THE CONCENTRATION OF AN ACETATE - REPLACING FACTOR

APPROVED:

Roger J. Williams

H. L. Lochte

H. R. Henze

W. A. Selvig

M. J. Copeland

George W. Watt

F. A. Matsen

APPROVED:

A. P. Brogan

Dean of the Graduate School

THE CONCENTRATION OF AN ACETATE - REPLACING FACTOR

DISSERTATION

Presented to the Faculty of the Graduate School of

The University of Texas in Partial Fulfillment

of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Milton Edmond Getzendaner, A. B., M. S.

Austin, Texas

June 1949

ACKNOWLEDGEMENTS

This work was suggested by and carried out under the direction of Professor Roger J. Williams. The author is grateful for the helpful suggestions, and truly appreciates the freedom given in the pursuit of the problem.

In addition, discussions with Drs. William Shive and Robert E. Eakin gave added stimulus to the research.

Thanks are also due to the author's parents, Mr. and Mrs. J. William Getzendaner, for making possible financial arrangements such that a full acedemic load could be carried during the last year of graduate work.

April 1949

TABLE OF CONTENTS

INTRODUCTION

INTRODUCTION

Although investigations in the general field of nutrition have led to the discovery and isolation of numerous compounds which are nutritional essentials, present work shows that there are still significant gaps in our knowledge of the absolute nutritional requirements for many forms of life. The literature is replete with reports of "growth factors" contained in naturally occurring materials of various kinds, which are as yet unidentified. The addition to basal media of crude extracts of these materials makes possible vigorous growth, whereas on a purely synthetic medium it would be negligible.

Lactic acid bacteria are among the organisms for which such growth factors have been found. Cheldelin, and Riggs (1) found that Wilson's liver fraction L contains a factor for Lactobacillus gayonii 8289. When a casein hydrolysate medium containing Norit and Lloyd's Reagent filtrates of peptone and yeast extract was supplemented with this liver fraction, the organism grew, while the unsupplemented medium was unsatisfactory. A heat-stable factor for acid production by certain lactobacilli has been found in several vegetable juices by Metcalf, Hucker and Carpenter (2). A substance in tomato juice absorbed on Norit has been found by Kuiken, Norman, Lyman, Hale, and Blotter (3) to be essential for Lactobacillus arabinosus. Also, Shorb (4) reported an unidentified factor necessary for Lactobacillus lactis which was found in canned tomato juice as well as in liver. This has been designated the "TJ" factor. The existence of a factor in liver necessary for hemolytic streptococci was reported by Wooley (5), and later Sprince and Wooley (6) found that it was also necessary for Lactobacillus casei.

They called the active material "strepogenin" and found it in largest amounts in crystalline insulin, trypsinogen, trypsin and casein (7). Scott and co-workers have found that two unknown factors are required for the growth of the same organism (8). They attempted to correlate these factors with those previously reported by other workers and found that their factor S, as measured by chick assay was the same as strepogenin (9). Still more recently they showed that Lactobacillus casei requires a factor, or factors, present in anti-pernicious anemia liver extracts in addition to strepogenin, for maximum growth (10). They found that tomato joice, shortly after canning gave the same response. Colio and Babb (11) found a factor in malt sprouts which was stimulatory for Lactobacillus casei and S. lactis R when very small inocula are used.

In 1937 Snell et al. (12) showed that acetate in the medium for growing lactic acid bacteria might show a function other than buffering. It was shown that the presence of acetate in a medium of peptone, glucose and mineral salts, supplemented with an ether extract of an aqueous potato extract, gave greater acid production by Lactobacillus delbruckii than when supplemented by the potato extract alone. Guirard et al. (13, 14) concluded that acetate may serve a dual purpose: (a) that of a buffer, to prevent the lactic acid formed during growth from lowering the pH of the medium to toxic levels, and (b) an unknown function, concerned with the production of early and luxuriant growth. It was reported that lactic acid bacteria responded to various pure substances supposedly involved in acetate metabolism. The occurrence of a substance or substances in natural materials which duplicated the growth-stimulating effects of acetate for the lactic acid bacteria was also reported (14,15). Concentration of the activity was undertaken and a 44-fold

concentration was effected, which resulted in a material having 440 times the activity of sodium acetate on a weight basis.

The research reported here was undertaken to concentrate the above described active material from natural sources for the purpose of isolating pure material for identification.

EXPERIMENTAL

EXPERIMENTAL

I. ASSAY PROCEDURE

Throughout the remainder of this dissertation, the term "acetate factor", (or merely "factor") will be used to designate the substance (or substances) which is being concentrated. It is present in natural materials, and in minute amounts replaces acetate in the culture medium of the test organism.

Lactobacillus casei (A.T.C. 7469) has been employed exclusively in this work for the quantitative estimation of acetate-replacing activity in the materials tested. This organism has been carried in this laboratory several years by monthly transfers on glucose yeast extract stabs containing 1% glucose, 1% yeast extract and 2% Agar. It was chosen because of its satisfactory use by Guirard (14). It may be presumed on the basis of her work that this factor will be effective as a nutrilite for organisms other than the one chosen for convenient use.

The acetate-free, phosphate-buffered medium employed by Guirard et al. (13, 14) was used. The composition of the basal medium is given in Table I. Solution 1 was preserved by autoclaving it for 10 minutes at 15 pounds pressure when first made, then steaming it for 10 minutes after each removal of an aliquot. Solutions 1, 2 and 3 were combined as indicated and adjusted to pH 6.5. Solutions 2 and 3 were kept in the refrigerator under toluene, and solution 3 was renewed monthly.

