A STUDY OF THE GROWTH STIMULATING PROPERTIES OF LIVER EXTRACTS, YEAST SOLUBLES AND ENZYMATIC HYDROLYSATE OF CASEIN FOLLOWING PARTITION CHROMATOGRAPHY ON PAPER

by

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Table of Contents

(Table of Contents--Continued)

ERRATA

p. 23, line 12 ; for 'tubidimetrically' read turbidimetrically

p. 23, line 17; delete sentence beginning ' These curves---' ,

insert--- These curves while showing good growth responses, are highly erratic with duplicate tubes exceeding± 5 % variability. Curves 4 and 5, representing growth from cells washed four and five times respectively show good agreement within duplicates but maximal response is less than $\frac{1}{2}$ the growth when cells wre washed once.

INTRODUCTION

The growth stimulating effect of enzymatic hydrolysates of casein, of liver extracts and Yeast solubles for certain strains of haemolytic streptococci was first observed by D. W. Woolley in 1941. When the organism was cultured in a media containing all known materials essential to the organism, slight growth was obtained until small amounts of one of the previously mentioned materials was added to the synthetic media. The growth stimulation was ascribed to the presence of unknown factors or factors which Woolley on further study concluded to be peptide in nature (see Literature Review). These peptide factors were given the name strepogenin, a term which must at this time be used only in the descriptive sense as applicable to all materials which will stimulate the growth of Streptococci and, as was later shown, Lactobacillus casei when cultured on a synthetic media under the conditions described under Procedure for Assay.

Strepogenin has been found in a wide variety of materials ranging from crystalline insulin to gelatin but by no means do all proteins demonstrate growth stimulation to an equal extent. This variation in intensity was explained on the basis on differences in concentration of the active material in the naturally occurring mixtures. But qualitative as well as quantitative differences in the growth stimulation of a number of materials were also observed and reported. These led Scott et al (21) and Stokes (24) to conclude that part of the stimulation of dried whey and yeast solubles were due to a content of glutamine plus the effect of some 'Unknown Protein Factor'. The result of their findings restricts the scope of stimulations previously ascribed to strepogenin to exclude that

due to glutamine. The problem of identifying the unknown protein factor remained unsolved.

Attempts to isolate strepogenin by Woolley and others have been successful only to the point of obtaining concentrates of high potency. No one has obtained a pure substance upon which chemical characterization could be performed or a specific method of analysis devised. However considerable data on the solubility characteristics of the factor in a variety of solvents has been reported. Among these, the high solubility of strepogenin in water and phenol was noteworthy. These facts suggested the use of some partition technique between these two solvents as a satisfactory hypothesis upon which to accomplish a separation. The intermittent countercurrent fractionation technique of Craig (82) or partition chromatography on paper, a method of Consden, Gordon and Martin (50) both offered attractive methods. The latter procedure was chosen solely on the grounds of the simplicity of the technique and the very high resolution which may be obtained through its use.

The bulk of this thesis contains a report of the application of this chromatographic technique to the problem of separating strepogenin like growth stimulating components of enzymatic hydrolysates of casein, dried Yeast Solubles and Liver extracts.

LITERATURE REVIEW OF STREPOGENIN

Early History

The term "streptogenin", later simplified to "strepogenin" was coined by D.W. Woolley (1) in 1941. He applied it to a factor or factors present in the alcohol insoluble fraction of liver extract, aqueous extracts of yeast, and rice bran, that caused stimulation of growth of certain haemolytic streptococci (American Type Culture Collection strain x 40) in a characterized media containing all known growth essentials for optimal growth of the organism. Attempts to isolate the factors from these sources were unsuccessful but furnished evidence for assuming them to be of peptide nature (2, 3). This led Sprince and Woolley to examine protein digests for growth stimulating properties and to find that crystalline proteins, particularly crystalline insulin and trypsinogen, were by far the richest sources of the factor (3). Pollack and Lindner (4) had observed the growth stimulating properties of peptone for Lactobacillus casei grown on a characterized media. Chu and Williams (5) had concluded this stimulation to be due to para-aminobenzoic acid plus glutamine plus pyridoxine. Sprince and Woolley (2) demonstrated that this peptone stimulant was similar to their streptococal factor (3). Smith (6) prepared a concentrate from yeast extract, which stimulated L. casei, with properties indicating a material similar to those of Sprince and Woolley (3) and Wright and Skeggs (7) who almost simultaneously demonstrated the presence of an L. casei stimulant in casein digests.

The inferential evidence of the peptide nature of strepogenin (3), coupled with its presence in digests of various proteins, led Woolley to examine the importance of the growth factor

in animal nutrition. He was able to show that casein and denatured crystalline trypsinogen significently increased the growth rate of mice (8) when added to a basal ration in which the nitrogen was supplied as acid-hydrolyzed casein fortified with tryptophane and cystine. His partial concentrates of strepogenin showed a similar growth stimulating effect which was entirely destroyed on acid hydrolysis. An extension of this work (9) led to the conclusion that the growth stimulating properties of proteins for mice on such a diet correlated closely with their content of strepogenin as determined microbiologically. Womack and Rose (10) were able to confirm these observations of the existence in casein of a growth factor for rats and reported the similarity of its properties to those of strepogenin.

Bosshardt (11) demonstrated the increased growth rate and increased protein utilization with mice fed a diet supplemented with Wilson's Liver Extract; and Jaffe (12) demonstrated a growth stimulating activity of Liver Extract and yeast with mice. Both these materials have been shown to have a high strepogenin potency. (9). A comparison of the growth promoting properties of casein, whole egg-powder, wheat germ, corn germ, soybean for weanling rats by Breese-Jones and Widness (13) led to the observation that these proteins promoted growth in the order stated; the same order as their relative strepogenin concentrations. An interesting and relevant observation was reported by Agren (14) who showed that the addition of cathepsin hydrolysate of casein to a stock diet did not increase the growth rate of rats but lowered the food consumption significantly.

Direct evidence for a peptide stimulating factor was

reported by Woolley (15) upon consideration of the competitive inhibition of strepogenin and a tomato-wilting agent, lycomarasmin isolated by Plattner and Clauson-Kaas (16) from Fusarium lycopersici. This wilting agent proved on later work (17) to be a peptide toxin containing glycine and an asparagine derivative, and possibly a third component of a-hydroxyalanine. A variety of peptides containing glycine and aspartic acid were synthesized (18) most of which showed inhibition of strepogenin and some tomato-wilting activity. When glutamic acid was substituted for aspartic acid, and glycylalanylglutamic and serylglycylglutamic peptides were produced, no wilting activity was obtained; but a definite, though small, growth-promoting activity similar in quality to strepogenin activity was obtained although much lower in potency than the active principle obtained from trypsin digests of crystalline insulin (19). These observations were confirmed by Krehl and Fruton (20) who investigated the larger field of bacterial utilization of peptides.

Evidence was supplied for the presence of a growth-stimulating factor for chicks similar to strepogenin by Scott, Norris and Heuser (21) in dried Brewer's Yeast. Assays of their yeast-fortified diet with L. casei for strepogenin demonstrated a linear relationship for chick activity and L. casei activity.

