE ASPARTIC ACID DEAMINASE SYSTEM OF TREATED BACTERIAL CELLS

Experiment number	Ammonia nitrogen (γ) found in presence of			
	No addition	Biotin	Isolated biocytin	Synthesized biocytin
1	22.0	44.0	47.5	42.0
		(0.001)[a]	(0.001)	(0.001)
2	36.5	59.5	58.0	59.5
		(0.001)	(0.001)	(0.001)
3	31.0	46.0	41.5	43.5
		(0.0001)	(0.0001)	(0.0001)

[a] Figures in parentheses indicate micrograms of biotin or biocytin as biotin per 2 ml. enzymatic reaction mixture.

TABLE III

RF VALUES OF ISOLATED AND SYNTHESIZED BIOCYTIN

Solvent	*RF* values of	
	Isolated biocytin	Synthesized biocytin
Water	0.94	0.91
Ethyl alcohol (70%)	.59	.58
n-Butyl alcohol (satd. with H_2O)	.12	.14
n-Butyl alcohol (satd. with 0.1 *N* HCl)	.18	.18
n-Butyl alcohol (50%), H_2O (40%), HAc (10%)	.10	.09
n-Butyl alcohol (satd. with 10% urea)	.05	.05
t-Butyl alcohol (satd. with H_2O)	.34	.35
Isoamyl alcohol (1 part), 5% KH_2PO_4 (2 parts)	.84	.83
n-Butyric acid (70%), H_2O (30%)	.85	.84
Isobutyric acid (satd. with H_2O)	.82	.82
Isobutyric acid ($^1/_2$ satd. with H_2O)	.68	.67
Phenol (satd. with H_2O)	.89	.90
m,p-Cresol (satd. with H_2O)	.86	.87
Pyridine (70%), H_2O (30%)	.76	.76

TABLE IV

RF VALUES OF BIOTIN, ISOLATED BIOCYTIN AND THE BIOTIN ACTIVITY OF YEAST EXTRACT

Solvent	*RF* values		
	Biotin	Isolated biocytin	Yeast extract
Water	0.95	0.87	0.91
n-Butyl alcohol (satd. with H_2O)	.46	.08	.11
n-Butyl alcohol (50%), H_2O (40%), HAc (10%)	.71	.09	.09
n-Butyl alcohol (satd. with 10% urea)	.08	.02	.04
t-Butyl alcohol (anhydrous)	.88	.86	.85
Isoamyl alcohol (1 part), 5% KH_2PO_4 (2 parts)	.78	.84	.83
n-Butyric acid (70%), H_2O (30%)	.85	.85	.85
m,p-Cresol	.67	.85	.90
Ethyl acetate	.05	.03	.04
Benzyl alcohol	.64	.10	.14

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Studies of Acid Stability.—1-γ amounts of isolated and synthesized biocytin in 1-ml. volumes of water were autoclaved for 1 hour at 120° with 1-ml. portions of 0.2, 0.4, 2.0 and 6.0 N H_2SO_4. Following autoclaving each hydrolysate was neutralized, diluted and assayed for microbiological activity with *Lactobacillus arabinosus*.[16] The results are summarized in Table V.

TABLE V

ACID STABILITIES OF ISOLATED AND SYNTHESIZED BIOCYTIN

Normality	Per cent. availability to *Lactobacillus arabinosus* after hydrolysis with acid of the indicated normality: Isolated biocytin	Synthesized biocytin
0	0	0
.1	23	21
.2	43	51
1	91	102
3	100	100

Studies of Enzymatic Stability.—1-γ amounts of isolated and synthesized biocytin were digested under benzene at 37° for 18 hours with 10-mg. quantities of various commercial enzyme preparations in 10 ml. of menstruum considered appropriate for the action of the particular enzyme. Appropriate controls without added biocytin also were prepared. The amount of free biotin, as determined by direct assay with *Lactobacillus arabinosus*, found in each sample containing added biocytin less the amount of free biotin found in the appropriate enzyme control was taken as a measure of the extent to which a particular enzyme hydrolyzes biocytin. The following enzymes, under the conditions used, did not hydrolyze over 1% of the added biocytin: pepsin in 0.1 N HCl, papain or takadiastase in pH 4 phosphate buffer, polidase, mylase or trypsin in pH 7 phosphate buffer.