To carry out a test, the standard was made up in five tubes, (16 - 20 mm. x 150 mm. without lip) containing 0.50, 1.0, 1.5, 2.0 and 5.0 mg. of anhydrous sodium acetate each in 5.0 ml. of aqueous solution. The unknowns were put into a suitable known concentration and introduced into tubes in

TABLE I

Composition of Double Strength Medium

			Amt. for 50 assay tubes
I	Solution 1		250 ml.
	Acid hydrolyzed casein* 10%	10 ml.	
	KH_2PO_4	5 g.	
	L-tryptophan	200 mg.	
	L-cystine	200 mg.	
	L-asparagine	200 mg.	
	Inorganic salts: solution A (25 g. each of KH_2PO_4 and K_2HPO_4 in 250 ml. of solution.)	10 ml.	
	Inorganic salts: solution B (10 g. $MgSO_4.7H_2O$ + 0.5 g. each of $FeSO_4.7H_2O$, $MnSO_4.4H_2O$ and NaCl in 250 ml. of solution.)	10 ml.	
	pH adjusted to 6.5		
	Water to make	1000 ml.	
II	Solution 2		5 ml.
	Adenine sulfate	100 mg.	
	Guanine hydrochloride	100 mg.	
	Uracil	100 mg.	
	Hydrochloric acid to affect solution		
	Water to make	100 ml.	
III	Solution 3		4 ml.
	Thiamine chloride	1.5 mg.	
	Calcium pantothenate	1.5 mg.	
	Riboflavin	1.5 mg.	
	Nicotinic acid	1.5 mg.	
	p-Aminobenzoic acid	1.5 mg.	
	Pyridoxine hydrochloride	75 mg.	
	Inositol	15 mg.	
	Folic acid	0.563 mg.	
	Biotin	0.012 mg.	
	Water to make	30 ml.	
IV	Glucose		5 g.

---

* Vitamin-free Labco Casein hydrolyzed with hydrochloric acid according to procedure described in the University of Texas Publication No. 4137, p. 82. The hydrolysate preserved under toluene and treated with Darco G-60 (10%) at pH 3.0 before incorporating into the medium.

the amounts 0.05, 0.15, 0.50, 1.50 and 5.0 ml. respectively. A pair of tubes was included in each test containing no supplement. Distilled water was added to bring the volume of solution in every tube to 5.0 ml., then 5.0 ml. of the double strength medium was added. The culture tubes were capped with aluminum caps and autoclaved at 15 pounds pressure for 10 minutes. After cooling, one drop of the innoculum described below was put into each tube with a blunt-tipped 2 ml. graduated pipette, and the test was incubated for 18 hours at 37 ± 1° C.

The organism used for innoculation was taken from a stab culture which was renewed every two weeks from the stock culture. It was allowed to incubate 48 hours at 37° C., then was refrigerated. The medium was the same as that used for the monthly transfers.

The innoculum was prepared by transferring the organism from the agar stab culture to a tube containing 10 ml. of medium of the following composition per liter: 5 g. Bacto-Peptone, 1 g. Bacto yeast extract, 10 g. anhydrous sodium acetate, 10 g. glucose, and 5 ml. each of inorganic salts A and B (Table I). After an 18 hour incubation, a loopful of the suspension was transferred to another tube containing 10 ml. of the same medium. Only the bacteria from this first serial transfer, after incubation, and a second serial transfer were used for innoculation, care being taken to adhere closely to 18 hour incubation periods. To facilitate timing, it was possible to keep the cultures in the medium in the refrigerator several hours before starting incubation, and for 4 - 5 hours after incubation before use. Immediately before use the cells were centrifuged to the bottom of the tube, the supernatant solution poured off, and 10 ml. of 0.9% saline added. After resuspending, they were again centrifuged, the supernatant

solution poured off and the organism resuspended in saline. A satisfactory suspension for innoculation was usually obtained by adding 0.2 ml. of the washed organisms to 10 ml. of saline. A faintly turbid suspension was used.

When higher concentrates of active material were obtained, it lost its water solubility. For testing, it was made up into alcoholic solution such that 0.1 ml. or less diluted to 10 ml. would give a suitable concentration for testing. Ethanol at that low concentration has been shown to be non-toxic for Lactobacillus casei (14).

After incubation for 18 hours, the turbidity of the solutions was read on a turbidimeter so set that distilled water was zero and an opaque object 100. The data from the acetate standard were plotted with turbidity versus weight of sodium acetate. Only the straight line portion of the curve was used from the graph. The weight of sodium acetate required to give the same turbidity as that obtained from the unknowns was determined. Comparisons of turbidity were limited to samples tested simultaneously.

From the values obtained by this procedure two quantities have been defined:

1. Potency = $\frac{\text{wt. of sodium acetate}}{\text{wt. of sample giving} \doteqdot \text{response}}$

2. Units of activity = potency x wt. in grams.

Thus, 1 mg. of material, 1γ of which gives the same turbidity as 1 mg. of sodium acetate, has a potency of 1000 and contains 1 unit of activity.

It was noted by Guirard (14) that drying of samples often resulted in loss of activity. To overcome this trouble, as well as to facilitate obtaining an accurate record of the total solids in each preparation, samples were routinely dried in tared weighing bottles in an atmosphere of

natural gas, under partial vacuum at temperatures up to 60° C. Under these conditions there was no loss of activity by any sample which could be traced to drying.

II. SOURCE MATERIAL

Since it was desirable to use a source material which would be readily available as well as relatively inexpensive, several materials were tested in addition to those reported by Guirard et al. (14, 15). Results of these tests are reported in Table II. Reference to this table shows that while the acetate factor is relatively rich in some pernicious anemia preparations, it is not consistently concentrated in them. This indicates that the two factors, assuming each to be unitary, are not identical.

TABLE II

The Potency of Various Materials

Material		Potency
Egg (hot water extract)		no activity at levels tested
Milk (whey solids)		2
Vitab Rice Bran Concentrate		3
Liver autolysate		50
Commercial Anti-pernicious anemia concentrates:		
Shart & Dohme	2 USP Unit ml.	22
	5 " "	0
	15 " "	3
Lilly	15 " "	19
	20 " "	20
	30 " "	46
Armour	15 " "	3
Upjohn	15 " "	7

It will be noted in Table II that the liver autolysate, which was prepared in accordance with unpublished information furnished by Professor William Shive, has a higher potency than any other material tested. This material

was therefore used as a source of the factor in question.

An extract was prepared from 4.40 kg. of hog's liver. After autolysis, it was steamed for 30 minutes, and filtered with suction aided with Hyflo Super-Cel (Johns Manville Co.). The solid was slurried with enough water to make a thin paste, steamed again and refiltered. The slurrying, steaming and filtering was repeated twice more, resulting in four extracts which were combined to make sample No. 2:107.4.* The total volume was 16.5 liters containing 189 g. of solids. This material had a potency of 30, constituting a total of 5670 units.