The heterogenous character of the growth-stimulating properties for L. casei of tryptic digests of casein and of liver fractions collectively attributed to strepogenin, became more evident with the report of Daniel, Scott, Heuser and Norris (22). These authors demonstrated that a strepogenin concentrate from casein prepared by lead precipitation of the inactive components and complete inactivation of the residue by Norit adsorption showed marked

growth-stimulating properties when quantities of antipernicious anemia concentrates of liver were added. Such growths were markedly in excess of that produced by liver concentrates alone. Later this group (23) demonstrated the presence of glutamine in the strepogenin concentrate and its almost complete removal by treatment with Norit. They were able to demonstrate the essentially similar nature of the response of L. casei to glutamine and to strepogenin concentrates earlier noted by Woolley (19), although glutamine was entirely inactive in growth response for mice (9) and hence cannot be identical with strepogenin. These same authors were able to demonstrate two additional, unidentified growth factors for L. casei in whey and alcohol soluble liver fraction. Further implications of the role of glutamine in nutrition of L. casei were demonstrated by Stokes, Koditschek, Rickes and Wood (24). These authors found evidence to indicate that asparagine, normally inhibitory for growth on L. casei, acted as a growth-stimulating factor on a strain of L. casei serially subcultured in strepogenin free medium so that strepogenin was not required. In addition, their work demonstrated the regeneration of strepogenin activity for L. casei by the addition of asparagine or glutamine after complete inactivation of a tryptic digest of casein or Liver Fraction L. by acid hydrolysis.

At the present stage, the problem of growth stimulatory materials for L. casei is far from solved. It is apparent that the assay of complex mixtures (such as Yeast extracts, Liver fraction, Casein hydrolysates) for growth activity with L. casei represents not the response to one stimulatory component but the response to several components. Results cannot be interpreted

with certainty until techniques for the fractionation of each of these sources are developed and ultimate identity of individual materials in each mixture is established. It was to this end that the technique of partition chromatography on paper was considered and undertaken since it offered one method of accomplishing the desired fractionation.

Variations in Growth Stimulations attributed to Strepogenin In Various Materials:

The details of the procedure used by Woolley, Pollack and Lindner, and by Scott, Norris and Heuser in assaying materials for growth-stimulation properties will be discussed in the experimental section. Briefly, the technique involves the culture of L. casei in an innoculum containing strepogenin, washing the cells obtained with sterile saline, and using a diluted saline suspension of these cells to innoculate two series of tubes; one containing graded amounts of standard with basal media, and the other graded amounts of unknown in basal media. Growths obtained after 16 to 20-hour incubation are measured turbidimetrically, and comparisons are made of the growth obtained in the unknown series of tubes with that obtained in the standard.

Proteins are examined after tryptic digestion for 16 hours at 37°C. and cleared of unreactive protein and polypeptides by adjustment of pH to 3.0, boiling and centrifuging. Assays are made of the resulting supernatant solution.

A growth-stimulating unit is expressed by Woolley (1,3) as the milligram amount of standard (Liver Fraction L.) per millilitre of media required to produce half-maximal growth of Lactobacillus casei. Potency of unknowns are then expressed as multiples or fractions of this unit quantity.

Table I shows the results obtained by various authors for strepogenin concentration in a variety of materials. Among these, it is noteworthy that crystalline insulin, crystalline trypsinogen, and crude casein, show the highest potencies, and that gelatin shows the lowest.

Physical and Chemical Properties of Strepogenin

All conclusions about the chemical and physical properties of strepogenin are based on the somewhat questionable evidence of response of Lactobacillus casei or of certain haemolytic streptococci to additions of the aqueous solutions of highly complex mixtures of partial protein hydrolysates or liver fractions after physical or chemical operations. The media used, in the light of current evidence, were lacking in other factors besides strepogenin; hence the properties must be viewed as inferential rather than conclusive.

Physical Properties

Adsorption: The most outstanding physical property of strepogenin is its resistance to adsorption from aqueous solution by Norit, Bentonite, activated alumina, silica gel, Fuller's Earth (17, 4). Freshly prepared barium sulphate (1, 2) showed good adsorptive qualities which like the activity of amounts of activated charcoal of the order of 300 to 500 per cent of total solids, indicated that adsorption was on the non-selective type in which the inactive solids to a level of 60% of total solids were also adsorbed. Attempts at elution of charcoal or barium sulphate with ordinary solvents did not evolve any useful elutants save that of Chattaway et al (26), an ammonia, ethanol, water mixture (12% w/v NH_3 in 50% v/v ethanol). Unfortunately, the results of these authors cannot be included in

TABLE I

Material	Potency of Growth Stimulation expressed as Wilson's Liver Fraction L units per mg. materials	Observer
Wilson's Liver Fraction L	1.0	Woolley (3)
Dried Beef Pancreas	0.5	"
Crystalline Trypsinigen	30.0	"
Crystalline Trypsin (denatured)	13.0	"
Commercial Trypsin (denatured)	10.0	"
Crystalline Chymotrypsin	16.0	"
Crystalline Ribonuclease	10.0	"
Crystalline Insulin	40.0	"
Crystalline Yeast Protein	0.6	"
Casein (Vitamin Free)	5.0	"
Gelatin	Less than 0.1.	"
Dried Brewer's Yeast	5.0	* Scott, Norris & Heuser (80)
Crude Casein	22.0	" " "
Dried Buttermilk	12.0	" " "
Purified Casein	10.0	" " "
Tryptone (Difco)	10.0	" " "
Skim Milk (Dried)	8.0	" " "
Whole Milk (Dried)	6.5	" " "
Blood Fibrin (Commercial)	6.0	" " "
Soybean Meal	5.0	" " "
Dried Whey	4.7	" " "
Lactalbumin	3.2	" " "
Dried Cereal Grasses	2.6	" " "

TABLE I (Continued)

Material	Potency of Growth Stimulation expressed as Wilson's Liver Fraction L units per mg. materials	Observer
Fish Meal	2.2	Scott, Norris & Heuser
Alfalfa Meal	1.5	" " "
Liver Meal	1.4	" " "
Dried Egg Albumin	1.2	" " "
Autoclaved Egg Albumin	0.5	" " "
Gelatin	0.05	" " "

* Values converted from expression of unit whereby Brewer's Yeast is given a value of 1000 mg./units per gm. by Scott et al, to the equivalent value in the unit used by Woolley.

this discussion since there is a strong likelihood that their test organism Lactobacillus casei (Helvetious) is a different strain from the one used by American workers.

Isoelectric Point: Studies of the migrational tendencies of the factor (peptone) by R.J. Williams (27) indicated the presence of both acidic and basic groups. The greatest concentrations were found in cathode cup when electrolysis was performed in buffers at a pH range of 3.5 to 4.5 indicating the isoelectric point to be in this range.

Dialysis: Dialysis of trypsinized casein at pH 4.5 in a closed cellophane bag against running water indicated that the factor was dialyzable with somewhat more than 40% of total potency recovered from the dialysates by this procedure. Pore size of the membrane used led Woolley and Sprince (3) to consider the molecular weight to be less than 500. The dialyzable material showed some properties differing from those of non-dialyzable residue in response to heavy metal precipitants. (See Chemical Properties).

Solubility: The material was reported as highly soluble in water at all pH values (2, 3, 4, 6, 7). A detailed list of other solvents in which the material is less soluble is shown in Table II.

Stability: The factor or factors are apparently stable in neutral or slightly acidic or alkaline pH values but show decomposition in strong acids (1, 2, 4, 6). This instability in strong acid or alkali is not as evident as was earlier supposed. Woolley reports great difficulty in decomposing his active serylglycylglutamic peptide even at 100°C. for 48 hours in 5N HCl (18). Heating to 100° C for two hours at neutrality (4) showed no decrease in potency. At values

SOLUBILITY

TABLE II

Solvent	Solubility: Insol. 0, Slightly sol. ∕, Very sol. ∕∕	Observer	Source	Comments
Butanol	0	1,2,3,4,	Trypsinized casein	With solvents, insoluble or only slightly soluble in water, most of tests of solubility were done on both the water and water saturated solvents.
Acetone	0	1, 6	Casein, Yeast	
Chloroform	0	1, 6	Casein, Yeast	
Acetic Acid	0	3,4	Casein, Peptone	
Methanol	0	3,4,6	Casein, Peptone	
Ethanol	0	2,3,4,6	Casein, Peptone, Yeast	
Pyridine	0	3,4,6	Peptone	
Aniline	∕	3,4	--	
Diethylether	0	6	Yeast	
Ethyl Acetate	0	1	Casein	
Dry Acid Ethanol	∕	1, 3	Casein	Precipitated on neutralization
ETOH ∕ n Butylamine 4:1 v/v	∕	1, 3	Casein	
Ammonium Sulphate ½ saturated	∕	1	Casein	
Ammonium Sulphate saturated	0	1	Casein	
Magnesium Sulphate saturated	∕	1	Casein	

above pH 12 a rapid destruction of potency occurs to approximately 50% which may indicate a tendency of the factory to racemize (4).

