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Isolation of a New Acetate-replacing Factor

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A new acetate-replacing factor for lactobacilli has been isolated from distillers' dried solubles.

An isolation procedure has been developed by which a new acetate-replacing factor for lactobacilli has been obtained in a nearly pure form. Evidence for the existence of a factor essential or stimulatory for the growth of a number of lactobacilli in a defined medium devoid of acetate has been presented by Skeggs, *et al.*[1] The factor may be quantitatively determined by microbiological assay with *Lactobacillus acidophilus* ATCC 4963.

The factor is an acid since it is retained by strong anion-exchange resins but not retained by strong cation-exchange resins. It is relatively stable to alkali, but unstable to acid and not readily absorbed by Norit or fullers' earth. It is readily soluble in water and ethanol, and sparingly soluble in chloroform and butanol.

This paper describes procedures by which the factor has been isolated in a nearly pure state from distillers' dried solubles. The successful isolation scheme was the following: methanol extraction of distillers' solubles; continuous chloroform extraction of the methanol soluble fraction; superfiltrol chromatography of the chloroform soluble portion; ethanol precipitation of inert material in selected eluates; adsorption chromatography of the ethanol soluble portion on a strong cation-exchange resin; repeated ion-exchange chromatography of selected fractions from the cation-exchange resin on a strong anion-exchange resin; and, finally, repeated countercurrent distribution of selected fractions from the anion-exchange resin.

Two steps in the isolation procedure were of particular value in increasing the potency of the factor. The first is the ion-exchange chromatography on Dowex-1 with elution by dilute formic acid. In this step the potency of the concentrates could be increased about fivefold. The elution was essentially quantitative and no loss of activity accompanied the procedure.

The second procedure of note was the use of countercurrent distribution. Early in the isolation work it was suspected that the factor was a compound of low molecular weight and high water solubility. The countercurrent method was favored by the finding that in the solvent mixture ethanol, water, chloroform (4:4:3) the factor had a distribution coefficient of approximately 1. The final purification involved successive countercurrent distributions. After each stage the band containing the highest microbiological activity was collected and redistributed and the less active side bands were reworked. A highly active concentrate of the factor was obtained as a clear oil. The weight distribution and microbiological activity curves were close to the theoretical distribution curve (Fig. 1).

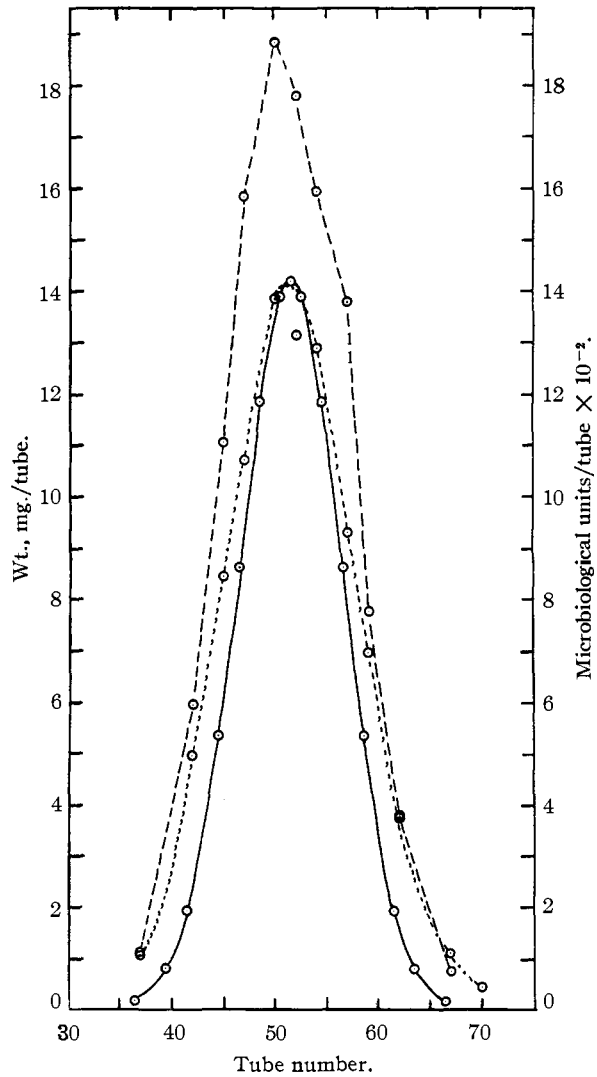

Fig. 1.—Final countercurrent distribution of 100 transfers: theoretical weight curve, — (solid line); experimental weight curve, ... (dotted line); total microbiological activity, - - - - (dashed line).

