A NEW GROWTH FACTOR REQUIRED BY BUTYRIBACTERIUM RETTGERI[1]

LEO KLINE[2] AND H. A. BARKER

Division of Plant Biochemistry, University of California, Berkeley, California

Received for publication June 1, 1950

Butyribacterium rettgeri is a gram-positive, nonsporulating, nonmotile, anaerobic or microaerophilic, rod-shaped bacterium that ferments carbohydrate and lactate, forming acetic and butyric acids and carbon dioxide as the main products (Barker and Haas, 1944). The fermentation produced by this organism differs from those caused by sporeforming butyric acid bacteria with respect to the absence of hydrogen production, the larger yield of volatile acids, and the smaller yield of carbon dioxide. Barker, Kamen, and Haas (1945) showed that the low yield of carbon dioxide obtained in a lactate fermentation was due to the total synthesis of acetic and butyric acids from the carbon dioxide.

During the course of an investigation into the mechanism of this carbon dioxide fixation, it was found that *B. rettgeri* failed to grow on a synthetic medium containing lactate as the fermentable carbon source, ammonium sulfate as a nitrogen source, and inorganic salts. The addition of casein hydrolyzate (acid or enzymatically hydrolyzed), a mixture of known vitamins, and a purine-pyrimidine mixture did not promote growth. The further addition of yeast extract permitted good growth (figure 1).

The following report deals with the demonstration that a new factor (B.R. factor) is required for this organism, with its occurrence in yeast extract and other materials, and with certain of its chemical and physical properties.

METHODS

The basal medium selected for the study and assay of the B.R. factor is given in table 1. In the absence of an added source of the factor no measurable growth was observed on this medium in incubation periods up to 1 week at 37 C. The mineral constituents were the same as those used by Haas (1947), who also showed that sodium acetate, when added with yeast extract, stimulated the initial rate of growth; therefore, sodium acetate was included in the basal medium. The vitamin and purine-pyrimidine supplements were patterned after the general requirements of the lactobacilli, and, though the individual requirements were not determined, these supplements were found to be definitely stimulatory when added in conjunction with yeast extract and were routinely included in the basal medium. Casein hydrolyzate, added to the otherwise complete medium,

[1] This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships by the National Institutes of Health, United States Public Health Service.

[2] National Institutes of Health predoctoral fellow 1947–1948. Present address: Western Regional Research Laboratory, Albany, California.

either as an enzymatic or acid hydrolyzate (plus tryptophan), also failed to promote growth but exerted a definitely stimulatory effect when added in conjunction with suboptimal amounts of yeast extract (table 2). Several commercial acid-hydrolyzed "vitamin-free" casein preparations containing 10 per cent solids were used to provide a 0.2 per cent concentration of casein hydrolyzate solids in the basal medium, and 0.01 per cent L-tryptophan was added concomitantly.

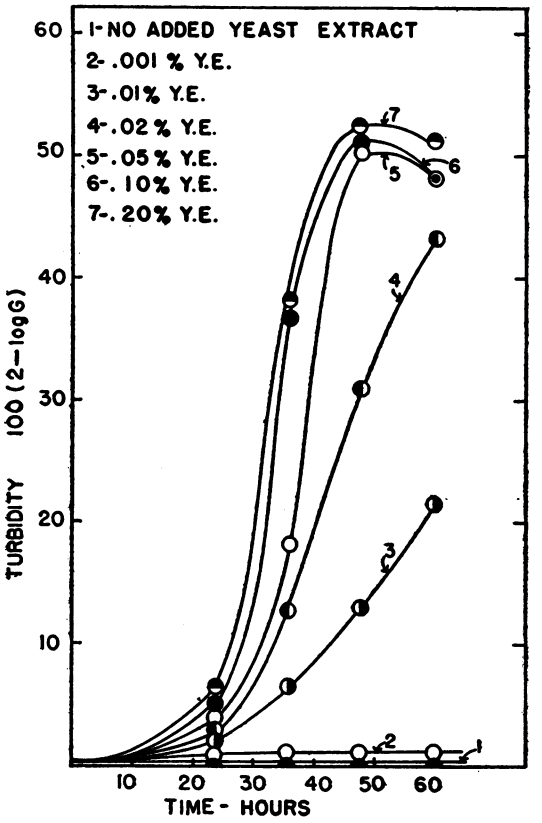

Figure 1. Growth on a lactate medium with the standard yeast extract as the source of the B.R. factor (cf. table 2 for composition of medium).