This stability in acid solution may explain the slight activity of acid hydrolysates of trypsinized casein reported by Peeler et al (23).

The solubility of the active principle from trypsinized casein in phenol was used by Woolley (3) in a partial purification of the material. In this, the dried dialysate was extracted with liquified phenol in the cold and later partitioned into dilute hydrochloric acid. Treatment of the material with phenol is reported to increase the solubility in ethanol which was formerly quite low.

CHEMICAL PROPERTIES

Reaction With Heavy Metal Cations: Attempts to form lead and barium salts (1,2,3) in alkaline medium did not effect the potency of aqueous solutions of trypsinized casein or peptone. Mercuric chloride in acid medium and silver in basic medium, however, led to the formation of an insoluble salt which was inactive but could be regenerated by treatment with hydrogen sulphide (3,4). Treatment of potent extracts (6) with alkaline copper sulphate formed inactive soluble copper salts which were again recoverable as potent material by treatment with hydrogen sulphide.

Reaction With Anionic Precipitants: Treatment of trypsinized casein or peptone with picric acid, nucleic acid or chloramine T reagent did not alter the potency of filtrates when freed of the reagents (1,3,4). Flavianic acid (4) produced an insoluble precipitate which showed some potency but was not selective. Trichloracetic acid had no effect (6), but treatment with phosphotungstic

acid yielded a solid precipitate which on decomposition in butanol: ether: water (1:1:2, v/v) led to partial recovery, although much loss of potency was encountered with use of this reagent (3,4). Preparation of the brucine salt was accomplished (4), but the regenerated product had little potency.

Acetylation: Treatment of highly potent concentrates with acetic anhydride led to a large reduction in potency of this product. Only 18% of prior activity was recoverable (3).

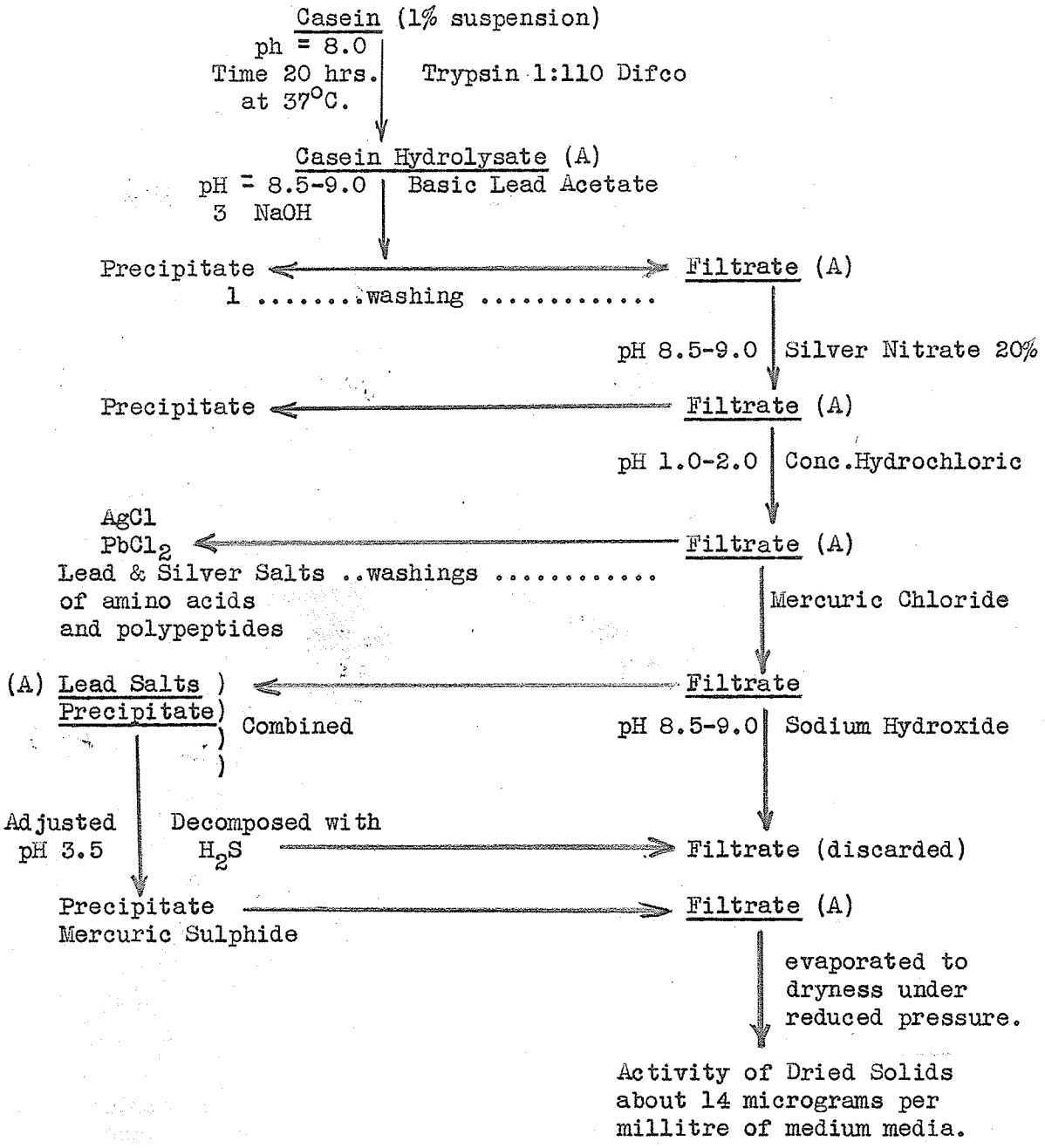
Ninhydrin: Treatment of concentrates with ninhydrin had little effect on potency at room temperature but at 100°C. for one hour, potency of concentrates from casein was reduced about 50%.

Nitrous Acid: Nitrous acid was first reported to have no effect on the potency of trypsinized casein or peptone (1,2) but was later shown to effectively reduce the potency to negligible value when allowed to react with the concentrates (3). Apparently, a free amino group is necessary for activity.

