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]

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[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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that no LBF was required by the test organism if very large amounts (30–50γ per 10 ml.) of calcium pantothenate were supplied. These relationships are illustrated in Fig. 1A, which shows that 8γ of calcium pantothenate permit maximum growth with 1 unit of LBF, whereas 10 units of LBF are required in the absence of pantothenate. Conversely, over 30γ of calcium pantothenate is required for maximum growth in the absence of LBF (Fig. 1B, *cf.* ref. 6). The concentrate of LBF-1A used in these studies was over 100 times as active, on the weight basis, as calcium pantothenate in supporting growth. As shown in Fig. 1, however, *L. helveticus* utilizes pantothenate very inefficiently; the amount of LBF-1A required for growth of this organism is quite similar to the amounts of calcium pantothenate required by lactic acid bacteria that use the latter vitamin efficiently.[7]

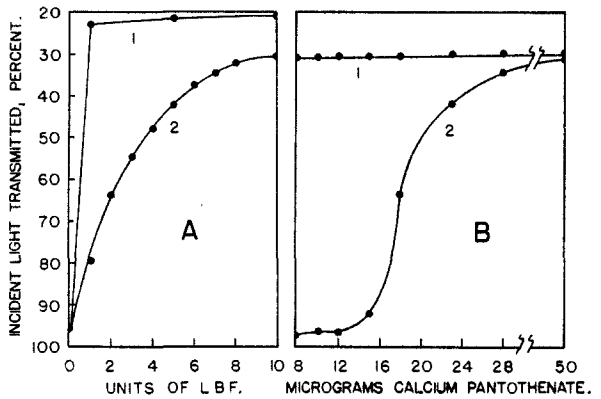

Fig. 1.—A, the response of *L. helveticus* 80 to LBF-1A in the presence (curve 1) or absence (curve 2) of 8γ of calcium pantothenate per 10 ml.; B, the response of *L. helveticus* 80 to calcium pantothenate in the presence (curve 1) or absence (curve 2) of 1 unit of LBF-1A concentrate per 10 ml. One unit of LBF-1A concentrate weighs approximately 0.04γ when dry weights are determined as previously described.[3] Because of chemical change accompanied by some loss of weight, the actual weight of 1 unit is greater than this by an indefinite factor not larger than 2.[12]

These results suggested that LBF was an active combined form of pantothenic acid. Direct evidence for this was secured by chromatography on paper of acid hydrolysates of LBF-1A. One of three strongly ninhydrin-positive zones corresponded exactly in R_f value to control spots obtained with β-alanine (Table I). Approximately 34% of β-alanine was found present by direct assay of the hydrolysate.

Relation of LBF to Coenzyme A (CoA).—The demonstration that LBF contained β-alanine directed our attention to incompletely characterized bound forms of pantothenic acid. These included CoA and its degradation products and the pantothenic acid conjugate (PAC[9]). Samples of CoA and of PAC were inactive for the test organism. However, treatment of CoA with intestinal phosphatase[10] released a fragment having the same microbiological activity as LBF. Treatment of either this fragment of CoA or of LBF itself with an enzyme from chicken liver[10] destroyed their LBF activity, with the release of free pantothenic acid. These results were reported in a preliminary communication,[4] to which reference should be made for experimental details. The conclusion was drawn that LBF was either identical with, or closely related to, the phosphorus-free conjugate of pantothenic acid liberated from CoA by treatment with intestinal phosphatase.

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TABLE I

SEPARATION OF HYDROLYTIC PRODUCTS OF LBF-1A BY PAPER CHROMATOGRAPHY[a]

Sample	Method for detecting zones: Ninhydrin	NaCN + nitroprusside R_f values	Iodine-azide
Series A			
LBF-1A[b]	None	0.90	0.89
Series B			
Hydrolyzed LBF-1A	0.15	None	None
	.36	0.39	0.38
	.45	0.47	0.47
	.52[c]	None	None
	.70[c]	None	None
β-Alanine	.14	None	None
β-Mercaptoethylamine	.46	0.48	0.48
Series C			
Calcium pantothenate + β-mercaptoethylamine, hydrolyzed	0.13	None	None
	.33	0.33	0.33
	.42	0.42	0.42
	.50[c]	None	None
	.67[c]	None	None

[a] The chromatograms of series A were developed with water-saturated *n*-butanol; those of series B and C with pyridine:water (4:1, by volume). [b] Bioautographs of LBF under these conditions gave an R_f value of 0.90. [c] Very faint zones.

Treatment of LBF-1A with the chicken liver enzyme released pantothenic acid equivalent to about 65% by weight of the concentrate, in fair agreement with the pantothenate content calculated from the β-alanine assay. It was thus apparent that the unidentified portion of the LBF-1A molecule must be of relatively small size, and the concentrates of fairly high purity.