The complete medium for assay purposes was prepared from component solutions, usually to 0.9 of its final volume in order to accommodate the solution to be tested for B.R. factor activity, and 9-ml aliquots were dispensed in 18-by-150-mm culture tubes. A sample preparation of 100 ml of medium was as follows:

50 ml double-strength lactate-salts solution
1 ml vitamin supplement (100-fold concentrated)
1 ml purine-pyrimidine supplement (100-fold concentrated)
2 ml acid-hydrolyzed casein solution (10 per cent solids)
2 ml 0.5 per cent L-tryptophan

2 drops 2 per cent aqueous methylene blue solution
0.05 g cysteine hydrochloride

Adjust to pH 6.8 and make up to 90 ml. Add 9 ml per tube, and then the solution to be assayed and water, or water alone, is added to give a 10-ml volume.

TABLE 1

Composition of assay medium

	G PER 100 ML		MG PER 100 ML
Na-lactate	1.0	Thiamine·HCl	0.20
KH_2PO_4	0.2	Pyridoxine·HCl	0.10
$(NH_4)_2SO_4$	0.1	Pyridoxamine·2HCl	0.05
$MgSO_4 \cdot 7H_2O$	0.02	Pyridoxal·HCl	0.05
$CaCl_2 \cdot 2H_2O$	0.002	Ca-pantothenate	0.20
$FeSO_4 \cdot 7H_2O$	0.004	Riboflavin	0.20
$MnSO_4 \cdot 2H_2O$	0.00015	Nicotinic acid	0.10
$NaMoO_4 \cdot 2H_2O$	0.00015	Nicotinamide	0.10
Na-acetate·$3H_2O$	0.03	*p*-Aminobenzoic acid	0.01
Cysteine·HCl	0.05	Biotin	0.005
Casein hydrolyzate*	0.2	Pteroylglutamic acid (Lederle folvite)	0.005
L-Tryptophan	0.01	Adenine sulfate	2.0
		Guanine·HCl·$2H_2O$	2.0
		Uracil	2.0
		Xanthine	2.0
		Thymine	2.0

* Nutritional Biochemicals Corporation "vitamin-free" casein hydrolyzate (acid) was found to be satisfactory.

TABLE 2

Effect of casein hydrolyzate on growth
100 (2 − log G)

CASEIN HYDROLYZATE SOLIDS	36 HOURS				60 HOURS		
	Yeast extract, %				Yeast extract, %		
	0	0.01	0.05	0.10	0	0.01	0.05
per cent							
0.05	0	11	32	38	0	36	42
0.10	0	16	37	39	0	45	46
0.20	0	18	41	50	0	47	49
0.30	0	22	43	52	0	49	48

The basal medium used above was the complete assay medium minus the casein hydrolyzate.

The cysteine served to provide the necessary reducing conditions and was added just prior to adjusting the pH of the complete medium and autoclaving. Sodium thioglycolate (0.05 per cent) or 0.02 per cent $Na_2S \cdot 9H_2O$ were also satis-

factory for this purpose. The sodium sulfide was best added after autoclaving to minimize precipitation of metallic sulfides.

After autoclaving for 15 minutes at 120 C, the tubes were inoculated immediately upon cooling with 2 drops from a 48-hour liquid culture. The inoculum was grown in the complete medium plus the minimum amount of a source of B.R. factor necessary to give good growth in 48 hours; 0.05 per cent of a particularly active yeast extract was routinely used for this purpose. Since this inoculum was found not to provide sufficient carry-over of the B.R. factor to result in measurable growth, the need for an inoculum of washed cells, or a diluted inoculum,

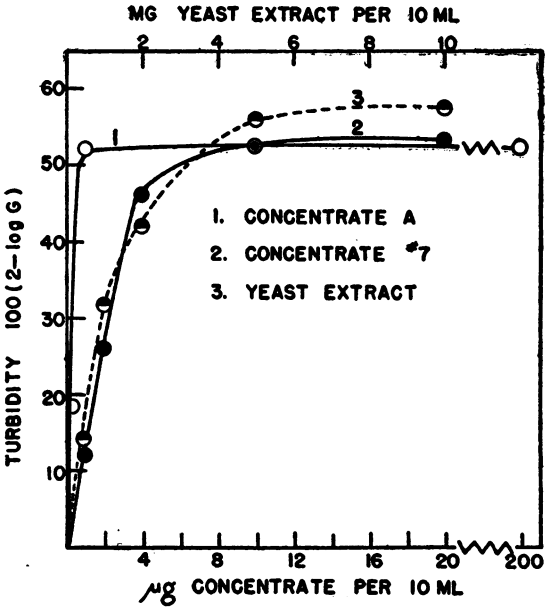

Figure 2. Growth on a lactate medium with different sources of the B.R. factor. Readings obtained after 48 hours' incubation at 37 C.

was obviated. The stock cultures were carried as stab cultures on the inoculum medium to which 1.5 per cent agar had been added. In the preparation of an inoculum from a stock culture three or four transfers in the liquid medium were made before the culture was satisfactory. Growth from the first or second transfer was commonly sluggish and did not permit reproducible assays.