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[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

A Method for the Rapid Cleavage of Sulfonamides[1]

BY H. R. SNYDER AND RICHARD E. HECKERT[2]

A mixture of 48% hydrobromic acid and phenol is an excellent reagent for the rapid cleavage of a sulfonamide when the object is the recovery of the constituent amine. The cleavage is not a simple hydrolysis; an oxidation–reduction reaction between the sulfur-containing fragment and the hydrobromic acid is involved. The phenol serves not only to remove bromine from the reaction mixture, thereby preventing its reaction with the liberated amine, but also to increase the solubility of the sulfonamide.

The foremost objection to the Hinsberg method[3] for the separation of primary, secondary and tertiary amines has been the difficulty with which the intermediate sulfonamides are hydrolyzed. Although a number of attempts have been made to find conditions under which this hydrolysis would proceed rapidly and in good yield, none has been entirely satisfactory. In the present investigation, a mixture of 48% hydrobromic acid and phenol has been found to be an excellent reagent for cleaving sulfonamides when the object is the recovery of the constituent amines.

The reagents commonly employed in the acid hydrolysis of sulfonamides are hydrochloric and sulfuric acids. Whereas sulfonamides are sparingly soluble in hydrochloric acid, they are usually soluble in strong sulfuric acid. Various concentrations of sulfuric acid[4] and sulfuric and acetic acids[5] have been used at moderate temperatures. Although satisfactory hydrolysis rates are normally observed, when aryl amines are produced in the hydrolysis the reaction is complicated by sulfonation. In concentrated sulfuric acid, the rearrangement of sulfonamides to amino-sulfones may also occur.[4b,6]

Concentrated hydrochloric acid was first thought effective only in sealed tubes at temperatures near 150°,[7,8] but Schreiber and Shriner[9] found refluxing 25% hydrochloric acid to be useful. Aniline and N-methylaniline were recovered from benzene and substituted benzenesulfonanilides in excellent yields by this method, but reflux periods of from 12 to 36 hours are normally required, and some o- and p-substituted arylsulfonamides react very slowly or not at all. There appear to be no examples of alkaline hydrolysis of sulfonamides involving, as a first step, cleavage of the sulfur–nitrogen bond.

Hydrobromic acid is often a more effective hydrolytic agent than hydrochloric acid, and it seemed desirable to test its action on a simple substance of this class. When benzenesulfonanilide was heated under reflux with freshly distilled 48% hydrobromic acid, p-bromoaniline rather than aniline was found as the principal constituent of the amine fraction. The reaction, further characterized by reduction of the sulfonyl group, undoubtedly is related to the cleavage of arylsulfonamides by hydriodic acid (d. 1.96), first described by Fischer.[10] At temperatures of 70 to 100° benzene- and p-toluenesulfonamides were cleaved in 25 to 30 minutes with the formation of the amines, the aryl disulfides and iodine. When phosphonium iodide was added to reduce the iodine, the thiophenols rather than the disulfides were obtained. Schönheimer[11] made use of this reductive cleavage of sulfonamides in the synthesis of polypeptides from amino acids and p-toluenesulfonamido acid chlorides. Under the

(1) After the completion of this work a similar method has been disclosed by D. I. Weisblat, B. J. Magerlein and D. R. Myers [U. S. Patent 2,562,222, July 31, 1951; see also D. Weisblat, Abstracts of Papers at XII International Congress of Pure and Applied Chemistry, p. 76 (Sept. 1951)].

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(4) (a) O. Witt and H. Truttwin, *ibid.*, **47**, 2786 (1914); (b) O. Witt and D. Uermenyi, *ibid.*, **46**, 296 (1913); (c) G. Schroeter and O. Eisleb, *Ann.*, **367**, 157 (1909).

(5) F. Ullmann and H. Bleier, *Ber.*, **35**, 4273 (1902).

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(11) R. Schönheimer, *Z. physiol. Chem.*, **154**, 203 (1926).