Experimental

Microbiological Determination.—The factor is determined by microbiological assay with *Lactobacillus acidophilus* ATCC 4963 as described previously in detail.[1] One mg. of an arbitrarily selected distillers' solubles fraction is termed one unit of activity.

Dry Matter Determination.—Total dry weight was determined on low potency samples by drying a suitable aliquot to constant weight at 80–100°. It became apparent that as fractionation proceeded an increasing proportion of

(1) H. R. Skeggs, L. D. Wright, E. L. Cresson, G. D. E. MacRae, C. H. Hoffman, D. E. Wolf and K. Folkers, *J. Bact.*, in press.

the dry matter was volatile at oven temperature. For this reason dry matter determinations (and therefore relative potency values) done this way on certain intermediate steps were of little value and are not given. Dry matter determinations in steps subsequent to the initial countercurrent distribution were carried out on suitable aliquots either by drying to constant weight in a vacuum desiccator over P_2O_5 at room temperature or by lyophilizing under usual conditions and are believed to be meaningful.

Methanol Extraction.—Two hundred pounds (90 kg.) of dried distillers' solubles (Hiram Walker Stimuflav) usually assaying about 3 units/g. was stirred with 100 gal. of methanol at 60° for one hr. under reflux. The mixture was filtered and the filtrate collected. The residue was re-extracted with one hundred gallons of methanol in the same manner. The two filtrates were combined and concentrated *in vacuo* to 20 gal. Ten gal. of water was added and the resulting mixture was concentrated *in vacuo* in order to remove most of the methanol. Total solids amounted to 45 kg.

Continuous Chloroform Extractions.—Two 20-gal. aqueous solutions from the processing of two 200-lb. batches were diluted with 20 gal. of water. This mixture was placed in a continuous liquid–liquid extractor containing 20 gal. of chloroform in the boiler and extracted continuously for 16 hr. At the end of this time the chloroform was removed from the boiling pot and concentrated *in vacuo* to 10 gal.

The 10 gal. of chloroform solution was extracted with five ten-gal. portions of 50% methanol. The five 50% methanol extracts were combined and evaporated to 4 l. Total solids amounted to 695 g.

Chromatography on Superfiltrol.—The main portions of four batches of crude concentrate prepared as described in the preceding section were combined, giving a volume of 7.77 l., containing 231,000 units of microbiological activity in 1457 g. of dry matter or a potency of 160 units/g. This solution was poured onto a column of 75 lb. of adsorbing agent prepared by mixing one part of Superfiltrol with two parts Celite 545. When the column had taken up the above solution, fractional elution was accomplished by the addition of water. Separate eluate fractions from the column were collected with the following volumes and microbiological activity: 1, 43 l., 218,000 units; 2, 20 l., 14,000 units; 3, 10 l., 0 units. Fractions 1 and 2 containing 232,000 units (of 231,000 applied to the columns) were combined and reduced to a small volume at reduced pressure.

Ethanol Precipitation of Inert Material.—A concentrate prepared as described in the preceding paragraphs was neutralized and filtered through a Buchner funnel with suction. To the clear filtrate was added 4 volumes of ethanol. The mixture was allowed to stand at 5° for 3 days. The precipitate that formed was filtered off and the ethanol solution was reduced *in vacuo* to a volume of 1420 ml. This solution contained 180,000 units of microbiological activity in 862 g. of solids or a potency of 210 units/g.

Adsorption Chromatography on Dowex-50.—A concentrate prepared as described in the preceding sections was divided into two equal portions of 710 ml. each. Each fraction was poured onto a chromatographic column containing 10 kg. of Dowex-50 resin previously converted to the H^+ form by washing with 2 *N* HCl and then with water until free of excess H^+ ions. When the resin had taken up the solution applied it was fractionally eluted with water. Fractions of 1 l. were collected and individually analyzed for microbiological activity. Pertinent data for one of these columns are summarized in Table I. Fractions 5 through 9 from each column were found to contain the major fraction of the microbiological activity. These fractions accounted for 166,000 units of microbiological activity out of 180,000 units applied to the columns, and contained 374 g. of solids or a potency of 440 units/g. The 10 l. of concentrate was reduced to a small volume *in vacuo*.

Ion-exchange Chromatography on Dowex-1.—A concentrate prepared as described in the above procedures was neutralized and applied to a chromatographic column containing 2 kg. of Dowex-1 freshly prepared in the OH^- form by washing with 10% NaOH solution and then with distilled water until free of excess OH^- ions. When the column had taken up the solution applied it was washed with water until the eluates were neutral. Fractional elution then was accomplished with 0.05 *N* formic acid. Eluate fractions of 2 l. were collected and individually analyzed for microbiological activity and solids content.

