from ethylene dichloride and finally an ethyl acetate–acetone–low boiling petroleum ether mixture (m.p. 246–248°).

Anal. Calcd. for $C_{25}H_{20}N_4O_{10}Cl_2$: C, 49.44; H, 3.32. Found: C, 49.65; H, 3.64.

The compounds 1-*p*-nitrophenyl-2-dichloroacetamido-3,3-dimethyl-1,3-propanediol-1-acetate ester and the 1-*p*-nitrophenyl-1-methyl-2-benzamido-1,3-propanediol-3-acetate ester diastereoisomers were used directly without purification.

Conversion to Products II, III, IV and V.—The acetyl groups of dichloroacetamido–acetate esters were selectively removed by treatment with an excess of dilute sodium hydroxide in aqueous acetone solution. The *p*-nitrobenzamides were hydrolyzed by heating on the steam-bath with excess 10% aqueous ethanolic hydrochloric acid for 3 hr. The hydrolysates were chilled and extracted with ether. The aqueous residue was made alkaline with ammonia and the free base extracted into ethyl acetate. Four such extracts were combined and evaporated. The products were recrystallized from appropriate solvents and converted to the dichloroacetamides by refluxing with an alcoholic solution of methyl dichloroacetate as described above.

DETROIT, MICHIGAN

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

Isolation of the *Lactobacillus bulgaricus* Factor from Natural Sources[1]

BY VINCENT J. PETERS, GENE M. BROWN, WILLIAM L. WILLIAMS AND ESMOND E. SNELL[2]

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Procedures for the isolation of one form of the *Lactobacillus bulgaricus* factor (LBF-1A) from the culture filtrate of *Ashbya gossypii* are described. These involve adsorption and elution from activated carbon and subsequent successive chromatography on Floridin, carbon, Superfiltrol and alumina. With minor modifications, the procedures are applicable to purification of other forms of LBF from natural materials and to purification of synthetic pantethine from crude reaction mixtures.

The discovery and partial purification of a widely distributed, unidentified growth factor for *Lactobacillus bulgaricus* was first reported in 1947.[3,4] Subsequent work showed that the same growth factor (termed LBF, or *Lactobacillus bulgaricus* factor) was required by many different lactic acid bacteria,[4,5] that it occurred naturally in several chromatographically distinct forms[6] and that several microörganisms that grew in its absence secreted it into the medium.[6] More recently, pantothenic acid was found[7–9] to have low LBF activity, and digestion of coenzyme A with intestinal phosphatase was shown to produce LBF as one degradation product.[7,10] Finally, the synthesis of two active products, termed pantetheine and pantethine, by condensation of methyl pantothenate with β-mercaptoethylamine was achieved.[11] Exhaustive tests against twenty-odd microörganisms[9] indicated that panthethine and one of the naturally-occurring forms of LBF (LBF-1A) possessed identical growth-promoting properties; as shown later, however, the two differ in that LBF-1A (as well as several other naturally-occurring forms of LBF) is a mixed disulfide formed by oxidation of pantetheine in the presence of an inert mercaptan.[10]

This paper describes the procedures used in isolation of LBF-1A, one of the forms of LBF that occurs in culture filtrates of *Ashbya gossypii*.[6] A summary of the procedures used is shown in Fig. 1; details of the various steps are described in the Experimental portion. Concentrates obtained in this way provided the material used in elucidation of the chemical nature of the growth factor.[11,12] Subsequently, these procedures also proved applicable to isolation of the growth factor from synthetic reaction mixtures.[11,13] Since these experiments were completed another procedure for the concentration of LBF has appeared.[14]

Experimental

Assay Procedure.—During the early part of this work, the method of Williams, *et al.*,[4] modified by doubling the vitamins of the basal medium, was used with either *L. bulgaricus* (Gere A) or *Lactobacillus helveticus* 80 as the test organism. After LBF was found to be a bound form of pantothenic acid,[7] a pantothenic acid-free medium with *L. helveticus* 80 was used with superior results. Incubation was for 17 to 24 hours at 37°; other details of this procedure are described elsewhere.[9] A single sample of yeast extract served as an arbitrary standard throughout; it was assigned an activity of one unit per mg. Dry weights were obtained by drying small aliquots containing from 3 to 10 mg. of solids at 100° for 12–18 hours. Material dried in this way is inactive for *L. helveticus*; consequently, the procedure may give an erroneous weight (and hence activity in terms of units per mg.) for fractions of higher purity.[13] As a guide to fractionation, however, the results were fully valid, and because of its convenience this procedure was used throughout.

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(2) Department of Chemistry, University of Texas, Austin, Texas.