III. SOLVENT EXTRACTION EXPERIMENTS

The partition coefficients of the active principle, and the total solid of a pernicious anemia preparation, between a buffered aqueous phase and n-butanol (Carbide and Carbon Chemicals Corp.) are shown in Table III. Buffer solutions were made up at the indicated pH's. Into separate test tubes were pipetted 0.40 ml. of each buffer, followed by 0.10 ml. of a 20 U.S.P. unit/ml. liver concentrate and 0.50 ml. of n-butanol. The mixtures were shaken vigorously several minutes, and allowed to separate overnight. A turbidness developed in tubes of pH 1.0 through 4.0, and was removed by centrifugation. Then 0.05 ml. of each solution was removed and diluted for testing.

---

* Samples and experiments are numbered according to the following scheme: the first number refers to the number of the author's notebook, the number after the colon gives the page number, and a number following the period is the numbered sample as found on the specified page.

TABLE III

Partition Between n-Butanol and water

pH of H_2O	Partition Coefficients*	
	Activity	Total Solids
1.0	8	0.50
1.5	4	0.40
2.0	3	0.79
2.5	3	0.31
3.0	2	0.33
3.5	2	0.26
4.0	2	0.58
5.0	1	0.54
6.0	0.2	0.37
7.0	0.12	0.29
8.0	0.12	1.01
9.0	0.33	0.37
10.0	0.33	0.35
11.0	0.40	0.35

* Partition coefficient = butanol solubility/water solubility.

The remainder of the butanol solutions were pipetted into separate weighing bottles, dried as previously described, and weighed. The weight of liver solids in aqueous solution was obtained by difference.

From this it was concluded that (a) the acetate factor is acidic, substantiating Guirard's observation (14, 15); (b) a large fraction of inactive material is either neutral or basic in character, so (c) extraction of aqueous liver extract with n-butanol at an acid pH should result in a concentration of activity. On the basis of these conclusions, experiments were carried out to effect concentration of the active material from crude liver extracts.

A continuous butanol extraction of 50 ml. of sample 2:107.2, referred to above, was carried out under reduced pressure. The pH of the solution was adjusted to 1.0 with hydrochloric acid, and it was saturated with butanol. A precipitate which formed was filtered off with the aid of

Super-Cel. The solution was put into a conventional continuous liquid-liquid extractor, in which the extracting solvent was circulated by boiling. The top of the condenser was connected to a water aspirator. After three hours of operation, the extract in the boiler was removed and fresh butanol added. Extraction was then continued for another five hours. Table IV summarizes the results.

TABLE IV

Results of Continuous Butanol Extraction

Material	Weight (g.)	Potency	Units
Unextracted	0.615	33	20.3
1st extract	0.157	92	14.5
2nd "	1.49	1	0.96
Residue			0.04

Another experiment was carried out using batchwise extraction. About 16 l. of the sample 2:107.4 was adjusted to pH 1.0 with hydrochloric acid, and the solution was saturated with n-butanol. A precipitate which formed was removed by filtration aided with Super-Cel. Three separate 700 - 800 ml. portions of butanol were stirred with the aqueous solution for half an hour, allowed to separate and then were removed. The butanol separated as an emulsion which, after removal, was broken by the addition of sufficient magnesium chloride or aluminum chloride. The aqueous phase from the broken emulsion was returned to the main batch. Results of the three extractions which were made are recorded in Table V. It was found that the precipitate removed with Super-Cel contained considerable activity. Extraction of this material by heating on a steam cone 20 minutes with butanol yielded active material, as indicated.

TABLE V

Results From Batchwise Butanol Extraction
of Sample 2:107.4

Material	Weight (g.)	Potency	Units
2:107.4	185	30	5550
1st extract	2.73	200	546
2nd "	2.25	200	450
3rd "	2.52	100	252
From Super-Cel			
1st extract	12.8	80	1020
2nd "	3.1	150	465
3rd "	5.1	113	577
Total Recovery	28.5	116	3310

To utilize the differences in partition between water and butanol under acid and basic conditions a continuous liquid-liquid extractor was used. The extractor was constructed from a one liter Pyrex Kjeldahl flask. A piece of glass tubing the same diameter as the neck, and 24 inches long was sealed to the neck, with a side-arm delivery tube at about 12 inches above the joint. A tube to conduct the organic solvent extended from above the top of the extractor to the bottom. The side-arm delivery tube led directly to the bottom of a 30 mm. tube 25 cm. long containing 100 ml. of a phosphate buffer solution at pH 8.0. The overflow from this tube was directed to the bottom of a similar tube. A pump operating about 10 ml./min. took the organic solvent from the top of the second tube and forced it up to the tube extending through the top of the extractor.

A liver extract obtained by autolysis was treated in this apparatus. The pH of 1.2 l. was adjusted to 1.0 with hydrochloric acid. The solvent used was an ether-butanol (1:1) solution. After 28 hours of extraction, during which time three fresh buffer solutions were used, a total of 20% of

the activity was recovered from the extract, with not more than a 2 - 3 fold increase in potency.

By means of the solvent extraction methods described above, concentrations of up to 10 fold were obtained. However, the recovery of activity was in general unsatisfactory. Because of the great losses of active material involved in concentrating it by these means, solvent extraction methods were abandoned.

IV ADSORPTION EXPERIMENTS

Preliminary experiments with low potency liver extracts indicated that it was possible to remove the acetate factor almost completely from solutions acidified to pH 3.5 - 4.5, by the use of a quantity of Darco G-60 nearly equal to the weight of solids treated. Elution by refluxing the charcoal with a solution of 5 parts water, 5 parts 95% ethyl alcohol and 1 part 28% ammonium hydroxide resulted in a 5 - 7 fold increase in potency but a low yield. When the pH was decreased to 3.0, the activity was removed almost quantitatively by the Darco, using a charcoal : solids ratio of 1:10.