After inoculation, a Na_2CO_3-pyrogallol seal was applied to effect anaerobiosis. The efficacy of the seal was indicated by the methylene blue (approximately 0.001 to 0.002 per cent) in the medium.

Incubation was at 37 C, growth was estimated turbidimetrically in the Evelyn colorimeter, and the results were expressed as 100 (2-log galvanometer reading). Readings obtained after between 36 and 48 hours of incubation were used to estimate the B.R. factor content, either in terms of a standard yeast extract assayed simultaneously or in terms of activity units. One unit of activity is de-

fined as the amount of the substance required to give half-maximum growth when present in 10 ml of medium. The specific activity of a substance was defined as the number of units of activity per mg of solids.

The adequacy of the medium is indicated by the fact that the addition of either 1 to 5 μg of purified concentrates or 5 to 10 mg of the standard yeast extract to 10 ml of the medium resulted in approximately equal growth rates and maximum growth (figure 2).

TABLE 3

Occurrence of "free" B.R. factor in various substances

SUBSTANCE	RELATIVE ACTIVITY	SUBSTANCE	RELATIVE ACTIVITY
Y. E. (Difco no. 1)	100	Beef extract (Difco)	0
Y. E. (Difco no. 2)	13	Beef extract (Liebig)	1
Y. E. (Difco no. 3)	10	Dried whole liver (Wilson)	4*
Y. E. (Difco no. 4)	2	Liver L (Wilson)	4
Y. E. (Fleischmann type 3)	60	Liver B (Wilson)	1*
Y. A. (Bakers' no. 1)	7	Liver S (Wilson)	0*
Y. A. (Bakers' no. 2)	12	Liver 1-20 (Wilson)	0
Y. A. (Bakers' no. 3)	15	Distillers' solubles	1*
Y. A. (Fleischmann 2040 yeast)	26	Corn steep liquor	0
Y. A. (drum-dried *T. utilis*)	2	Asparagus-butt juice	5
Y. A. (Difco dried)	5	Pea pod press juice	4
P. notatum autolyzate	20	Casein ("vitamin-free")	0*
B. subtilis autolyzate	20	Egg yolk	0*
Peptone (Difco no. 1)	16	Soybean phospholipid	0*
Peptone (Difco no. 2)	3	Oxgall (Difco)	0
Malt extract (Difco)	3		

The standard yeast extract (Difco no. 1) was assigned a value of 100. This standard had a specific activity of about 0.8 units per mg.

Y. E. = yeast extract; Y. A. = yeast autolyzate.

* Activities of these substances are reported in terms of the original solids. All others are in terms of soluble solids only.

PROPERTIES OF THE B.R. FACTOR

Inactive compounds. A number of compounds, reported to function as growth factors for various organisms, were tested and found to be inactive. These include adenylic acid (yeast), coenzyme I, indole-3-acetic acid, glutathione, inositol, choline, oleic acid, glutamine (Seitz-filtered), strepogenin (obtained from Dr. D. D. Woolley), pimelic acid, casein, sodium taurocholate, hemoglobin (Difco), "tween 80," cholesterol, *alpha*-tocopherol, 2-methyl-1,4,naphthoquinone, oxgall (Difco), linoleic-oleic acid mixture (Armour neofat-3), vitamin B_{12} (Merck crystalline).

Occurrence. It will be shown shortly that the B.R. factor occurs in natural substances partly in an active form and partly in an inactive form, from which the active moiety is liberated by autoclaving in 1 N acid or alkali. A number of

substances, including the ingredients of common media, were tested as sources of the "free" factor (table 3). The results indicate that the "free" factor is not abundant in the materials tested. The best sources, in addition to the standard yeast extract, are Fleischmann type 3 yeast extract and the various autolyzates. Subsequent studies showed that several materials, initially low in "free" activity, became highly active when subjected to the afore-mentioned hydrolysis.

Activity of ash. The possibility that the activity of the yeast extract might be due to mineral constituents, particularly the trace elements, was investigated by wet-ashing the standard yeast extract and adding the neutralized ash solution aseptically to the autoclaved assay medium. The ash solution was added to a level equivalent to the mineral constituents provided by a 0.3 per cent yeast extract addition. The ash was found to be completely devoid of growth-promoting activity when added alone and to have no effect on the growth when added in conjunction with yeast extract.

TABLE 4

Stability of the B.R. factor to heat, acid, and alkali

ADDITION TO BASAL MEDIUM	100 (2 − log G) TESTED AT 0.03%	
	24 hr	30 hr
Control*	18.9	40.4
Autoclaved in 1 N H_2SO_4	20.8	47.8
Autoclaved in 1 N NaOH	21.3	42.6

* Control represents the activity of the unheated solution of the standard yeast extract. The 1 N acid and alkali solutions containing 2 per cent yeast extract were autoclaved 30 minutes at 120 C.