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Source Material.—Of several organisms tested,[6] *Ashbya gossypii* liberated the largest amounts of LBF into the medium. Culture filtrates resulting from the growth of this organism with aeration at 25–30° for from 5 to 12 days in a medium of variable composition, but containing grain stillage,[15] animal stick liquor[15] and glucose were used as the source material. Their activity varied in different fermentations from 3 to 13 units per mg. of solids.

Fractionation Procedure.—From the results of many attempts at purification of LBF, the fractionation procedure illustrated in Fig. 1 was evolved. Individual steps are discussed briefly below.

Steps 1–2 (Adsorption on and Elution from Charcoal).—The procedure is illustrated by the following small scale operation. Three liters of clarified growth medium (40.2 g. of solids, 9 units per mg.) were stirred for 15 minutes with 75 g. of activated carbon (Darco G-60). The carbon was filtered off, the cake resuspended in 1 liter of water and again filtered. The combined filtrates (24.3 g. of solids, 1.1 units per mg.) were discarded. The moist carbon was refluxed for one-half hour with 750 ml. of *n*-butanol, filtered hot and again refluxed with 250 ml. of butanol. The combined eluates (1250 ml., 1.15 g. of solids, 116 units per mg.) were extracted twice in succession with 625 ml. of water each time. The combined aqueous phases (1.0 g. of solids, 60 units per mg.) were discarded; the butanol phase (0.148 g., 350 units per mg.) was used for subsequent chromatographic work.

Fourteen per cent. of the activity originally present was recovered in the final butanol phase. Much of the loss results from the initial presence of several different forms of LBF[6,16,17] which appear to be different mixed disulfides of pantetheine.[10,16] These different compounds do not fractionate in the same manner. The most active fraction obtained above contains primarily a single form of LBF.[6] This form has been referred to as LBF-1A.

Application of this procedure on a pilot plant scale[18] gave similar yields of activity; however, the final butanol-soluble material obtained varied in activity from 20 to 250 units per mg. of solids, depending upon the activity of the original growth medium and unidentified variables. Despite their lower activity, such concentrates were used for further chromatographic fractionation because of their availability on a large scale.

Chromatographic Separations.—The first material of high potency was obtained by procedure A (Fig. 1). This procedure was not so successful when applied to butanol extracts of lower initial potency; for the latter, procedure B was used. Since operation of all columns is substantially similar, only procedure B is described below. Details of the preparation of various columns used are given in Table I. All were operated at a flow rate of 2–3 ml. per minute.

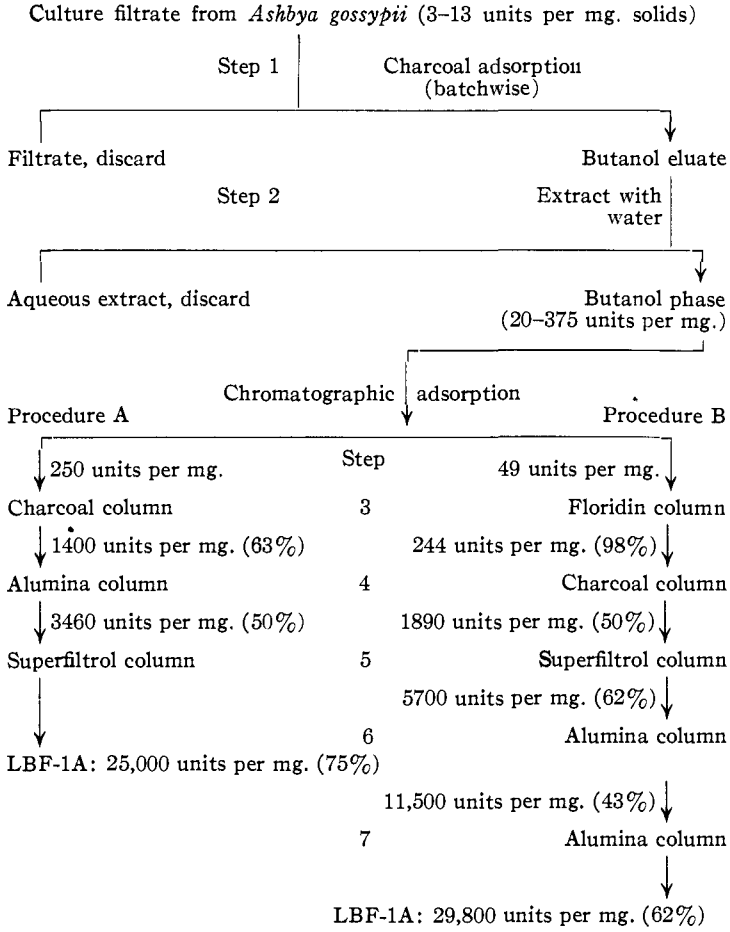

Fig. 1.—Diagram of procedures for isolation of LBF-1A. The figures for per cent. yield and activity indicate the percentage of the material placed on the column that was eluted at the given potency.