To remove the active material from the charcoal various eluting agents were tried. Best results were obtained by refluxing the charcoal with ethyl acetate. By this method potencies of 500 to 1000 were obtained, starting with material having a potency of 30. This resulted in about 50% recovery of the physiological activity. A sample experiment follows to illustrate the procedure used.

A 25 ml. sample of 2:107.4 was adjusted to pH 3.0 with hydrochloric acid. This sample contained about 300 mg. of solids, comprising 7.7 units. A slight precipitate which appeared on acidification was removed by centrifugation. One-tenth gram of Darco G-60 was stirred mechanically with

the liver extract for 30 minutes, followed by filtration and washing of the charcoal with water. The charcoal, still moist with water, was extracted by refluxing it 20 minutes with about 1.5 ml. of ethyl acetate (Merck Reagent). After filtering, the charcoal was washed on the filter with a small quantity of hot ethyl acetate. The eluate from the charcoal was dried and found to weigh 5.2 mg. It was then dissolved in 2.6 ml. of ethyl alcohol, making a solution of 2 mg./ml. Of this solution 0.05 ml. were diluted to 10.0 ml. to be tested, making a solution of 10γ/ml. The potency of the solids was 944, giving 4.9 units recovered, or 64%.

The charcoal was further treated with ethyl acetate in a Soxhlet extractor for 2 hours. This resulted in the recovery of 9.8 mg. of material having a potency of 80, which is 0.78 units.

Subsequent experiments showed that by the use of a lower charcoal: solids ratio, a more potent material could be recovered, but the percentage recovery dropped off. To increase the amount of active material recoverable from charcoal, the procedure of Frieden et. al. (16) for recovering adsorbed folic acid was used.

V. CONCENTRATION PROCEDURE

The aim of the work reported in the previous sections was to develop a method for obtaining and concentrating liver extract. The method developed will be described by giving a sample concentration (experiment No. 4:50).

A 25-pound batch of hog's liver was obtained from a supply house after it had been ground and frozen. While still in a semi-frozen state it was suspended by means of a Waring Blendor, small portions at a time, in 11 liters of distilled water. It was then autolyzed, after which it was steamed for 15 - 30 minutes. With the addition of about 300 g. of Super-Cel, the

material was filtered on large Buchner funnels with suction. The resulting solid was mixed with water to make a thick slurry, which was steamed and filtered. This was repeated to give a total of four filtrations. The total amount of Super-Cel used in all filtrations was about one kg. The solution, 44.5 liters, was stored overnight in 12 liter flasks filled into the neck under a layer of toluene to prevent bacterial action. The total solid content of the extract was determined to be 524 g.

The toluene was removed from the flasks of liver extract, and they were combined in a 20 gallon aluminum vessel. A sample was removed for testing (sample No. 2). The solution was stirred mechanically for half an hour with 98 g. of Darco G-60. Then 120 g. of Super-Cel were added with just sufficient stirring to disperse it well. The charcoal was filtered off with suction and washed twice with small quantities of tap water. The filtrate from the charcoal adsorption was returned to the vessel, and a sample removed for testing (sample No. 3). Then 235 ml. of 28% ammonium hydroxide were added. The pH was adjusted to 2.7 by the addition of approximately 450 ml. of concentrated hydrochloric acid. This acidified solution was treated with 131 g. of charcoal and stirred as above. At the end of half an hour of stirring, 400 g. of Super-Cel were added and the mixture filtered. The Darco was washed twice with small quantities of water. While still moist, it was transferred to a 3 liter flask for extraction.

In the manner described above, the aqueous liver extract was treated with 65 g. of Darco. After the half-hour stirring, 200 g. of Super-Cel were added and the charcoal was filtered and washed with water. A sample of the filtrate was removed (sample No. 9). The remainder of the filtrate was discarded.

To get the best yield of active material, immediately after filtering from the liver extract the charcoal was eluted with ethyl acetate (Merck Reagent) while it was still moist. This was done by refluxing it gently for half an hour with about one and a half volumes of ethyl acetate, then filtering it with suction. The residue was pressed firmly into the funnel and then was washed with about half a volume of hot ethyl acetate. The Darco from the first adsorption was extracted three times in this way, combining all of the extracts (sample No. 4). The second batch was extracted four times, the extracts being kept separate (sample Nos. 5, 6, 7, and 8 respectively). Finally, the third charcoal was extracted twice with ethyl acetate, and the eluates combined (sample No. 10).

Each of the eluate fractions described above was concentrated to a small volume in vacuo. A capillary leading to the bottom of the boiling flask admitted a slow stream of gas to prevent bumping, and a tube leading into the flask through a side arm admitted fresh extract to keep the volume nearly constant. When the total volume was less than 5 ml., the thick syrup was poured into a weighing bottle and dried in the usual manner, in an atmosphere of natural gas under reduced pressure. When sample 5 was concentrated, a white waxy solid precipitated out which was relatively insoluble in either ethyl acetate or 95% ethyl alcohol. It was filtered off and washed with a small amount of alcohol (sample No. 5A).

Table VI summarizes the results obtained from the complete concentration as described. The sample numbering corresponds to the description in the text. Inspection of the table shows that although the first charcoal treatment removed 3100 units from the solution, the activity of the solution was increased by 9300 units. This might possibly be due to the removal of some

TABLE VI

Summary of results of concentration experiment No. 4:50

Sample No.	Weight (g.)	Potency	Units
2	524	115	60,500
3	---	133	69,800
4	1.5481	2000	3,100
5	0.8977	45000	41,000
5A	0.0307	4850	120
6	0.4231	43000	18,300
7	0.2617	18000	4,790
8	0.1774	21500	3,820
9	---	1.9	1,000
10	0.3166	6900	2,180

inhibitory substance by the first treatment. Samples 5 and 6, the most potent, contained 85% of the total activity compared to sample 3. Including all of the active material removed from the second batch of charcoal (samples 5, 6, 7, and 8), the recovery of units of activity amounted to 97%. Perhaps this is a somewhat high value, because a summation of all of the units recovered from charcoal was greater than found at the start.