Stability to heat, acid, alkali, and enzymes. The B.R. factor, when heated in the crude form, e.g., yeast extract solution, was relatively stable to autoclaving in 1 N acid or alkali. The data given in table 4 show that 30 minutes' autoclaving was without significant effect on the activity. Similar results have been obtained for autoclaving in neutral solution.

The stability of the activity in the standard yeast extract to enzymatic digestion was investigated with Fairchild's trypsin and "takadiastase." Sterile aliquots of a 1 per cent aqueous solution of the yeast extract were subjected to 48 hours' digestion at 30 C with sterile (Seitz-filtered) solutions of the enzymes. The enzyme concentration in the digestion mixtures was 0.2 per cent in each case; the trypsin digestions were at pH 8.5 and the takadiastase at 6.5. No diminution of the activity was found to result from the treatments. The stability to enzymatic digestion of the activity in a purified preparation having a specific activity 50 times that of the yeast extract (prepared by preferential removal of the activity from the yeast extract by ether extraction) was tested with similar results.

Liberation of the activity from inactive precursors. Autoclaving in aqueous 1 N

acid or alkali for 30 minutes or longer was found to be very effective in transforming many relatively inactive materials, e.g., certain yeast and liver preparations, into sources comparable to, or better than, the standard yeast extract (table 5). Although the optimum conditions for the liberation of the activity

TABLE 5

Liberation of the B.R. factor by acid or alkaline hydrolysis

SUBSTANCE	TREATMENT	LEVEL ASSAYED	100 (2 − log G) 32 hr
		per cent	
Yeast extract (Difco no. 1)	None	0.03	43
Yeast extract (Difco no. 4)	None	0.03	2
Yeast extract (Difco no. 4)	Autocl. 30 min in 1 N H_2SO_4	0.03	31
Yeast extract (Difco no. 4)	Autocl. 30 min in 1 N NaOH	0.03	47
Liver L (Wilson)	None	0.05	2
Liver L (Wilson)	Autocl. 30 min in 1 N H_2SO_4	0.05	36
Liver L (Wilson)	Autocl. 30 min in 1 N NaOH	0.05	55
Liver 1-20 (Wilson)	None	0.08	3
Liver 1-20 (Wilson)	Autocl. 30 min in 1 N H_2SO_4	0.08	30
Liver 1-20 (Wilson)	Autocl. 30 min in 1 N NaOH	0.08	27
P. notatum autolyzate	None	0.02	7
P. notatum autolyzate	Autocl. 60 min in 1 N HCl	0.02	53
Distillers' solubles	None	0.20	3
Distillers' solubles	Autocl. 30 min in 1 N H_2SO_4	0.20	19

TABLE 6

Effect of pH on liberation of activity from liver L

(Two per cent solutions at each pH were autoclaved 30 minutes)

ADDITION TO BASAL MEDIUM	100 (2 − log G) TESTED AT 0.01% LEVEL 41 HR
None	0
Liver L, unheated	3.1
Liver L, autoclaved at pH 7	2.1
Liver L, autoclaved at pH 5	1.9
Liver L, autoclaved at pH 3	2.1
Liver L, autoclaved at pH 2	2.1
Liver L, autoclaved at pH 1	14.9
Liver L, autoclaved at pH 0	48.5

were not investigated, it was found that autoclaving for 1 hour was considerably more effective than the 30 minutes' treatment. The influence of the pH of autoclaving on the liberation of activity is shown in table 6. Little or no release of the activity occurred until the hydrogen ion concentration was increased to pH 1 and approximately a 3-fold further increase was effected at pH 0. The influence of higher acid concentrations was not investigated.

Enzymatic liberation was attempted with three enzyme preparations: Fair-

child's trypsin, takadiastase, and pancreatin. Sterile 1 per cent solutions of liver L were allowed to incubate 24 hours at 30 C at pH 8.5 with the trypsin and pancreatin and at pH 6.3 with takadiastase. The enzyme solutions were sterilized by Seitz-filtration and were employed at a concentration of 0.2 per cent in the digestion mixtures. The assays on the incubated mixtures showed that none of the enzyme preparations effected even a slight liberation. These results were somewhat surprising in view of the well-known efficacy of such enzymes in liberating bound growth substances. It is possible that the enzymatic liberation of

TABLE 7

Extraction of B.R. factor from aqueous solutions of yeast extract with ether as a function of the pH*