Step 3B. Floridin Column.—30.6 g. (49 units per mg.) of a concentrate from step 2 (Fig. 1) in 150 ml. of anhydrous butanol was drawn into the column, which was developed first with 5,000 ml. of anhydrous butanol, followed by 1670 ml. of water-saturated butanol. The active material was recovered in 97–98% yield, concentrated about fivefold (Table II).

TABLE I

PREPARATION AND OPERATION OF CHROMATOGRAPH COLUMNS

Fractionation step	Adsorbent	Method of packing column	Ratio of adsorbent to solids	Diameter of column, cm.
3B	Floridin (60–100 mesh)[a]	Slurry in *n*-butanol	25:1	7.6
4B	Darco G-60[b]	Dry	40:1	7.6
5B	Superfiltrol[c]	Slurry in *n*-butanol	25:1	7.6
6B	Alumina (acid washed)	Slurry in acetone	40:1	2.0
7B	Alumina (acid washed)	Slurry in acetone	200:1	2.0

[a] An activated fullers earth (from the Floridin Co., Warren, Pa.). [b] An activated carbon (from the Darco Corp., New York, N. Y.). [c] A fine mesh, activated fullers earth (from the Filtrol Corp., Los Angeles, Cal.). This was mixed with twice its weight of diatomaceous earth (Celite 545) before use, to speed the rate of column development.

TABLE II

FRACTIONATION OF LBF-1A ON FLORIDIN COLUMNS (STEP 3B)

Starting material: 30.6 g. (49 units per mg.) from step 2 in 150 ml. of *n*-butanol

Column No.	Effluent fraction[a]	Volume, ml.	Solids per ml., mg.	Activity, units per mg.	Total initial activity, %
I	1	5000	...	...	0
	2	1670	3.6	242	98
II	1	5000	...	...	0
	2	1100	5.4	244	97

[a] Fraction 1 was anhydrous butanol; fraction 2, water-saturated butanol.

Step 4B. Charcoal Column.—The effluent fractions 2 from the two columns of Table II were combined and sufficient 2 *N* KOH added to bring the *p*H of an aliquot diluted

(15) Grain stillage is an aqueous residue of the yeast alcoholic fermentation; animal stick liquor an infusion of slaughter house wastes.

(16) G. M. Brown and E. E. Snell, *Proc. Soc. Exp. Biol. Med.*, **77**, 138 (1951).

(17) C. L. Long and W. L. Williams, *J. Bact.*, **61**, 195 (1951).

(18) Slight modifications were made where necessary to adapt the procedure to the available equipment.

with water to 7.2.[19] After concentration *in vacuo* to 110 ml., one-half of this material was drawn into a charcoal column, previously wet with anhydrous butanol. The column was developed first with anhydrous butanol and subsequently with water-saturated butanol. About 50% of the initial activity was recovered in fractions 3 and 4; the remainder in less active fractions (Table III).

TABLE III

FRACTIONATION OF LBF-1A ON CHARCOAL COLUMNS (STEP 4B)

Starting material: 5.74 g. (244 units per mg.) from fraction 2, step 3B, in 60 ml. of *n*-butanol

Effluent fraction[a]	Volume, ml.	Solids per ml., mg.	Activity, units per mg.	Total initial activity, %
1	695[b]	0	0	0
2	685	.66	230	7.4
3	715	.40	2025	42
4	685	.04	4400	8.6
5–9	4400	.11	920	48.8

[a] Fractions 1–7 were anhydrous butanol; cuts 8–9 (1950 ml., 0.15 mg. per ml., 810 units per mg.) water-saturated butanol. [b] This volume represents the approximate "hold-up" of the column, as determined by the volume of butanol required to wet the dry-packed adsorbent.

Step 5B. Superfiltrol Column.—Fractions 3 and 4 from step 4B were combined with similar cuts from additional columns and concentrated *in vacuo* to a small volume. The resulting material in 85 ml. of butanol was drawn into the column, which was washed well with large amounts of anhydrous butanol, then developed with water-saturated butanol. About 60% of the active material was recovered in a single fraction, purified about threefold ((Table IV).