VI. EXPERIMENTS WITH CHROMATOGRAPHY

A. Paper Chromatography

A series of experiments was carried out to test the possibility of using paper chromatography as an assay method or for small scale purification. The method of capillary ascent described by Williams and Kirby (17) was employed. The procedure involved placing 0.01 ml. of a butanol solution of the material about one inch from the end of a strip of Whatman #1 filter paper 2 x 18 inches. After the spot dried, the strip was suspended in a one-liter graduated cylinder with the end containing the sample just dipping into a thin layer of the developing solution. The cylinder was made practically air-tight with a cap of "Parafilm". Development was allowed to continue

18 hours, or until the solvent attained a satisfactory height. Then the strip was removed and dried in the air. It was examined under an ultra-violet light to show up fluorescent areas so they could be marked. A strip about 8 mm. wide was then cut lengthwise from the marked chromatogram. For analysis the sample strip was cut into pieces separating the various areas outlined and the remainder divided into suitable fractions of the total length. Sufficient tubes of medium were prepared with no supplement, and one piece of the sectioned strip was put into each. The tubes were capped, autoclaved and innoculated as previously described.

In all, six separate chromatograms were run. The solvents employed were water-saturated butanol, water-saturated benzyl alcohol, ethanolamine, cyclohexanol saturated with water, dioxane-water (10:1), and wet n-butanol-ether (1:4). In all cases, definite as well as diffuse fluorescent areas were to be found, but the activity was not associated with any such area. Most of the solids traveled with the leading edge of solvent, as indicated by the color. Table VII gives the R_f values obtained for the active material. In cases where it was spread along the chromatogram for some distance, values are given to indicate the spread.

TABLE VII

R_f* Values For Acetate Factor Developed By Various Solvents Using Capillary Ascent

Solvent	R_f Value
wet butyl alcohol	.90 - 1.0
wet benzyl alcohol	.50 - .95
ethanolamine	.85 - 1.0
wet cyclohexanol	.95
dioxane-water (10:1)	.85 - 1.0
butyl alcohol-ether (4:1)	.75 - .95

*R_f value = ratio of distance traveled by the particular constituent, to the total distance traveled by the solvent.

One paper chromatogram was developed with ninhydrin as described by Williams and Kirby (17). It gave no color reaction, indicating the absence of α-amino acids in the eluate from Darco G-60 which was used.

B. Activated Adsorbents

Preliminary experiments were carried out with activated adsorbents by the disk method described by T. I. Williams (18). A hole 4 mm. in diameter was made in the center of a $3\frac{1}{2}$ x $4\frac{1}{2}$ inch glass plate. A thin disk of adsorbent 3 inches in diameter was formed between a solid plate and the perforated one. The sample solution was introduced through the hole in the upper plate, followed by the eluants. After the disk was developed, the top plate was carefully removed, and a 90° wedge was marked off on the disk. Concentric arcs, having the spot just under the hole in the upper plate as the center, were scraped off into separate test tubes and eluted with 95% ethyl alcohol. In this way five or six samples were obtained. Colored bands were carefully isolated as separate samples. Aliquots of the eluates were used for testing. Samples of 1 mg. each were chromatographed on disks of silica gel (Dayco 8595 I2, the Davison Chemical Corp., Baltimore, Md.), aluminum oxide, (according to Brockman, Merck 42861), and adsorptive powdered magnesia (# 2641).

For exploratory experiments where it is important to use a small quantity of material, the use of disk chromatograms serves very well. However, if quantitative recovery of the active material is necessary, the method is limited. This is especially true when colorless compounds are being sought, since there is no possibility of continuous elution with the collection of separate samples of eluate. This method serves as a very rapid test of the eluting powers of solvents for visible constituents, since an entire run may take only 10 - 30 minutes. Moreover, since the adsorbent can be divided

and eluted as described above, the location of colorless fractions can be determined, if suitable assays are available.

The results of these tests showed that magnesia or aluminum oxide would probably serve as satisfactory adsorbents, and that solutions containing ethanol caused rapid elution of the active material, while with acetone present, the rate was moderate.

Since the above work showed promise that chromatography would result in further concentration, the use of columns of activated adsorbents was undertaken. From the first, trouble was experienced in finding solvents in which the material was sufficiently soluble, but which would cause a slow rate of elution, allowing useful separations. Hence, a mixture of solvents was used including n-butanol, nitroethane, acetone, carbon tetrachloride and Skellysolve C or benzene. The column was either packed dry and then washed with carbon tetrachloride-Skellysolve C (1:1), or the adsorbent was slurried with the solvents and packed. After the sample had been introduced and the solution just disappeared below the surface of the adsorbent, carbon tetrachloride was used as the first eluant. After the rapidly moving bands had been eluted from the column, a slightly better eluant, such as acetone, was used with carbon tetrachloride in small quantities to carry through slower moving fractions.

A series of chromatograms was tried using first a 4 x 35 mm. column and then a larger one, 10 x 80 mm. This work indicated that with either alumina (Alcoa Activated alumina F 20) or magnesia or both mixed together, it was possible to get about a 50% recovery of units with an increase of potency of 5 - 10 fold over the starting material. The active material eluted through with the first solids.

A larger column was prepared using a tube 2.5 x 22 cm. which was packed with a 3:2 mixture of alumina and magnesia. Both of the adsorbents had been previously dried in an oven at about 200° C. for 6 hours. A 0.42 g. sample of fraction 3:94.4 was put into the column. No active material was eluted through the column in the usual manner, so the column was extruded and the active material was eluted from the top fraction in poor yield with 95% ethyl alcohol, and water-ethyl alcohol (95%)-ammonium hydroxide (28%) in the proportion of 5:5:1. Because of the unsatisfactory results, the use of active adsorbents was discontinued.

C. Partition Chromatography

Recent success has been attained in the separation of aliphatic carboxylic acids by means of partition chromatography in the hands of Ramsey and Patterson (19) and Peterson and Johnson (20). There are several lines of evidence indicating that the acetate factor is an acid. Guirard (14, 15) found that the factor migrated to the anode in an electrical transport apparatus. The eluate from Darco G-60 is insoluble in water, but soluble in basic solution. Sublimation of the acid takes place readily, while the sodium salt is not volatile (see p.26). Because of its acid properties it seemed likely that partition chromatography could be adapted for this purification.