ADDITION TO BASAL MEDIUM	100 (2 − log G) AT		ORIGINAL ACTIVITY (APPROX.)
	24 hr	33 hr	
			per cent
None	0	0	
0.01% yeast extract	7.8	19.1	
0.025% yeast extract	15.8	40.3	
0.05% yeast extract	19.4	50.5	
Ether ext. pH 2, at 0.05%†	15.8	34.4	46
Ether ext. pH 6.5, at 0.05%	14.3	34.4	46
Ether ext. pH 10, at 0.05%	0.7	0.9	0
Extract. residue pH 2, at 0.05%	10.5	19.9	24
Extract. residue pH 6.5, at 0.05%	10.2	18.7	22
Extract. residue pH 10, at 0.05%	17.9	49.5	>90
Ext. + residue, pH 2, each 0.05%	17.7	49.1	>90
Ext. + residue, pH 6.5, each 0.05%	20.8	52.7	100
Ext. + residue, pH 10, each 0.05%	20.8	50.9	100

The percentage of the total solids extracted at pH's 2, 7, and 10 was 5.2, 1.8, and 1.3, respectively. The percentage of the total nitrogen extracted was 2.5, 4.5, and 1.6, respectively.

* Continuous extraction for 64 hours.

† Both extracts and residues were made up to original volume of yeast extract and the assay level is in terms of the original yeast extract.

the B.R. factor might be accomplished under conditions not offered in this experiment.

Miscellaneous properties. It has been shown that the activity of the standard yeast extract cannot be ascribed to its mineral constituents; therefore the organic nature of the factor is self-evident. The activity, as it occurs in the standard yeast extract, was found to be completely dialyzable. Steam distillation of the yeast extract solution adjusted to pH 2 did not result in the volatilization of any activity. Other studies have shown that both aqueous and organic solvent solutions of the activity may be safely evaporated to dryness on the steam bath without appreciable loss of activity.

Extraction of the activity from aqueous solution with immiscible solvents; demonstration as a weak acid. Before discussion of the results of various extraction and partition experiments with organic solvents, it is necessary to point out that the B.R. factor was found consistently to behave like a weak acid. When aqueous aliquots of the standard yeast extract, adjusted to pH 2, 6.5, and 10, were subjected to continuous extraction with diethyl ether for 64 hours, it was found (table 7) that the activity was extracted at pH 2 and 6.5 but not at pH 10.

Since the extraction residues in the acid range contained roughly one-fourth of the original activity, it appeared that either an active but nonextractable form

TABLE 8

Extractability of inactive precursor from untreated liver L and of active B.R. factor from acid-autoclaved liver L with ether

ADDITION TO BASAL MEDIUM		ASSAY LEVEL	100(2−log G) 39 hr
		per cent	
None			0
Liver L, acid-autoclaved, but not extracted		0.005	45
		0.02	48
		0.04	49
Liver L, extracted without acid-autoclaving	Extract	0.005	0.2
	Extract	0.02	1.3
	Residue	0.005	0.4
	Residue	0.02	0
Liver L, extracted and then both extract and extraction residue subjected to acid-autoclaving	Extract	0.005	2.0
	Extract	0.02	3.4
	Residue	0.005	44
	Residue	0.02	49
Liver L, acid-autoclaved before extraction	Extract	0.005	46
	Extract	0.02	49
	Residue	0.005	4.3
	Residue	0.02	14

existed in the yeast extract or that the extraction was incomplete in 64 hours. The inadequacy of the latter explanation was demonstrated by a subsequent experiment in which separate extractions were allowed to proceed for 6, 25, 48, and 74 hours. It was found that 6 hours sufficed to remove all the extractable activity. Autoclaving the aqueous solution of the standard yeast extract in 1 N acid before ether extraction was found to increase the percentage of the total activity that was ether-extractable from less than 70 per cent to more than 95 per cent. This indicates the existence in the yeast extract of an active but non-ether-extractable form requiring degradation to render it extractable.

Solvent studies with liver L, containing only the inactive precursor form, showed that this form was not extracted from aqueous solution with ether. After

acid-autoclaving, however, over 90 per cent of the liberated activity was extractable (table 8).

Aliquots of a 5 per cent solution of the standard yeast extract at pH 6.5 were subjected to continuous extraction for 12 hours with ethyl acetate, benzene, petroleum ether (bp 32 to 57 C), and diethyl ether. Approximately three-fourths of the activity was extracted by benzene, ethyl acetate, and diethyl ether. The petroleum ether, by contrast, removed only one-fourth of the activity. Less than 1.5 per cent of the solids were removed by the benzene and ethyl ether, suggesting the use of these solvents to obtain purification. Ethyl acetate removed about 4 per cent of the solids and the petroleum ether 0.8 per cent.