TABLE IV

FRACTIONATION OF LBF-1A ON SUPERFILTROL COLUMNS (STEP 5B)

Starting material: 1.0 g. (1890 units per mg.) from step 4B in 85 ml. of *n*-butanol

Effluent fraction[a]	Volume, ml.	Solids per ml., mg.	Activity, units per mg.	Total initial activity, %
1	2800	..	0	0
2–3	154	0.16	0	0
4	40	3.7	1000	7.6
5	42	5.0	5700	62
6	37	2.5	2000	9.0
7	20	1.5	1000	1
8–10	570	0.28	1420	18

[a] Fraction 1 is the anhydrous butanol wash; fractions 2–10 water-saturated butanol.

Step 6B. First Alumina Column.—Fractions similar to fraction 5 of step 5B, but from other columns were concentrated *in vacuo* almost to dryness, dissolved in acetone and drawn into the column, which was then washed successively with acetone, acetone–butanol, anhydrous and water-saturated butanol. Results (Table V) showed a fivefold increase in activity with 43% recovery in the most active anhydrous butanol cuts.

Step 7B. Second Alumina Column.—Fractions 6–8 of Table V and several cuts from a similar column were concentrated *in vacuo* to a sirup, dissolved in acetone, and fractionated on a column similar to that of step 6B, but with a higher ratio (200:1) of alumina to solids. Several fractions containing approximately 30,000 units of LBF activity per mg. of solids were obtained (Table VI).

Discussion

The active compound present in these concentrates of LBF-1A appears to be a mixed disulfide

(19) This step prevents considerable inactivation that otherwise occurs during the vacuum concentration of butanol solutions of LBF. It also renders behavior of the material on charcoal columns, otherwise very erratic, nicely reproducible.

TABLE V

FRACTIONATION OF LBF-1A ON ALUMINA COLUMN I (STEP 6B)

Starting material: 440 mg. (2500 units per mg.) from step 5B in 32 ml. of acetone

Effluent fraction[a]	Volume, ml.	Solids perml., mg.	Activity, units per mg.	Total initial activity, %
1–2	300	0.55	0	0
3	80	.46	0	0
4	112	.28	800	1.3
5	180	.30	3000	10.7
6	15.6	1.4	11,300	22.7
7	27.5	0.46	12,000	12.1
8	40	.20	11,000	8.0
9	118	.24	4000	10
10–14	160	4.6	1220	36.3

[a] Fractions 1 and 2 were acetone wash; 3–5 were 90% acetone, 10% *n*-butanol; 6–9 anhydrous butanol; and 10–14 water-saturated butanol.

TABLE VI

FRACTIONATION OF LBF-1A ON ALUMINA COLUMN II (STEP 7B)

Starting material: 71.3 mg.. (11,500 units per mg.) from Step 6B in 20 ml. of acetone

Effluent fraction[a]	Volume, ml.	Solids per ml., mg.	Activity, units per mg.	Total initial activity, %
1–21	950	..	0	0
22	18	0.05	6,000	0.6
23	7	1.0	29,800	24.4
24	10.7	1.0	29,800	37.0
25	9	0.35	26,000	9.2
26	52	.12	14,000	10.9
27	23	.20	16,500	9.2
28	120	.04	7,200	4.5

[a] Fractions 1–21 were acetone, acetone–butanol and anhydrous butanol fractions all found to be inactive; fractions 22–26 are 98% butanol–2% water eluates; 27–28 are water-saturated butanol eluates.

formed between pantetheine and a neutral mercaptan of low molecular weight.[10] Since its exact formula is unknown, its absolute purity also is not known. However, the compound has the same activity per pantothenic acid residue as does pantethine[9] and on the basis of comparative weights per LBF unit, as well as its content[11,12] of β-alanine and panthothenic acid, the concentrate must contain LBF-1A at a purity of 50% or better.

The purified growth factor is a colorless, extremely viscous sirup; when dried in air to constant weight at 100° it undergoes some pyrolysis,[13] loses its growth-promoting activity, and no longer yields –SH groups on reduction, as determined by the Kassel–Brand procedure.[20] The compound is readily inactivated by heating with aqueous acids or alkalies. Its chemical nature is considered more fully in succeeding papers.[10,12,13]

During the course of developing and applying the above procedure, a number of facts were discovered which, if applied from the start, would permit a much more efficient isolation to be achieved. Among these is the avoidance of losses during concentration of butanol solutions of the growth factor by proper adjustment of the alkali

(20) B. Kassel and E. Brand, *J. Biol. Chem.*, **125**, 115 (1938).