TABLE I

TYPICAL DATA ON THE CONCENTRATION OF THE FACTOR BY ADSORPTION CHROMATOGRAPHY ON DOWEX-50

Fraction	Total solids, g.	Microbiological activity Units/fraction	Units/g.
Start	431.11	90,000	0.21
1	0.07	0	0
2	0.05	0	0
3	1.81	0	0
4	52.55	12,500	0.24
5	98.00	31,900	.33
6	49.30	22,700	.46
7	21.17	15,200	.72
8	11.96	7,500	.63
9	8.03	4,500	.56
10	6.08	0	0
Recovery		94,300 (104%)	

Pertinent data are summarized in Table II. The major portion of the activity applied to the column was collected in fractions 18 through 23 where 142,000 units were obtained.

TABLE II

TYPICAL DATA ON THE CONCENTRATION OF THE FACTOR BY ION-EXCHANGE CHROMATOGRAPHY ON DOWEX-1

Fraction	Microbiologial activity
Start	146,000
1–17 (water wash)	0
18	8,200
19	21,600
20	32,000
21	40,600
22	27,600
23	12,000
24	0
Recovery	142,000 (98%)

The above described fractions 18 through 23 were reduced to a small volume *in vacuo* and the solution neutralized. The solution, now in a volume of 210 ml., was applied to a similarly prepared Dowex-1 column, washed with water and eluted as before. Of the 142,000 units applied to the column, 144,000 units of microbiological activity were now encountered in fractions 18 through 28.

Preliminary Countercurrent Distribution.—A concentrate prepared as described in the preceding sections was reduced *in vacuo* to a volume of less than 75 ml. This solution containing 35,000 units of activity was then subjected to countercurrent distribution in a system where the two phases were formed from the equilibration of chloroform, ethanol, and water (3:4:4 by volume). The apparatus used was a 15-tube unit of the Craig type.[2] The volume of each phase per tube was 75 ml. When the first mobile phase had reached the end, it was transferred from the apparatus. This process was repeated until 30 separate fractions had been collected. These separate fractions were individually assayed for microbiological activity. Pertinent data are summarized in Table III. Fractions 5 through 30 were arbitrarily combined, reduced to a small volume *in vacuo* and lyophilized to dryness. Microbiological assay showed the presence of 34,500 units of activity in 4.6 g. of dry matter or a potency of 7,150 units/g.

Where the fractions appeared to be stable it was convenient to stockpile concentrates in this lyophilized state and to draw on this supply in subsequent studies.

Preparation of High Potency Concentrate by Countercurrent Distribution.—A distillers' solubles concentrate containing 111,000 units in 8.4 g. of dry matter or a potency of 13,200 units/g. was made up to a suitable volume in the phases of the two-phase chloroform–ethanol–water system (3:4:4 by volume) and loaded in five adjacent tubes of a 200 tube all glass Craig countercurrent distribution machine.

(2) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *Anal. Chem.*, **23**, 1236 (1951).

TABLE III

TYPICAL DATA ON THE CONCENTRATION OF THE FACTOR BY COUNTERCURRENT DISTRIBUTION

Fraction	Microbiological activity, units
Start	35,100
1–3	880
4–6	2,960
7–9	5,100
10–12	6,300
13–15	6,750
16–18	5,460
19–21	3,270
22–24	1,930
25–27	1,090
28–30	430
Recovery	34,500 (98%)

The apparatus was arranged to run with 10 ml. of each phase in each tube. The distribution was carried through 265 transfers with effluent collected in a flask at the end. The tube contents were then combined by groups of five and assayed for microbiological activity. The combined tube content from tubes 131–135 and tubes 136–140 showed the highest potency. These contained, respectively, 14,700 units in a weight of 247 mg. or a potency of 59,500 units/g. and 16,700 units in a weight of 224 mg. or a potency of 74,500 units/g. These two fractions were combined with others of similar potency to give a total of approximately 106,600 units in a weight of 1.769 g. or a potency of 60,000 units/g. This preparation was distributed in the same solvent system and equipment by the previous technique until a total of 299 transfers had been made. The material of highest potency was found in plates 141 to 165. These were combined to give 85,600 units in 905 mg. or a potency of 94,000 units/g. This material was loaded into two tubes of the 200-tube countercurrent machine. The ends of the system were connected after the first 200 transfers. Then the contents of tubes 1 through 50 and 151 through 200 were removed (retaining tube contents of 51–150 in place) and fresh solvent phases were inserted. Operation was continued until a total of 600 transfers had been made; then tube contents were again replaced as above and operation was continued to a total of 1200 transfers. The material of highest potency occurred in tubes 150 to 179 where 34,500 units in 273.2 mg. or a potency of 126,000 units/g. was obtained.