The equilibrium distribution of the activity between equal volumes of water and immiscible solvent (partition coefficient) was determined with a number of solvents. For this purpose a partially purified concentrate was used which had been prepared by benzene extraction of the activity from an acid-autoclaved

TABLE 9

Partition coefficients of the B.R. factor

SOLVENT	COEFFICIENT $\frac{\text{Activity in solvent}}{\text{Activity in water}}$	ORIGINAL ACTIVITY RECOVERED
		per cent
n-Butanol	>23/1	75
Ethyl acetate	7/1	80
Chloroform	3/1	65
Ether	1/2	50
Benzene	1/3	50
Cyclohexane	1/4	97
n-Hexane	1/7	>95
Skellysolve A	1/12	>91
Iso-octane	1/12	>90

aqueous yeast extract solution. Approximately 14 μg of the concentrate solids represented one unit of activity (as compared to about 1,200 μg for the standard yeast extract). The aqueous concentrate was adjusted to pH 3 before partitioning to convert the activity entirely to the acid form (the pK_a of the activity was estimated to be close to 5). The distribution of the activity between the aqueous and nonaqueous phases at equilibrium is shown in table 9 for a number of solvents. It was found that the partition coefficient, activity in solvent/activity in water, was roughly proportional to the dielectric value of the organic solvent. Thus the activity went almost completely into butanol; ethyl acetate and chloroform contained two-thirds or more, whereas the paraffinic solvents removed 10 per cent or less.

Existence of the B.R. factor in several forms. Thus far the results point to the existence of the following forms of the factor: (1) an inactive precursor that is not ether-extractable, (2) an active form also not ether-extractable; and (3) the ether-extractable activity. However, filter paper chromatograms have shown

that more than one form of the extractable activity exists. The purified concentrate used for the chromatographic studies was a composite of four separate preparations from (1) acid-autoclaved liver L; (2) acid-autoclaved yeast extract; (3) autolyzed and acid-autoclaved Fleischmann 2040 yeast; and (4) autolyzed and neutral-autoclaved Fleischmann 2040 yeast. The mixture had been further purified by extraction with benzene from alkaline solution to remove impurities, followed by acidification and extraction of the activity with benzene. The benzene extract was evaporated to dryness on the steam bath and the residue taken

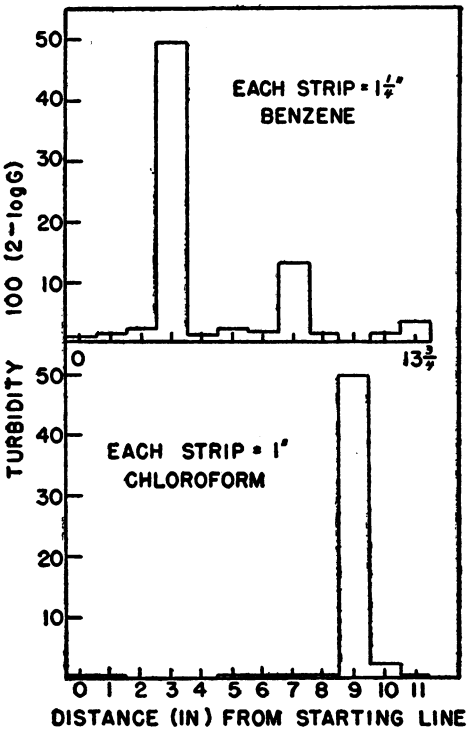

Figure 3. Filter paper chromatograms of a purified preparation of the B.R. factor (3.7 μg = 1 activity unit). Developing solvents: chloroform and benzene (each saturated with water and containing 0.1 per cent acetic acid). Filter papers, after drying, were cut into strips of the size indicated. Each strip was immersed for 10 minutes in 10 ml of assay medium.

up in water and filtered; 3.7 μg of the filtrate solids represented one unit of activity, approximately a 300-fold purification as compared to the standard yeast extract. The filter paper chromatograms were developed with chloroform or benzene, saturated with water, and containing 0.1 per cent by weight of glacial acetic acid. As shown in figure 3, the concentrate appeared homogeneous when developed with chloroform, but with benzene a small amount of a second component was disclosed as well as a trace of a third that moved rapidly with the solvent front.

Extraction of the activity directly from the powdered yeast extract. Continuous

Soxhlet extractions for 24 hours of the powdered standard yeast extract (without preliminary drying) were performed with a number of solvents. The amount of activity extracted is shown in table 10. Benzene and petroleum ether removed only about 10 per cent of the activity and less than 5 per cent of the solids. Diethyl ether and ethyl acetate removed about 20 to 30 per cent. Acetone, which removed 40 per cent, also removed 20 per cent of the solids. Extraction with the alcohols, particularly ethanol and methanol, appeared more or less completely to remove the activity since the extraction residues were found to contain less than 5 per cent of the original activity; however, the remainder did not immediately show up in the extracts. The activity in the methanol extract (after evaporation of the methanol and resolution in water) gradually increased on standing, and it was found that autoclaving an alkaline solution of the ethanol extract caused