Methods of Concentration: Woolley reports two procedures (3) by which a concentration of the growth-stimulating factor of Liver Fraction L was obtained. These procedures are outlined in Tables 3 and 4. It is, however, very difficult to interpret these concentration procedures in the light of the present information which indicates that the stimulation of Liver Fractions may be due to a number of compounds rather than one single factor. If this concept is adopted, it would be necessary for phenol extraction or heavy metal precipitation techniques to achieve simultaneous concentration of all growth stimulants of the liver extract. Although these procedures did not yield a product sufficiently pure for chemical characterization, they were successful in achieving a concentration

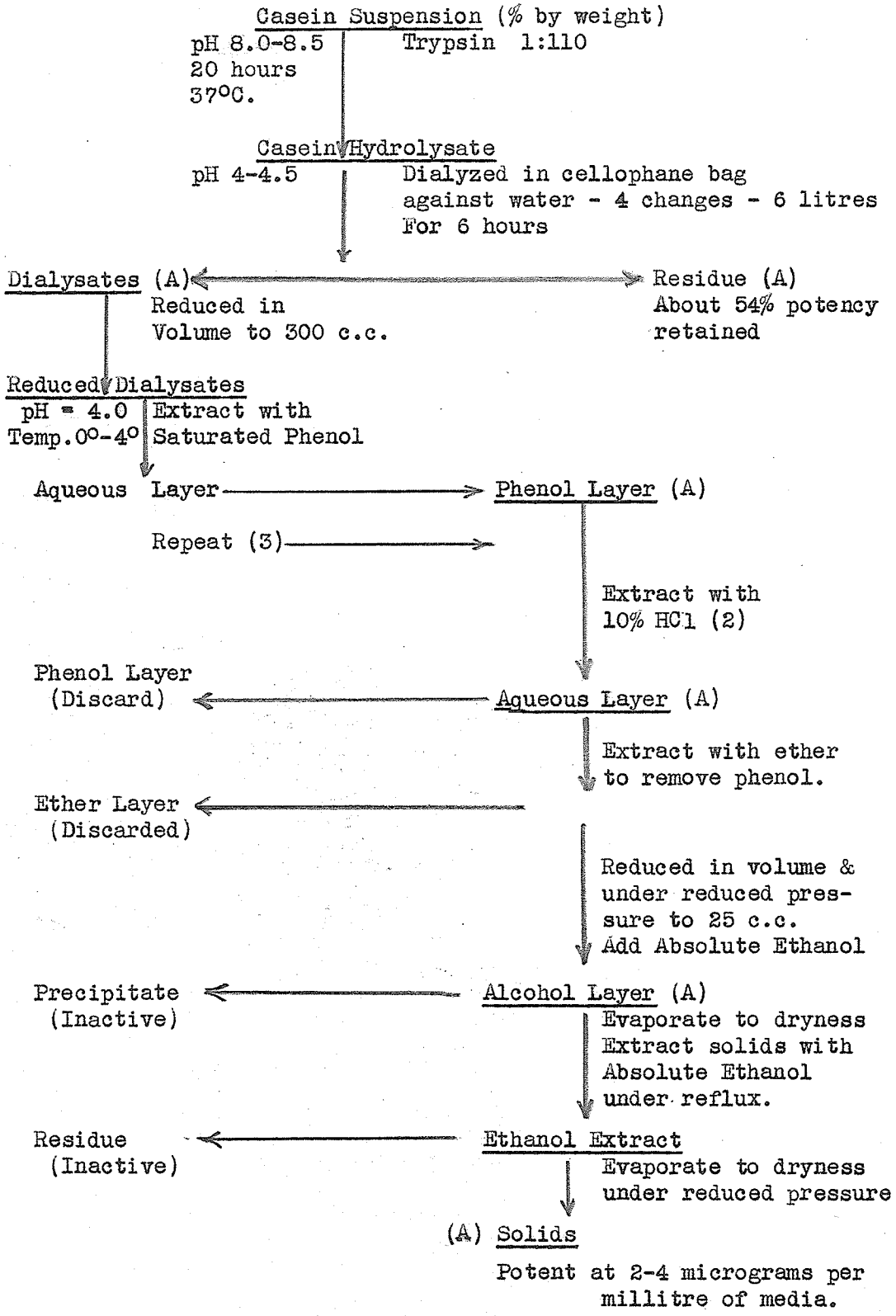
METHODS OF CONCENTRATION (3) TABLE III

HEAVY METAL PROCEDURE:



(A) Indicates Active Solutions.

PHENOL METHOD: TABLE IV



(A) Active

of some active component since Woolley reports (3) the final products to be potent at a concentration of 10 micrograms per millilitre of media.

REPORT OF EXAMINATION OF ASSAY PROCEDURE

Method of Assay

The procedure of determining the potency of growth-stimulating properties of the various materials used is similar in most vitamins or amino acids (28,29,30). The method depends upon the assumption that the growth requirements of the microorganism used are well known. Then, in a media devised to be deficient in a single growth essential, response of the organism to additions of that substance will be proportional to the concentration of the substance added although by no means will that response be a linear function of the concentration of the addend particularly when the concentration of the substance is increased to the point of eliciting a maximal growth response of the organism. In this problem all known growth essentials are supplied in a characterized basal media on which Lactobacillus casei will grow but not at maximal rate. More than 72 hours is required to obtain a good growth response on a media lacking strepogenin while maximal growths are obtained in the presence of strepogenin in 20-22 hours (2). Thus, if growth responses are measured in 20-22 hours, variations in response to concentration of sources of strepogenin when co-related with a standard response curve can be used to obtain a measure of the relative concentration of strepogenin in the mixture. Thus, except for the importance of the time of growth, the procedure of assay is analogous to most other microbiological assay procedures.

Test Organism Used

Lactobacillus casei No. 7469 American Type Culture Collection was obtained from Georgetown University Medical School, Washington, D.C., and used exclusively in this work.

Maintenance of Test Organism

The method used was that of Scott, Norris and Heuser (21) in which the organisms were grown in a broth consisting of 10 ml. of casein basal media, 50 mg. of charcoal treated Difco Bacto-Peptone * and 20 mg. of steamed Yeast solubles**.

The organisms were grown for 24 hours in this broth at 37°C. Loop transfers of the well-shaken broth cultures were made to sterile agar media in cotton plugged tubes and grown in stab for 24 hours at 37°C. Tubes demonstrating good growth along the stab line were stored at 5°C. for assay use. Because of the demonstrated alteration of the organism on long storage (31), no culture stored longer than 30 days was used in an assay but the entire store replenished with fresh organisms at this time.

* Prepared by suspending 10 gm. of Difco Bacto-Peptone in 100 ml. of water and adding 10 gm. of Norit. This mixture was stirred for one-half hour and filtered. The filtrate was diluted to one litre with water and preserved under toluene. 5 ml. of this stock solution was used in the preparation of broth.

** Prepared by dissolving 2.0 mg. Difco Dried Yeast Solubles in 100 ml. of distilled water and autoclaving for 15 minutes at 15 lbs. Stored in cotton-plugged flask. 1.0 ml. of this solution was used in broth.

For an assay, a loop transfer was made from the agar**** stab to sterile innoculation***** in a 40 ml. centrifuge tube, all subsequent operations being performed in this tube. The stab was used once and then discarded to avoid danger of contamination. The innoculum was incubated for 20 hours at 37°C and then the cells packed by centrifuging at 2000 r.p.m.

Washing Cells

After centrifugal packing, the cells were washed free of innoculum by suspension and centrifuging in equal volumes of sterile saline (0.85%)

*** Agar stabs were prepared by warming and shaking the following components:

Difco Bacto Peptone	1.0 gm.	Water to make 100 ml.
Sodium Carbonate	0.5 gm.	10 ml. of melted solution poured
Agar (Difco White, Special)	1.5 gm.	into cleaned test tubes plugged
Yeast Solubles	1.0 gm.	and autoclaved for 15 minutes
Glucose	1.0 gm.	at 15 lbs. pressure.

**** Innoculum was prepared by using 10 ml. of casein basal medium, 20 mg. of Difco Yeast Solubles and 2.0 mg. of Liver Fraction L. (Nutritional Biochemicals). Preparation was steamed for 10 minutes and allowed to cool to room temperature before innoculating.