Small-scale experiments, each using about 20 mg. of Darco eluate (sample No. 4:50.6) were carried out in a 13 mm. column. A combination of the conditions used for aliphatic acids was adapted for this purpose. Celite 545 (Johns Manville Co.) was used as the support for water buffered with sodium phosphate as the stationary phase. Benzene was used as the mobile component, with butanol added to increase its eluting power.

After a satisfactory procedure for concentration of the factor was

developed, larger samples were used in proportionately larger tubes. The final and largest sample was 338 mg. of sample 4:50.5. A detailed procedure is given below for this experiment (No. 5:72).

A buffer solution was prepared using 3.25 g. of potassium dihydrogen-phosphate (Merck Reagent) in 500 cc. of water and the addition of sufficient 5 N sodium hydroxide to make a pH of 8.4.

Thiophene-free benzene was prepared. This was done by shaking benzene with small quantities of concentrated sulfuric acid in a separatory funnel until fresh acid no longer acquired a color. The benzene was then shaken with sodium hydroxide pellets, and finally distilled over sodium hydroxide pellets in an all-glass apparatus. All of the benzene used in partition chromatography was prepared in this way. Solutions of n-butanol in benzene used as eluants were 2% (BB2), 5% (BB5) and 16% (BB16). These solutions were saturated with water before use.

A chromatographic tube 3.0 x 30 cm. was used with a perforated porcelain disk at the bottom, covered with a thin pad of cotton. To 54 g. of Celite 545 spread in a thin layer in a large evaporating dish were added 48.6 ml. of the buffer solution. It was sprinkled over the top of the Celite with a 25 ml. graduated pipette. The dampened Celite was stirred rapidly with a spatula until it appeared homogeneous, then was put into a 500 ml. glass-stoppered Erlenmeyer flask. Sufficient benzene was added to the flask, and the packing material was shaken vigorously to make a thin slurry.

A small portion of the slurry was poured onto the cotton mat. This was packed tightly into place by means of a cork, just smaller than the tube, on the end of a piece of brass rod. Then more of the slurry was added. By pumping the tamper up and down it could be worked to the bottom of the loose

Celite and then, allowing a small quantity to remain under the cork, it was pressed firmly into place. The tamper was rotated during packing, as was the glass tube. The column was packed by repeating this process, pressing only a small amount of material into place at one time. The completed column was 26 cm. high. It was kept covered with benzene until used.

A solution containing 338 mg. of sample 4:50.5 in ethyl alcohol was evaporated to dryness. The solid material was stirred with 0.75 ml. of n-butanol, then 14.25 ml. of benzene were added slowly with stirring. The precipitate which resulted was centrifuged and treated with 0.10 ml. of butanol followed by 1.90 ml. of benzene. Again the undissolved solid was centrifuged and treated with 0.10 ml. of butanol and 1.90 ml. of benzene. Finally, the residue from the third extraction was stirred with 0.05 ml. of butanol, and 0.95 ml. of benzene were added. After centrifuging, the solid was dried (sample A). All four of the supernatent solutions were combined.

The column was allowed to drain until the benzene just disappeared below the surface. The combined 5% butanol solutions were then added to the top of the column by means of a funnel. Measurement of the volume of effluent was started at the time of addition of the sample. When the surface of the column was exposed after the sample passed into the column, benzene was added. A separatory funnel was supported above the column. The exit tube passed through a stopper fitting snugly into the top of this column. The eluants were introduced by this means, automatically maintaining a suitable level. A flow rate of 1 - 2 ml. per minute was maintained.

Table VIII summarizes the results of the chromatogram. There were 11,150 units at the start. About 10,000 units were put onto the column, and 5,050 units were recovered in the concentrated fractions. This is a 50% recovery of activity. The active material consists of 21.5 mg., dry weight,

having an average potency of 235,000. The acetate factor was concentrated

TABLE VIII

Experiment No. 5:72. Results of Partition Chromatogram Using pH 8.4 Buffer and Benzene-Butanol

Sample No.	Effluent ml.	Weight mg.	Potency (x 10-3)	Units	Remarks
4:50.5		388	33	11,150	Starting Material
5:72.A		26.6	4	106	Insoluble in BB5
1	245	121.2	4	458	Color appeared at 65 ml. BB5 started at 205 ml.
2	90	14.0	11	154	Contains brown ring which started from top with BB5.
3	40	8.2	12	100	
4	60	5.9	21	125	
5	120	6.5	175	1400	
6	185	6.9	400	2760	BB16 started at 110 ml.
7	55	8.1	110	890	Contains brown ring

7-fold by this chromatogram. The solid containing the active material was a dark brown, waxlike material.

All of the materials having a potency of 100,000 or greater, resulting from partition chromatography and vacuum sublimation (p.26) were combined. This made a total of 101 mg. when dried. It was mixed with 0.2 ml. of butanol, then 5.8 ml. of benzene were added with stirring. After centrifuging, the resulting solid was treated with 0.05 ml. of butanol. Then 0.95 ml. of benzene were added and after stirring, the precipitate was centrifuged. The solid was again treated with butanol (0.025 ml.) and 0.50 ml. of benzene. The precipitate was centrifuged and dried (sample No. 94A).

The supernatant solutions resulting from the above operations were combined, and benzene was added to bring the volume up to 10.0 ml. This gave a solution of 2.75% butanol in benzene. Of this, 0.01 ml. was removed for testing (sample No. 94B).

A chromatographic column was prepared in a tube 1.7 x 40 cm. Twenty grams of Celite 545 were moistened with 18.0 ml. of buffer solution at pH 8.0. The buffer solution was prepared in exactly the same way as previously described (p. 22), except that only enough 5 N sodium hydroxide to bring the pH to 8.0 was used. The Celite was stirred, slurried with benzene and packed as previously described. The packing was about 35 cm. long.

This column was operated in the same manner as the one described above. Table IX summarizes the data obtained. The sample was followed immediately by benzene. After 105.5 ml., the rapidly moving colored material had been eluted through, and BB2 was started as eluant. When 117 ml. of this eluant had been introduced, BB5 was used. The most potent fractions were eluted from the column by the time 115 ml. of BB5 solution had been collected, then BB16 was used to conclude the run.