TABLE 10

Extraction of the activity from the powdered standard yeast extract with organic solvents*

SOLVENT	SOLIDS EXTRACTED†	ACTIVITY FOUND IN EXTRACT	ACTIVITY FOUND IN RESIDUE
	per cent	*per cent*	*per cent*
Diethyl ether	1	20	75
Petroleum ether (32–57 C)	5	3	90
Benzene	3	15	90
Ethyl acetate	7	32	75
Acetone	20	40	60
n-Butanol	48	57	3
Methanol	68	17	5
Ethanol, 95%	74	28	2

* This material is quite hygroscopic and probably contained several per cent of water. It was not desiccated before extraction.

† The actual solids extracted were determined only on the diethyl ether extract. For the others, the figure was estimated by weighing the oven-dried extraction residues: the estimated "solids" extracted, therefore, include any water contained in the original material.

a great increase in activity. These results suggested that extraction with the alcohols may have resulted in esterification of the active compounds.

It was thought that the incomplete extraction of the activity by diethyl ether, for example, might be attributable to the existence of most of the activity in the salt form. To test this hypothesis, aliquots of a solution were adjusted to pH 1.5, 4.2, 7.0, 9.0, and 10.5 and dried *in vacuo* from the frozen state. These dried samples were extracted with ether in the same manner as above, with the surprising result that no activity was extracted at any pH and the full activity was found to be contained in the extraction residues. These results suggest that the small amount of water normally present in the hygroscopic yeast extract is essential to the partial extraction of the activity with organic solvents.

Determination of the pK_a. It has been shown that the activity is extracted with ether from aqueous solution at pH 2 and 6.5 but not at 10. An estimate of the

pK_a of the active compounds was obtained using a liver L solution that had been autoclaved at pH 0.1 for 1 hour to effect liberation of the activity. Twenty-five-ml portions of the acid-autoclaved liver L in a 1 per cent solution were adjusted to the following pH values: 1.0, 2.0, 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, and 9.0, and partitioned with 25-ml portions of washed, redistilled chloroform. The aqueous portions at pH 6.0, 6.5, and 7.0 were buffered with 0.04 M phosphate buffers. Assays were performed in duplicate on the aqueous and chloroform phases after partitioning, and the total activity recovered was 100 ± 3 per cent with all samples. The percentage of the total activity present in the chloroform phase is plotted in figure 4 as a function of the pH. The curve closely resembles that for the titration of a monobasic weak acid. At partition equilibrium it can be assumed that the condition of true partition of the acid form of the activity between the two phases existed and that equilibrium between the salt and acid form in the aque-

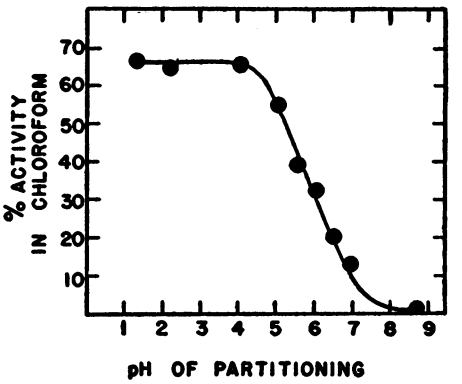

Figure 4. Partition of the activity of acid-autoclaved liver L (160 μg = 1 activity unit) between equal volumes of chloroform and water as a function of the pH of the water phase.

ous phase existed according to the law of mass action. Applying this law as expressed by the Henderson-Hasselbalch equation, the derived equation was:

$$pK_a = pH - \log\left(\frac{W}{S} \cdot C - 1\right)$$

where C is the observed partition coefficient of the free acid, S is the total activity in the chloroform phase, and W is the total activity in the aqueous phase. The values obtained at the several pH values were as follows:

pH	pK_a
5.1	5.1
5.6	5.2
6.1	5.5
6.6	5.1

The average value of approximately 5.2 suggests that the B.R. factor is a carboxylic acid.

Purification of the B.R. factor. The general procedure was based mainly upon

the observation that a 25- to 50-fold purification was possible by extracting from aqueous solution with benzene or ether. Various materials served as starting sources but the most suitable were Difco yeast extract, liver L, and *Penicillium notatum* autolyzate, after they had been subjected to acid-autoclaving. By means of successive solvent extractions, without attempting efficient recoveries of the activity, several preparations were obtained having specific activities of 225, 270, and 500; i.e., 4.5, 3.7, and 2.0 μg, respectively, in 10 ml of medium gave half-maximum growth. The best preparation represented a purification of greater than 500 over source materials.