Method of Assay Tube Innoculation

The degree of dilution of the washed cells using one drop of final suspension to innoculate one assay tube determines the number of organisms used with each assay. Since the extent of growth depends upon the number of organisms used, there was shown an optional dilution factor which produced a balance between the desired extended range of growths with increasing concentrations of factor and an adequate number of organisms present to produce optimal growth in assay tubes in the incubation time used. It has also been shown that growth characteristics vary when a heavy innoculum is used (45). From cell dilution experiments (see results of Media Test), it was found that a dilution to a galvanometer deflection of 90 in an Evelyn Colorimeter using a 620 mu. filter (31) against saline control, approached this optimal value.

After dilution, the cells were transferred to a sterile 20 ml. syringe fitted with a No. 22 needle, supported vertically. A steady rate of dropping of about 10 drops per minute was obtained with this simple assembly. One drop of this suspension was used for each assay tube. Care was taken to ensure the drop was deposited into the medium without touching the sides of the tube.

Measurement of Bacterial Growth

Several methods of measuring bacterial growth were considered including total nitrogen determination of cells by the method of Mueller (32) and William (33), the method of volume of packed cells of Schmidt and Fischer (34), but both were superseded by the opacity procedure of Mestre (35). In this, the turbidity of the culture is determined with a photoelectric colorimeter fitted with a 620 mu. filter (31). The method of determination

of optical density of a culture suffers from the numerous inherent errors of turbidity measurements (36). The chief difficulty in measuring bacterial growth by this procedure lies inthe tendency of the rapidly growing cells to sediment hence making opacity determinations variable. This error was minimized by thorough shaking before taking a reading. The turbidity procedure was chosen instead of direct titration with standard base of Feeney, Strong and Earle (37) partially because Chattaway et al (38) have reported several instances of lack of correlation between growth and acid production and because serious doubts of the procedure when applied to bacterial growth under the somewhat rigorous conditions of the assay have been raised by other authors (39). This procedure does not lend itself to extended observation of rate of bacterial increase in a single assay tube as periods of incubation are increased to 80 hours and was not used for that reason.

Basal Media

The composition of the media used is shown in Table V. It is essentially that of Landy and Dicken (4) as modified by Sprince and Woolley (2) to increase the concentration of manganese and corresponds closely to that of Pollack and Lindner (41) if the amino acid concentration of casein as determined by Block and Bolling (46) is considered. The media is, however, somewhat simplified in accordance with the finding of Dunn, Shankman, Camien and Block (43,44,45) with the result that inositol and choline, originally considered growth stimulating but shown by Dunn et al (44) to be neither essential to long term or short term growth, are omitted. Another change resulted from the inability to obtain "complete"

COMPOSITION OF BASAL MEDIA FINAL DILUTION TABLE V

Ingredient	Concentration
Glucose	1.0%
Sodium Acetate anhydrous	0.6%
Casein Hydrolysate	0.5%
Cystine	0.02%
l-tryptophan	0.005%
Adenine Sulphate	10 micrograms/ml.
Guanine Hydrochloride	10 micrograms/ml.
Uracil	10 micrograms/ml.
Xanthine	10 micrograms/ml.
Riboflavin	0.2 micrograms/ml.
Thiamine Hydrochloride	0.1 micrograms/ml.
d-calcium pantothenate	0.1 micrograms/ml.
Pyridoxine Hydrochloride	0.1 micrograms/ml.
p-aminobenzoic acid	0.1 micrograms/ml.
Biotin	0.4 milli micrograms/ml.
Nicotinic acid	0.4 milli micrograms/ml.
Folic acid	0.02 milli micrograms/ml.
Salt Solution A.	10 ml/500 ml media
* Salt Solution B.	10 ml/500 ml media
# Water (glass distilled)	to 1000 ml

* Salt Solution A.
K_2HPO_4 - 25 gm.
K H_2PO_4 - 25 gm.
Water - 500 ml.

\# Salt Solution B.
$MgSO_4 - 7H_2O$ - 10.0 gm.
$FeSO_4 - 7H_2O$ - 0.5 gm.
$MnSO_4 - 4H_2O$ - 0.5 gm.
Water - 500 ml.

hydrolysis of casein by the sulphuric acid procedure advocated by Landy and Dicken following the procedure of Sahyun (42). The procedure adopted consisted of hydrolysis for 72 hours in 5N hydrochloric acid under reflux and removal of acid by vacuum distillation. It was found that this procedure left little growth-stimulating properties to the casein hydrolysate even at the 72-hour incubation.

Preparation of Standard Curves

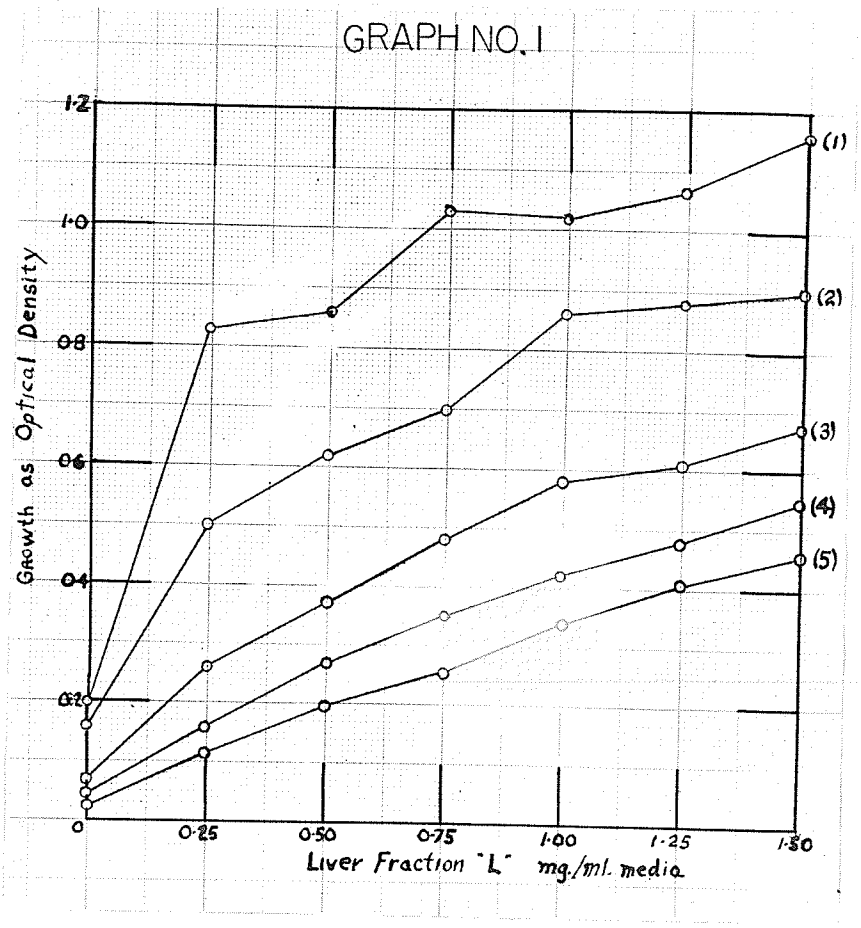
Basal Media was prepared in stock solutions at double the concentration shown in Table V and a 5.0 ml. volume dispensed into each of ten colorimeter tubes. Volumes of standard Wilson's Liver Fraction L. or Yeast solubles were then added in 0.5 ml. increments up to 5 ml. and the volume in each tube made up to 10.0 ml. with glass distilled water. The standard series was, unless otherwise stated, prepared in duplicate with control tubes included in each series. Control tubes consisted of: (1) Innoculated tubes containing basal media only: (2) Non-Innoculated controls containing basal media only: and (3) innoculated tubes of basal media and maximal concentration of standard for adjustment of Evelyn Colorimeter to zero optical density.