Table IX shows that of 14,850 units of activity in the starting material,

TABLE IX

Experiment 5:94. Results of Partition Chromatogram of High Potency Material Using a Buffer at pH 8.0 and Benzene-Butanol

Sample	Effluent ml.	Sample Weight mg.	Potency (x 10^{-3})	Units	Remarks
B		101	147	14,850	Starting material
1	42.5	26.3	30	789	
2	10.5	30.0	10	300	Most of color through in 1st and 2nd cuts.
3	70.0	2.5	48	120	BB2 started after 53 ml.
4	120	6.2	435	2,800	BB5 started after 100 ml.
5	35	2.9	2000	5,800	A yellow-colored band in this fraction
6	90	6.3	800	5,040	BB16 started after 60 ml.
7	100	10.4	60	624	

13,640 units were recovered in the most potent fractions, a 92% recovery.

VII. VACUUM SUBLIMATION

Early in the work of concentrating the acetate factor it was found possible to sublime it under high vacuum. The first successful experiment was run on 5 mg. of material having a potency of about 1000. Using a mercury vapor pump backed by a Cenco Hyvac pump, it was possible to get a pressure of less than 1 mm. of mercury as measured on a McCleod gage. The sample was dried in the bottom of a tube about 18 mm. in diameter. A cold-finger inside the tube extended to within about 5 mm. of the bottom, and was kept full of dry ice. A paraffin bath was used to heat the sample. At 100 ± 10° C. no sublimate was observed in two hours. Approximately 10% of the activity was sublimed in four hours while the temperature was 200° C. The residue contained about 2% of the activity.

In addition to demonstrating the ability of the factor to undergo vacuum sublimation, this experiment indicates something of its stability to heat. Thus it is seen that heating the material to 100° for 2 hours followed by heating to 200° for 4 hours did not completely destroy the activity.

As more potent materials were obtained, sublimation was tried with them. It was found that the more potent starting material gave better recovery. A sublimation carried out following drying of the sample in alkaline solution removed very little of the active factor, but took out a fairly large quantity of inactive material. Then on dissolving the sample in acid solution and drying it again, the active compound sublimed readily.

In one experiment, 50 mg. of sample 4:50.6 were used. It was introduced into the sublimation tube in alcoholic solution, and 1 ml. of 0.5 N alcoholic

sodium hydroxide was added. This was evaporated to dryness in a stream of gas. The tube was rotated to spread the solid evenly on the bottom and lower wall. At a pressure of 1 - 5 mμ of mercury it was heated to 100° ± 5° C. for three hours. This resulted in 6.6 mg. of sublimate of slightly active material.

The residue was dissolved in 0.5 ml. of 95% alcohol and 0.5 ml. of 5 N hydrochloric acid were added. This was taken to dryness as before, the pressure was reduced and the temperature raised to 100° C. After maintaining these conditions for three hours, 9.4 mg. of sublimate had been collected. It contained about half of the units of activity of the starting sample. The residue from the last operation was subjected to a temperature of 125° for two hours and yielded 9.3 mg. of sublimate containing about 5% of the activity.

It was found by other experiments that the percentage recovery could be increased by taking the residue into solution and drying it again then subjecting it to conditions for sublimation.

Because of the low recovery of active material by sublimation, and the fact that partition chromatography was successful, sublimation was not used earlier in the procedure. Also, the nature of the apparatus limits the amount of material that can be processed at one time. However, it is possible that this technique might be used successfully to further purify the material resulting from chromatography. One important fact in favor of this is that the amount of solid is reduced to a quantity which is amenable to sublimation.

DISCUSSION

Among the most important problems to be solved in connection with isolation of the acetate factor, the assay procedure may well rank first. When the work was started, the medium and procedure described seemed entirely adequate. Although there were variations, interpretation of the growth response tests was not too difficult. However, as the factor was obtained with increased potency, greater inconsistencies appeared in connection with assaying. In some cases the same sample differed in potency by two or three fold between two tests. Fortunately in most cases, this was not too serious. Usually the values were fairly consistent within a single test, so it was possible to tell which samples were most potent, and to determine the relative values. In a single assay, all of the samples which pertained to an entire experiment were tested to get significant comparison. In addition, throughout all of the testing except at the very first, the same liver extract sample was tested in every assay as a secondary standard. This served to indicate whether the entire test was consistent with previous ones on the basis of the sodium acetate standard.

The reason for the variations in response of Lactobacillus casei to the acetate factor is unknown. There has been some indication that the trouble might originate with the organism itself. The culture was handled as described under "Assay Procedure" because assays innoculated with bacteria treated in this manner were generally more satisfactory than when the innoculating organisms were treated otherwise. Possibly even more satisfactory conditions could be found.

Since more trouble was experienced with high potency material, it might be suspected that the response noted in crude extracts results from

the presence of one or more compounds, other than the one being concentrated, which stimulate growth of the organism. On the other hand, this might be an indication of a synergistic effect in which the synergist is more or less removed as the potency is increased. At various times in the concentration procedure observations were made which would tend to support both of these possibilities. There have also been indications of some inhibitory substance present in the liver extract. All of these probably contribute to the difficulty in testing.

The perfection of the assay to the point where it is as satisfactory as that of the B vitamins may necessarily be postponed until the acetate factor is obtained as a pure compound or compounds, or some of the other variables are elucidated.

In the concentration procedure for the acetate factor, the greatest loss of units of activity occurred during the first partition chromatography step. The recovery of activity in material suitable for further processing, was about 50%. This might be accounted for in several ways. First, the loss may be due to separating two or more active compounds as suggested above. Or the loss may be due to separating the active compound from a synergist, thus losing the increased effect. Perhaps the loss is merely due to incomplete separation of the active compound from the rest of the material. Probably all three of these contribute to the incomplete recovery.

To concentrate the acetate factor from a 25 - 100 pound batch of liver in a concerted process from raw liver through the second chromatography step would probably take about a month and a half. The time required would depend to some extent on the equipment available for filtration of the large volume of solution. The residue from both the liver and charcoal filtrations

is of such a nature as to make filtration slow. Very likely up to twice as much Super-Cel could be used in each step with no deleterious effect on the concentration. This should make the process much faster than it was originally.