Evidence Concerning the Nature of the Unidentified Amine of LBF Hydrolysates.—The occurrence with β-alanine of additional ninhydrin-reactive compounds in acid hydrolysates of LBF-1A was mentioned above. Of these, β-alanine and the unidentified compound of R_f value 0.45 (Table I) were present in the largest amounts, as judged by the size and depth of color of the zones obtained. Because of the amounts present, it appeared probable that the compound at R_f 0.45 was the chief unidentified component of the molecule. Since LBF-1A is a neutral compound, and is not destroyed by nitrous acid,[8] it was evident that the amino group of this compound was coupled to pantothenic acid by an amide linkage, and that the resulting compound did not contain additional acidic or basic groups. In an attempt at separation of this unidentified amine, acid hydrolysates of LBF-1A (1.2

mg.) and of CoA (≏ 300 γ of pantothenic acid) were made strongly alkaline with KOH and steam distilled. Each distillate was acidified, concentrated to dryness *in vacuo*, taken up in 0.05 ml. of water, spotted quantitatively on paper and developed with pyridine–water (4:1). Each chromatogram showed the ninhydrin-reactive zone of R_f value 0.45, confirming both the close relationship of LBF to CoA and the suppositions made above concerning the nature of the unidentified amine.

Lack of materials made an indirect identification of the amine necessary. Of a large number of available amines of low molecular weight tested, ethanolamine, 1-amino-2-propanol and 2-amino-1-propanol gave R_f values (from pyridine–water, 4:1) equal to that of the unidentified amine. However, crude condensates of each of these with pantothenic acid were without LBF activity.

Identification of β-Mercaptoethylamine (Thioethanolamine) as a Fragment of LBF.—The suspicion that LBF-1A might contain sulfur first arose because of the disagreeable odor of its acid hydrolysates. This disappeared on standing. Unhydrolyzed LBF was found to give positive tests with the iodine–azide[11] and the cyanide–nitroprusside reagents, thus confirming the presence of sulfur. The latter reagent is specific for sulfhydryl or disulfide linkage. Furthermore, the sulfur-containing compounds, as indicated by either reagent, migrated on paper to the same position indicated for LBF-1A by bioautographic studies (Table I). Thus the sulfur was not an impurity. In acid hydrolysates, the R_f values of the sulfur-containing compounds corresponded to those of the two major unidentified ninhydrin-reactive compounds (Table I), indicating that the unidentified amines were mercaptoamines or the corresponding disulfides. Again, the zone at R_f 0.45 was the major one. With both LBF-1A and hydrolyzed LBF-1A, nitroprusside reagent alone failed to give any color; this developed only when cyanide was used in conjunction with nitroprusside. LBF-1A, and the sulfur-containing amines liberated from it by acid hydrolysis, thus appear to be disulfides.

The correspondence of the R_f value of the unidentified sulfur-containing amine with that of ethanolamine and the possible natural occurrence of thioethanolamine (β-mercaptoethylamine) as a decarboxylation product of cysteine, led us to examine this product as a first candidate for the unidentified amine of R_f value 0.45. A sample synthesized at our request by Dr. E. L. Wittle of Parke, Davis and Co. behaved identically on paper to the major ninhydrin-reactive, sulfur-containing zone of LBF hydrolysates (Table I). No R_f value corresponding to the free –SH compound was obtained; β-mercaptoethylamine and its oxidation product, bis-(β-aminoethyl) disulfide, behaved identically on paper, and like LBF hydrolysates, gave no nitroprusside test unless reduced on paper with cyanide.

On the basis of this evidence, β-mercaptoethylamine and pantothenic acid were condensed. As reported in a preliminary communication[5] and more fully in an accompanying paper,[12] the purified product, named pantethine, corresponded closely to LBF-1A in chemical properties and showed the same growth-promoting activities for *L. helveticus* 80. Its growth-promoting activities for twenty-odd additional organisms also corresponded exactly to those of LBF-1A.[6] Despite this close relationship, however, the two are not identical as originally believed.[5,6] The nature of the relationship between them became apparent in work with synthetic pantethine and is described separately.[13]

The Nature of the Unidentified Mercaptoamine at R_f 0.38.—The mercaptoamine at R_f 0.36–0.39 (Table I), found together with the disulfide of β-mercaptoethylamine in LBF hydrolysates, has not been identified. The same zone, together with the two faintly ninhydrin-positive, sulfur-free zones (Table I), are found when equimolar quantities of the disulfide of β-mercaptoethylamine and of calcium pantothenate are mixed and hydrolyzed under the same conditions used for LBF-1A. These unidentified products therefore arise secondarily through interaction among the degradation products of LBF and are not primary hydrolytic products. Because of this, their nature was not investigated further.

Experimental

Methods and Materials.—The LBF-1A was obtained by the purification procedures described previously[3] and from indirect evidence was about 50% pure. For LBF assays, the methods of Craig and Snell[6] were used with *Lactobacillus helveticus* 80 as the test organism. Bioautographs employed the same organism and medium, the latter solidified with 2% of agar. Whatman No. 1 filter paper was used for chromatograms; these were developed in large glass cylinders by the ascending technique.

Acid hydrolysates of LBF and of coenzyme A concentrates were prepared by autoclaving at 120° for 6 hours with 2 *N* HCl in sealed glass tubes.

β-Alanine was determined with *Saccharomyces fragilis* 2360 in the medium of Atkin, *et al.*,[14] modified by omission of asparagine to prevent inhibition of the response to β-alanine.[14]

Acylation of LBF.—Two ml. of acetic anhydride, 2600 units of LBF-1A and 8 ml. of pyridine were heated on a steam-bath for 0.5 hour. Benzoylation was similarly effected with benzoyl chloride in pyridine at room temperature.

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