The tubes were innoculated with one drop of saline suspension of washed cells and allowed to grow at 37°C. for 20 hours. At the conclusion of the incubation period, the tubes were read in an Evelyn Colorimeter to determine the turbidity. The resulting transmission values were plotted (as optical densities) against the concentration of Liver Fraction L used in each tube. Examples of such curves are shown in Graph #3.

Tests on Basal Media

Effect of Cell Washing: Innoculum was prepared, incubated for 22 hours at 37°C. and cells separated by centrifuging and decantation of broth. The cells were washed by suspension with shaking in a volume of sterile saline equivalent to original broth volume and centrifuged free with resuspension in saline. One ml. of this suspension was removed and diluted to give a galvanometer deflection of 90. The diluted cells were then used to innoculate a duplicate series of 10 standard tubes containing from 0 to 2.0 mg. of Wilson's Liver Fraction L. per ml. of media. In all, six washings were performed and six duplicate standard series prepared and incubated with control tubes for 22 hours at 37°C. Growth was determined tubidometrically.

The results of this test are shown in Graph No. 1 in which growth is plotted as optical density of the culture against concentrations of Liver Fraction L. Curves 1 and 2 represent growths with an innocula of cells washed once and twice respectively. These curves, while showing high growth responses (maximal densities in excess of 1.0), also are highly erratic in response with duplicate tubes far exceeding ± 5% but the maximum density was less than one-half that of growths when cells were washed once. Since both small variability and high maximal response are desirable conditions for the assay, a compromise had to be accepted at the degree of cell washing which gave the highest maximal response simultaneously with a low variability in duplicate tubes. This optimal condition was reached with thrice washed cells (Curve 3) in which the maximal response was of the order of 0.6 with the variability within ± 5%.

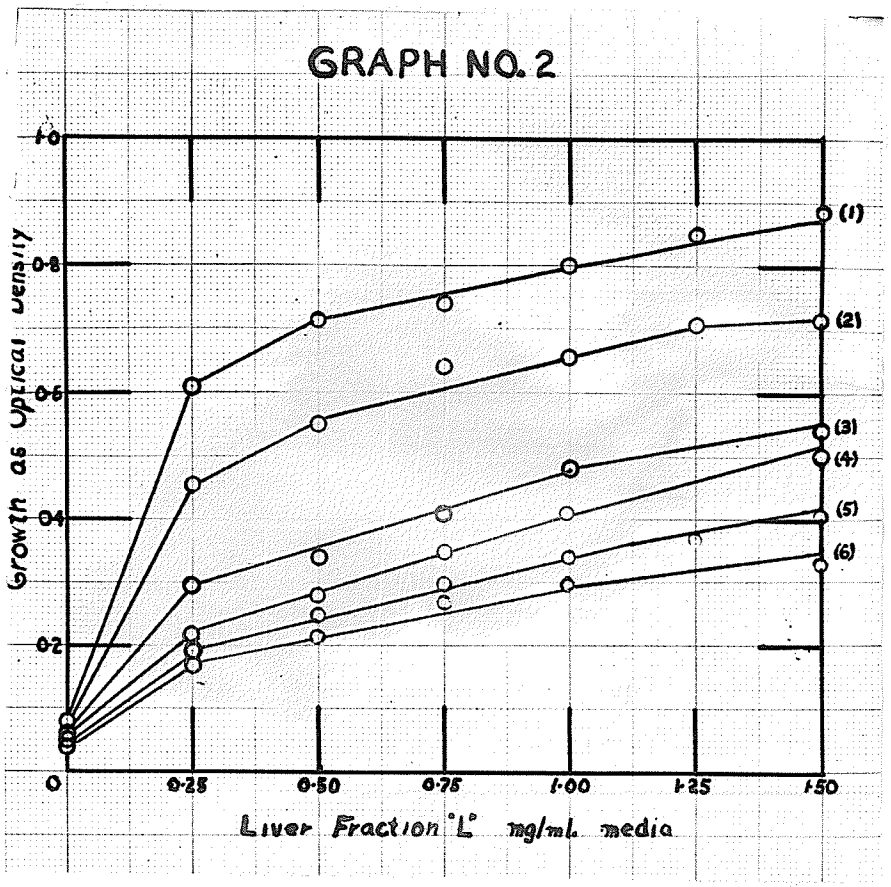


Response of L. casei to concentrations of Liver Fraction L. Curves 1 - 5 represent responses obtained when inoculating cells are washed from 1 to 5 times respectively with saline.

Effect of Cell Dilution

Innoculum was prepared, incubated for 22 hours and washed in saline three times. Aliquots were then taken and diluted 1:10, 1:20, 1:30, 1:40, 1:50 and 1:60 with sterile saline and used to innoculate a standard series of tubes containing media and graded amounts of Wilson's Liver Fraction L.--growth measured turbidometrically after 20 hours' incubations. Results are shown in Graph 2. In this graph, curves 1 and 2 represent responses obtained with concentration of Liver Fraction L (as shown) when the innoculating cells were washed but diluted 1:10 and 1:20 respectively. With these dilutions, while maximal responses are high, and variability with ±5%, there is only a small increase in optical density with large increments of Liver Fraction L. With curves 5 and 6 representing dilutions of 1:50 and 1:60 respectively, while a more acceptable range of growths with increased concentrations of Liver Fraction L is obtained, the response to the highest concentration of Liver is considerably reduced. For the assay, the condition of as extensive a range of optical densities with increasing concentrations of Liver as is possible is the most desirable condition providing the condition is satisfied simultaneously with large amounts of growth.

But curves 1, 2, and 5, 6 indicate that these conditions are mutually exclusive. Therefore, a compromise had to be accepted at a cell dilutions of 1:30 or 1:40 (curves 3 and 4) whose maximal responses are admittedly low, but whose range of optical densities with increasing concentration of Liver Fraction L demonstrate a desirable gradient.



Response of L. casei to concentrations of Liver Fraction L. Curves 1 - 6 represent responses obtained when innoculating cells are diluted 1:10 and 1:60 respectively with saline.

The 1:40 dilution was chosen as optimal cell dilution. The density of this saline suspension corresponding to a galvanometer reading of 90 when saline is used as a blank with a 620 mu. filter.

Effect of Increase of Media Components: Tubes were prepared in quadriplicate consisting of: (1) 5.0 ml. basal media;

(2) $\frac{1}{2}$-maximal concentrations of Liver Fraction L-i.e. 0.75 mg. ml.

(3) aqueous solutions of amino acids.

The amino acid solutions were prepared at concentrations such that addition to the media of 1.0 ml of the solution yielded a media which had double the concentration of that amino acid. Hence by preparing tubes with 1.0 ml., 2.0 ml., or 3.0 ml. of those solutions media could be obtained having 2X, 3X or 4X the basal media concentrations of that amino acid. A control tube was prepared at normal concentration of amino acids and growth in the fortified tubes compared with this control. Variation in growth with the amino acids examined are reported in Table VI as the average optical density of the quadriplicate values. The percentage increase or decrease in optical density with each concentration which should reflect the tendency of the individual additions to promote or inhibit growth is calculated and listed for convenient comparison only.

Examination of these results led to the following conclusions:

(1) With all amino acids save Leucine, serine, aspartic acid, glutamine, and glutamic acid, variations in growth with increasing concentrations are not sufficiently different from the control to warrant any conclusion other than they do not exert any effect on the growth of L. casei.

(2) With serine and glutamic acids, a tendency to promote growth in the concentrations studied is evident. This is in agreement with the observations of Woolley (18).

(3) The pronounced growth stimulation of glutamine (added to the media after Seitz filtration) is in accord with the observations of Scott, Norris and Heuser (22) and Woolley (19). The inhibtion shown by asparagine agrees with the results of Rickes, Koch and Wood (25).

(4) The inhibitory effect of high concentrations of leucine has not been previously reported.