Another time-consuming operation is the partition chromatography. Experience with this process has indicated that the scale of operation reported here is nearly optimum. It is very difficult to pack the Celite column so that horizontal bands will be formed. As might be expected, the larger the diameter of the column, the more pronounced is the irregularity. If a large batch is to be carried through this step, more than one column could be operated simultaneously. In addition to saving time, this would probably make the cuts from the various columns more nearly identical.

No batch of liver was taken through the entire concentration procedure in consecutive operations. For this reason the yield of the most potent fractions of the second partition chromatogram does not represent all that might be expected from 25 pounds of liver. While 13,640 units were obtained, making a yield of 20% of the activity contained in the first extract, as much as 25% - 30% might be obtained from a unified operation.

The flow-sheet below gives the operations with data to indicate their magitude and the results to be expected when starting with 25 pounds of liver.

FLOW - SHEET

25 lbs. hog's liver
blend with 11 l. water
autolyze
steam ½ hour, filter with Super-Cel
slurry residue with water, steam and filter 3 times
discard residue

Filtrate - 45 l.; 524 g. solids; potency 115; 60,500 units
stir ½ hour with 98 g. Darco G-60
filter with Super-Cel
discard residue

Filtrate - 70,000 units
add 235 ml. of 28% ammonium hydroxide
adjust pH to 2.7 with conc. HCl (450 ml.)
stir ½ hour with 131 g. Darco G-60
filter with Super-Cel

Filtrate
stir ½ hour with 65 g. Darco G-60
filter with Super-Cel
discard filtrate

Charcoal
While moist, extract twice by refluxing ½ hour with ethyl acetate and filtering
discard charcoal

Extract
concentrate in vacuo and dry

Sample 1 - 0.32 g.; potency 7000; 2,200 units

Charcoal
while moist, extract 4 times by refluxing ½ hour with ethyl acetate and filtering

Sample 2 - extracts 1, 2
1.32 g.; potency 44,000
60,000 units

Sample 3 - extracts 3, 4
0.43 g.; potency 18,000
8,500 units

Sample 2 - put through partition chromatogram buffered at pH 8.4

Sample 3 - put through partition chromatogram buffered at pH 8.4

Sample 1 - combine with fractions from 2 and 3 having potency of about 10,000. Put through partition chromatogram buffered at pH 8.4

Combine all fractions having potency from 20,000 - 100,000 and chromatograph at pH 8.4

Combine all samples having potency greater than 100,000 (150 mg.) and put through partition chromatogram at pH 8.0 Yield should be about 15 mg. of potency 1,200,000; 18,000 units.

[illegible]

SUMMARY

SUMMARY

1. For concentrating the acetate factor, fresh hog's liver was used as starting material, and every step in the process of concentration was developed by the use of microbiological testing which proved to be one of the most difficult features of the investigation. The initial extract was prepared by autolysis, steaming and filtering.

2. Darco G-60 was used to remove the factor from the liver extract at pH 2.7. A previous treatment with the charcoal at its natural pH did not remove appreciable activity, but increased the potency of the extract. Refluxing the charcoal with ethyl acetate while it was still moist removed the factor with almost quantitative recovery.

3. The material recovered from charcoal was further purified on a partition chromatogram, by the use of a column of Celite 545 holding water buffer solution at pH 8.4. Thiophene-free benzene and benzene-butanol solutions were used as eluants. Materials having potencies around 10^5 were obtained.

4. The most active fractions from the chromatogram at pH 8.4 were combined and put through a similar column at pH 8.0. This resulted in 15 mg. of material having a potency of about 10^6.

5. The most active single fraction obtained by chromatography was one having a potency of 2×10^6, which represents a concentration of about 3×10^5 fold over raw liver.

6. Experience indicates that vacuum sublimation may be effective as the next step in the purification.

BIBLIOGRAPHY

BIBLIOGRAPHY

1 Cheldelin, V. H. and Riggs, T. R., Arch Boichem. 10, 19 (1946)

2 Metcalf, D., Huckner, G. J. and Carpenter, D. C., J. Bact. 51, 381 (1946)

3 Kuiken, K. A., Norman, N. H., Lyman, C. M., Hale, F. and Blotter, L., J. Biol. Chem. 151, 615 (1943)

4 Shorb, Mary S., J. Biol Chem. 169, 455 (1947)

5 Wooley, D. W., J. Exp. Med. 73, 487 (1941)

6 Sprince, H., and Wooley, D. W., J. Exp. Med. 80, 213 (1944)

7 Sprince, H., and Wooley, D. W., J. Am. Chem. Soc. 67, 1734 (1945)

8 Scott, M. L., Norris, L. C. and Heuser, G. F., J. Biol. Chem. 166, 481 (1946)

9 Scott, M. L., Norris, L. C. and Heuser, G. F., J. Biol. Chem. 167, 261, (1947)

10 Daniel, L. J., Scott, M. L., Heuser, G. F. and Norris L. C., J. Biol. Chem. 174, 71 (1948)

11 Colio, L. G. and Babb, V., J. Biol. Chem. 174, 405 (1948)

12 Snell, E. E., Tatum E. L. and Peterson, W. H., J. Bact. 33, 207 (1937)

13 Guirard, B. M., Snell, E. E. and Williams, R. J., Arch. Biochem. 9, 361 (1946)

14 Guirard, B. M., Thesis: The Nutritional Role of Acetate for Lactic Acid Bacteria, The University of Texas (1945)

15 Guirard, B. M., Snell, E. E. and Williams R. J., Arch. Biochem. 9, 381 (1946)

16 Frieden, E. H., Mitchell, H. K. and Williams R. J., J. Am. Chem. Soc. 66, 269 (1944)

17 Williams, R. J. and Kirby, H., Science 107, 481 (1948)

18 Williams, Trevor I. "An Introduction to Chromatography", p. 36 Blackie and Son Ltd., London and Glasgow (1946)

19 Ramsey, L. L. and Patterson, W. I., J. Assn. Official Agr. Chem. 31, 139 (1948)

20 Peterson, M. H. and Johnson, M. J., J. Biol Chem. 174, 775 (1948)

The vita has been removed from the digitized version of this document.