Fractions from tubes 150 through 179, inclusive were combined and distributed again in the same solvent system. A total of 100 transfers was made in this distribution. Combinations of two tubes were made. Pertinent data are summarized in Table IV. When the weight distribution was

TABLE IV

PERTINENT DATA ON THE COUNTERCURRENT DISTRIBUTION OF HIGH POTENCY FACTOR

Tubes	Weight, mg.	Microbiological activity Units/fraction	Units/g.
37–38	2.1	220	104,000
42–43	9.9	1190	120,000
45–46	16.9	2210	131,000
47–48	21.4	3170	148,000
50–51	27.7	3770	136,000
52–53	26.3	3560	135,000
54–55	25.8	3190	123,000
57–58	18.6	2760	148,000
59–60	13.9	1550	111,000
62–63	7.5	760	101,000

plotted in the usual method the curve was very close to the theoretical distribution curve but not close enough to indicate complete purity. The contents of plates 45 to 58 inclusive, were combined and the solvents removed at reduced pressure. The nearly pure factor was a clear, almost colorless oil which weighed 150 mg. It was highly active in the microbiological assay, having a potency of about 140,000 units/g.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE UNIVERSITY OF CALIFORNIA AT LOS ANGELES]

Mold Metabolites. VIII. Contribution to the Elucidation of the Structure of Helvolic Acid[1]

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Helvolic acid, a $C_{32}H_{42}O_8$ metabolic product of *Aspergillus fumigatus*, has been thoroughly characterized through identification of its functional groups. That the molecule contains an α,β-unsaturated carboxyl group, two acetoxyl groups, one α,β-unsaturated ketonic function, an isolated ketonic function, one isolated double bond and four rings (probable) has been demonstrated. The relative positions of some of these functions have been deduced. Aromatization of the substance gave a minute yield of a hydrocarbon which possesses an ultraviolet absorption spectrum characteristic of the 11-naphtho[2,1-*a*] fluorene ring system. This observation coupled with the fact that Diels isolated a hydrocarbon containing this same ring system by aromatization of cholesterol suggests that helvolic acid possesses a steroid skeleton. The data can be interpreted in terms of a highly hypothetical steroidal structure for helvolic acid.

A solid material possessing antibiotic properties was isolated by Waksman, *et al.*,[2] from the culture filtrates of *Aspergillus fumigatus* and was given the name fumigacin. Chain, *et al.*,[3a] and Menzel, *et al.*,[3b] demonstrated that fumigacin was a mixture of gliotoxin (a known compound) and a previously unknown antibiotic which the former group designated as helvolic acid (I). This substance has been partially characterized chemically[3a,b,4] and biologically[3b,4c,5] and X-ray diffraction studies of both the acid and its methyl ester have been reported.[6]

(1) This work was generously supported by a grant and a sample of helvolic acid from the Upjohn Co., Kalamazoo, Michigan.

(2) S. A. Waksman, E. S. Horning and E. L. Spencer, *J. Bact.*, **45**, 233 (1943).

(3) (a) E. Chain, H. W. Florey, M. A. Jennings and T. I. Williams, *Brit. J. Exp. Pathology*, **24**, 108 (1943); (b) A. E. O. Menzel, O. Wintersteiner and J. C. Hoogerheide, *J. Biol. Chem.*, **152**, 419 (1944).

(4) (a) J. H. Birkinshaw, A. Bracken and H. Raistrick, *Biochem. J.*, **39**, 70 (1945); (b) W. H. Elliott, P. A. Katzman, S. A. Thayer and E. A. Doisy, *Fed. Proc.*, **6**, 250 (1947); (c) H. W. Flory, *et al.*, "Antibiotics," Vol. I, Oxford University Press, London, 1949, p. 332; (d) T. I. Williams, *Biochem. J.*, **51**, 539 (1952); (e) H. S. Burton, E. P. Abraham and H. M. E. Cardwell, *ibid.*, **62**, 171 (1956).

(5) E. A. Hall, F. Kavanagh and I. N. Asheshov, *Antibiotics and Chemotherapy*, **1**, 369 (1951).

(6) D. M. Crowfoot and B. W. Low, *Brit. J. Exp. Pathology*, **24**, 120 (1943).