Relation of the B.R. factor to other growth substances. The inability of the known growth factors to substitute for the B.R. factor is the main reason for presuming that a new growth substance is under consideration. A lack of coincidence of the properties of the B.R. factor with those reported for the known growth factors also lends support to this presumption. In particular, the extreme stability to heat, acid, and alkali combined with the readiness with which certain forms dissolve and partition into immiscible solvents serves to differentiate it.

The properties of the B.R. factor indicate that it is a nonamphoteric monobasic acid. It is probably not a simple fatty acid since substances of this type failed to substitute for it and the value of the partition coefficient is inconsistent with such a structure.

Strepogenin-type substances and vitamin B_{12} concentrates were inactive; moreover, these substances appear to be very dissimilar to the B.R. factor in their properties.

Comparison of the B.R. factor with the large number of unidentified growth factors reported in recent years reveals similarities in a few instances, but the establishment of definite relationships must await a more complete knowledge of the properties and interchangeability of the various factors. Factors that have some resemblance to the B.R. factor are reported as follows: (1) A factor stimulating the initial rate of growth of *Lactobacillus casei* reported by Guirard, Snell, and Williams (1946). The factor replaced, and was more active than, acetate. It was extracted by ether and was acidic but appeared to be unstable to autoclaving.

(2) A factor stimulating the oxidation of pyruvate by washed cells of *Streptococcus faecalis* was reported by O'Kane and Gunsalus (1947, 1948) and was characterized as having extreme stability to heat, acid, and alkali. It was also reported to be soluble in some organic solvents, but only limited data were reported in this regard.

(3) A factor essential for the growth of a protozoan, *Tetrahymena geleii*, was characterized by Stokstad *et al.* (1949). This substance, called protogen, was reported to be quite stable to heat, acid, and alkali, and it partitioned readily into *n*-butanol but not into chloroform. The factor appeared to exist in more than one form.

(4) A factor stimulating the growth of rats and chicks was reported by Novak and Hauge (1948), who proposed the name of vitamin B_{13}. It was stable to autoclaving in acid and alkali and was qualitatively reported to be soluble in water,

acetone, chloroform, ethanol, ethyl acetate, and benzene. Acidic properties were not mentioned.

A recent note by Snell and Broquist (1949) suggests that protogen, the pyruvic oxidase factor, and the acetate-replacing factor are identical.

SUMMARY

Butyribacterium rettgeri has been shown to require a new factor for growth on a medium containing lactate as the fermentable carbon source. The factor is not replaceable by any of the growth factors of known composition or by recently reported substances such as strepogenin and vitamin B_{12}.

A synthetic medium was devised for the assay of the new factor which permitted a comparable growth response to either 5 to 10 mg of a crude source such as yeast extract, or 1 to 5 μg of a purified preparation in 10 ml of the medium.

A number of physical and chemical properties are described. The factor occurs largely in the bound form and is liberated by autoclaving in 1 N acid or alkali. It is stable to enzymes, heat, acid, and alkali and partitions readily into a number of immiscible organic solvents. Extraction of the activity from aqueous solution as a function of the pH suggests that it is a weak acid with pK_a close to 5.2.

REFERENCES

BARKER, H. A., AND HAAS, V. 1944 *Butyribacterium*, a new genus of gram-positive, non-sporulating anaerobic bacteria of intestinal origin. J. Bact., **47**, 301–305.

BARKER, H. A., KAMEN, M. D., AND HAAS, V. 1945 Carbon dioxide utilization in the synthesis of acetic and butyric acids by *B. rettgeri*. Proc. Natl. Acad. Sci. U. S., **31**, 355–360.

GUIRARD, B. M., SNELL, E. E., AND WILLIAMS, R. J. 1946 The nutritional role of acetate for lactic acid bacteria. II. Fractionation of extracts of natural materials. Arch. Biochem., **9**, 381–386.

HAAS, V. 1947 Dissertation. University of California, Berkeley, California.

NOVAK, A. F., AND HAUGE, S. M. 1948 Isolation of the unidentified growth factor (vitamin B_{12}) in distillers' dried solubles. J. Biol. Chem., **174**, 647–651.

O'KANE, D. J., AND GUNSALUS, I. C. 1947 Accessory factor requirement for pyruvate oxidation. J. Bact., **54**, 20–21.

O'KANE, D. J., AND GUNSALUS, I. C. 1948 Pyruvic acid metabolism. A factor required for oxidation by *S. faecalis*. J. Bact., **56**, 499–506.

SNELL, E. E., AND BROQUIST, H. P. 1949 On the probable identity of several unidentified growth factors. Arch. Biochem., **23**, 326–328.

STOKSTAD, E. L. R., HOFFMANN, C. E., REGAN, M. A., FORDHAM, D., AND JUKES, T. H. 1949 Observations on an unknown growth factor essential for *T. geleii*. Arch. Biochem., **20**, 75–82.