This series of experiments was undertaken to assess what effect increases in the amino acid concentration would have on the growth of L. casei in the knowledge that all sources of strepogenin examined have appreciable concentrations of free amino acids present. Particularly is this true of Liver Fraction L which has been shown by Tishkoff et al (47) to contain significant amounts of free valine, phenylalanine, leucine and tyrosine as well as lower concentrations of histidine, alanine, glycine and glutamic acids. Since this work shows that certain amino acids may of themselves exert stimulating or inhibiting effects, assessment of the growth stimulation of liver extracts containing unknown growth factors as well as free amino acids, should be made under conditions whereby the effects of the amino acids may be applied to the results or under conditions where these amino acids are removed from the assay. The latter condition is met in the assay of Strepogenin following paper chromatography.

Standard Growth--Concentration Relationships

Standard curves utilizing Wilson's Liver Fraction L. Difco Yeast Solubles and

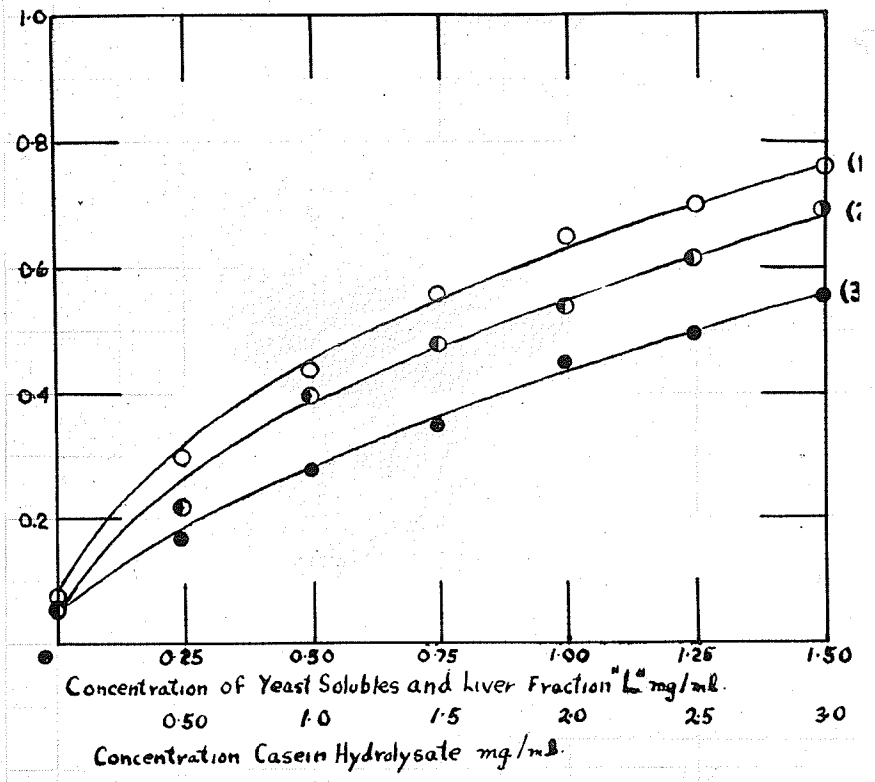
TABLE VI

Media with 1.0 mg/ml Wilson's Liver Fraction L. Supplement Amino Acids	Optical Density						
	Without Supplement	2X	%	3X	%	4X	%
Valine	.50	.49	-2	.49	-2	.48	-4
Tryptophane	.51	.51	0	.51	0	.52	+2
Leucine	.46	.46	0	.35	-24	.32	-31
⁂ Asparagine ⁂	.46	.46	0	.43	-6	.40	-12
Serine	.46	.52	+12	.51	+10	.42	-8
Alanine	.46	.45	-2	.47	+2	.45	-2
Isoleucine	.46	.48	+4	.47	+2	.48	+4
Aspartic	.46	.56	+21	.57	+24	.32	-31
Glycine	.47	.45	-4	.47	0	.46	-2
Arginine	.46	.45	-2	.46	0	.47	+2
Methionine	.46	.46	0	.48	+4	.50	+8
Histidine	.46	.45	-2	.44	-4	.45	-2
Threonine	.45	.45	0	.45	0	.46	+2
l-Proline	.43	.42	2	.45	+4	.45	+4
l-Lysine	.45	.42	-6	.44	-2	.44	-2
l-Hydroxyproline	.45	.42	-6	.44	-2	.42	-6
# Glutamine #	.45	.51	+13	.65	+40	0.95	+100
Glutamic	.46	.49	+6	.55	+19	0.65	+40

⁂ 0.5 mg/ml added

0.01 mg/ml added

GRAPH NO.3



Typical response curves for L. casei to concentrations of Yeast (1) Liver Fraction L. (2) and Tryptic Digest of Casein (3)

enzymatic hydrolysate of casein as sources of strepogenin were prepared frequently but are not cumulative in nature for presentation as composite curves, embodying the mean values of all the runs attempted. However, since these curves exhibited characteristic differences from one another which ~~was~~ were considered significant and since the individual character of a curve with any one of these sources was reproducible at will, the curves are shown in Graph 3 for one such comparison and will serve to illustrate.

The reasons for the variation of these curves are difficult to determine with any certainty. Possibly the differences may be due to the difference in concentration of other ingredients than strepogenin in these complex mixtures or to a difference in the nature of the growth-stimulating materials in each of these sources. However, the use of Yeast solubles as a reference standard rather than Liver Fraction L. is suggested by these curves because of the much greater range of optical densities relating growth and concentration obtained from the lowest effective concentration to the concentration electing maximal response. It would seem to indicate this range of growth is due to an absence of inhibitory substances present in casein hydrolysate or Liver Fraction L. It was thus decided to use Difco Yeast Solubles as a reference standard.

EFFECT OF LYOPHILIZATION OF LACTOBACILLUS CASEI

In order to avoid the repetitious work of carrying L. casei culture in agar stab, an attempt was made to prepare lyophilized cells of L. casei. The procedure used was basically that of Nyman, Gunsulus and Gortner (48) in which cells were grown in nutritive broth for 24 hours at 37° C. The cells were then centrifuged free of broth and suspended in 1% Gelatin solution of equal volume to the original innoculum. 0.1 ml.

portion of this solution was then transferred to sterile 7 x 30 mm. tubes and dried over anhydrous calcium sulphate at high vacuum for 24 hours. At the end of this period, the cotton plug was pushed half way down the tube, a crystal of anhydrous silicic acid placed above the plug and the tube sealed in an oxygen flame. For use, the tubes were cut below the cotton plug and one drop of sterile medium added before loop transfer to innoculum.

It was found that cells lyophilized by this procedure had altered their characteristics from the parent culture. The treated cells grew profusely and rapidly in basal media entirely lacking a source of strepogenin in 16 hours whereas cells from the parent culture showed no growth in this period. The addition of Liver Fraction L. to the basal media had no detectable effect of the growth of lyophilized cells.

This reaction of lyophilized cells while sufficient to render the technique useless for the assay of strepogenin, was sufficiently marked to arouse some speculations on the explanation of the phenomena.

The current concept of the nature of any growth factor in microbial nutrition is the dynamic concept expressed by B.C.J.G. Knight (49) as follows: (quote)

".....a substance required as a component of one of the essential metabolic processes, which might appear in three different roles as a component of the nutrients. It might appear (1) as an essential nutrient when its rate of synthesis by the cell was so slow as to be insignificant; (2) as a growth stimulant when its rate of synthesis was somewhat faster but still slow enough to be a limiting factor or (3) as a substance not required at all for nutrition

because the cell could synthesize it so fast that it was not a limiting factor in growth."