concentration, as described earlier; this precaution would undoubtedly lead to more active fractions from step 2 of the procedure, but was discovered only subsequent to preparation of the concentrated butanol eluates. The highly variable activity of culture filtrates from *A. gossypii* may result in part from liberation of variable amounts of LBF from coenzyme A by phosphatase action[7] and might be raised considerably by addition of suitable precursors of the LBF molecule (*e.g.*, β-alanine, sulfur compounds) to the fermenting medium. The relationship of LBF to coenzyme A and pantothenic acid, however, was discovered[7] only subsequent to completion of this phase of the fractionation. Finally, a considerable increase in the efficiency of certain of the chromatographic separations might be achieved by a more detailed investigation of the composition of the developing solvents, etc. Because study of the purified fractions described above sufficed to permit a correct assignment of structure to the active principle, pantethine[11-13] and because the latter is now available as a synthetic product, such refinements of the present isolation procedure have not been attempted. With only slight modifications, the procedures developed above for purification of LBF-1A have proved useful for the purification of other naturally-occurring forms of LBF, and of synthetic pantethine from crude reaction mixtures.[11,13]

AUSTIN 12, TEXAS

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN]

The Chemical Nature of the *Lactobacillus bulgaricus* Factor[1]

BY GENE M. BROWN AND ESMOND E. SNELL[2]

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Evidence bearing upon the chemical nature of LBF is as follows: (a) In large amounts, pantothenic acid replaces LBF-1A (or other forms of this growth factor) for *Lactobacillus helveticus* 80; however, LBF-1A is over 100 times as active as pantothenate in promoting growth. A close relationship of the two growth factors is thus indicated. Like pantothenic acid, LBF is inactivated by acetylation and its activity regenerated by mild alkaline hydrolysis. (b) Acid hydrolysates of LBF-1A contain large amounts of β-alanine and an unidentified amine. The latter also contains sulfur, present in a disulfide linkage and was identified as the disulfide of β-mercaptoethylamine. (c) Treatment of LBF-1A with a liver enzyme liberates large amounts of pantothenic acid. (d) LBF-1A is a neutral compound and is not destroyed by nitrous acid. The mercaptoamine must therefore be combined with pantothenic acid by an amide linkage. (e) In accordance with these structural considerations, synthesis of the disulfide of N-(pantothenyl)-β-mercaptoethylamine gave a compound (named *pantethine*) equal to LBF-1A in growth-promoting activity for *L. helveticus* 80. Treatment of coenzyme A with intestinal phosphatase liberates a compound with high LBF activity. β-Mercaptoethylamine was present in acid hydrolysates of the coenzyme. The fragment of coenzyme A with LBF activity is thus closely related to, or identical with, pantethine or LBF. Besides β-alanine and β-mercaptoethylamine, three additional ninhydrin reactive zones occur in hydrolysates of LBF-1A. One of these also contained sulfur. These three compounds are secondary products, formed by interaction among the hydrolytic products of LBF-1A.

The preceding paper[3] described preparation of highly purified concentrates of one form (LBF-1A) of the *Lactobacillus bulgaricus* factor (LBF). Preliminary communications have described incompletely the relationship of this substance to coenzyme A[4] and the synthesis[5] of a compound, pantethine, that has LBF activity equivalent to that of the isolated natural material.[6] Details of the studies with the natural material that led to postulation of the structure of pantethine are the subject of this paper.

Effect of Acylation on LBF Activity.—LBF-1A was completely inactivated by acetylation, but 70% of the activity was restored by hydrolysis with 1 *N* KOH in methanol at room temperature for 3 hours. Methanol saturated with ammonia regenerated the activity more slowly. These data indicate the presence of one or more free hydroxyl groups in LBF and are reminiscent of early results obtained with concentrates of pantothenic acid.[7] Benzoyl chloride inactivated LBF; the activity was not regenerated by the hydrolytic procedures described above. More vigorous procedures cannot be employed because of lability of LBF to acids and alkalies.

Neutral Character of LBF.—Repeated attempts to fractionate LBF-1A by partition between butanol and various buffers confirmed earlier findings[8] that the compound was essentially neutral.

Relation of LBF to Pantothenic Acid.—In efforts to improve the original assay procedure,[8] it was noted that considerably less LBF was required for growth by *L. helveticus* 80 if the pantothenic acid content of the medium was increased from 4 to 8γ per 10 ml. Furthermore, *L. helveticus* did not require pantothenic acid for growth in a medium supplemented with LBF-1A concentrates. The amounts of LBF-1A thus required were too small to supply the amounts of pantothenic acid required to give growth responses in the presence of sub-optimal amounts of LBF. Simultaneously, a coöperating group under Dr. O. D. Bird at Parke, Davis and Co., reported to us

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