In the strepogenin problem, the growth factor would seem to be of the type described in (2) of Knight's definition. L. casei (non-lyophilized) will grow in the basal media used in this work but at a much reduced rate such that maximal growth is only obtained at 72 hours incubation. In the presence of strepogenin, this maximal growth is obtained in from 16 to 20 hours. Thus the growth of the organism in the absence of strepogenin is determined by the ability of the organism to synthesize the material but with an exogenous source of the material a much more rapid rate of growth is possible. On this concept, the lyophilized cells, growing in the absence of exogenous strepogenin, may have been altered by the technique such that (a) either these cells no longer require strepogenin, i.e. this substance would no longer be a component of an essential metabolic pathway or (b) it remains an essential metabolic component but one which is now being synthesized by the organism at a sufficiently rapid rate that exogenous strepogenin is no longer a limiting factor of growth. If the former alternative were the case, the lyophilized cells grown in absence of strepogenin should not show evidence of the presence of strepogenin within these cells.

A large volume (250 ml) of basal media was prepared and innoculated with lyophilized cells. No strepogenin source was added. This culture was incubated for 24 hours and the cells collected by centrifuging. The washed cells were then ground in a mortar with fine carborundum powder under distilled water. No effort was made to hydrolyse the cells protein; but merely to effect an aqueous extract of the cell contents. The extract, after filtration, was autoclaved at

15 lbs. for 20 minutes. This sterile extract was tested for potency by adding it to basal media and innoculating with L. casei cells which had not been lyophilized. These cells showed no growth on basal media alone in 20 hours but showed excellent growth in the tubes to which the extract of lyophilized cells had been added.

From this, it was concluded that the lyophilized cells contained strepogenin. The alternative explanation of the non-dependency of these cells on exogenous sources of strepogenin cannot be explained on the basis that strepogenin was no longer a component of a metabolic pathway. The presence of strepogenin within the cell envelope can have arisen then only by the processes of synthesis, a reaction which the unlyophilized cells can perform but which in the lyophilized organism can be performed at a much more rapid rate; a rate which is now sufficiently rapid as to make the cell independent of exogenous supplies of the factor.

SEPARATION OF PARTITION CHROMATOGRAPHY

Review of Literature:

Partition chromatography on paper is a technique developed by Consden, Gordon and Martin (50,51,52,53) as an extension of their original process developed from the theory of Fenske and Cannon (55,56,57) on the separation of components of complex mixtures by virtue of their differences in partition coefficient between immiscible solvents. Martin and Synge simplified the process of Varteressian et al wherein fractionation was accomplished at equilibrium positions from two counter moving solvents, to a process whereby one solvent was immobilized on silica gel (54), the second solvent moving at a steady rate across this immobilized phase. Because of interference by adsorptive properties of silica gel, starch columns and later cellulose columns having little adsorptive properties for amino acids were used by Consden, Gordon and Martin; finally filter-paper alone was used as the support for the non-mobile phase. In this technique amino acid and peptide mixtures are placed near the top end of a strip of filter paper suspended in a gas-tight box. Solvent (phenol, butanol or collidine) saturated with water is allowed to run by capillary action along the paper, the whole exposed to an atmosphere saturated with water and the respective solvent used. As elution proceeds, the paper absorbs water from the atmosphere. The solution of amino acids running over this paper is dispersed by virtue of the fact that some of the components are more soluble in water than in the solvent used, hence transfer quickly to the aqueous phase. Others, less soluble in water, tend to require a longer run for complete transference to the aqueous phase. However,

a stage is reached when the solvent front has advanced a suitable distance (S), when the individual amino acids as shown by ninhydrin development take up representative and reproducible positions (C_f) with respect to one another and with respect to the position of the solvent front. This position is measured from the base line. The ratio of these two distance $\left(\frac{C_f}{S} = R_f\right)$ in the nomenclature of Consden et al is distinctive for a given material and can be used to identify the materials.

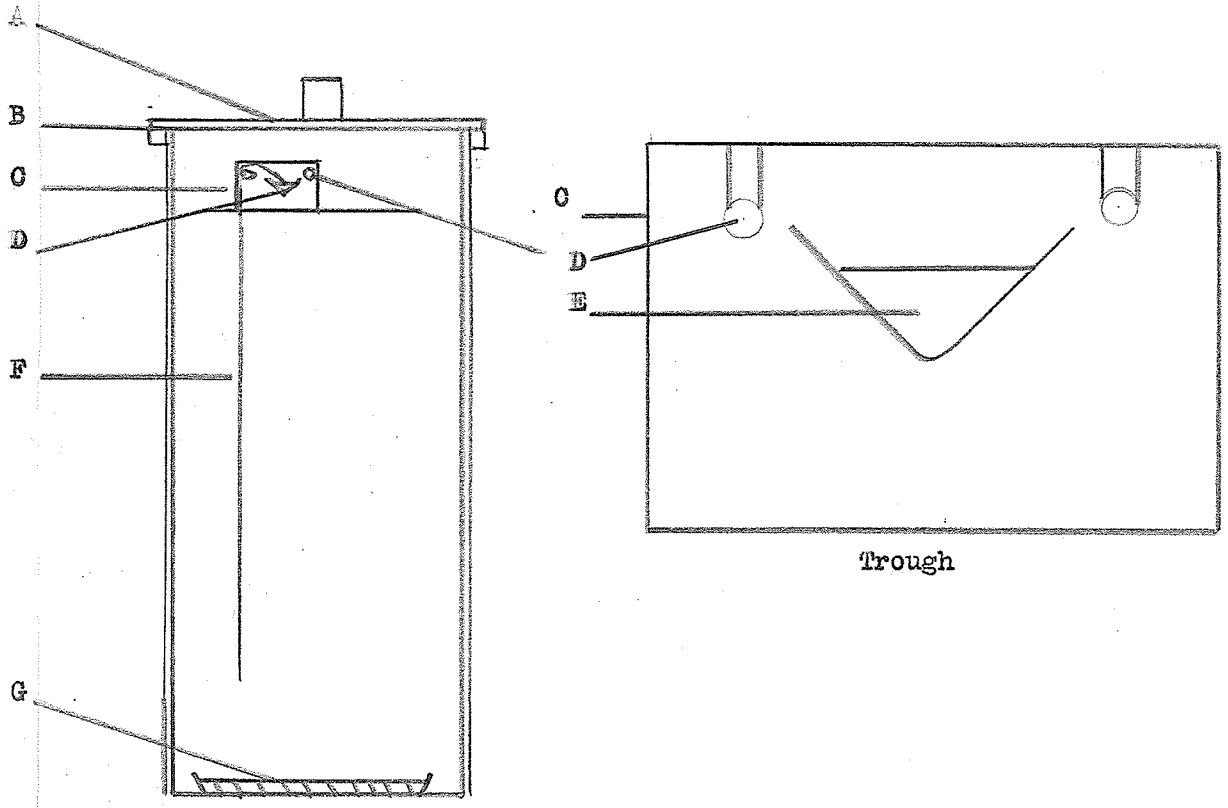
The R_f values of the same amino acid can vary with the solvent used and the method of operating the chromatogram, i.e., either by descending movement of solvent of Consden and Dent (50,60) or the ascending technique of Williams and Kirby (58). But with the same technique, in the same solvent, R_f values are highly reproducible. However, the technique will separate materials only when a difference in partition coefficient exists, hence those materials having the same coefficient will occupy the same position in a chromatogram. Here, the two-dimension technique of Consden (59) later developed by Dent (60) whereby a given mixture is eluted in one direction along a square of filter paper by one solvent, then at right angles by a second solvent supplied a means of separating groups of amino acids in this category.

The excellent reviews of Consden (61) and Dent (60) supply a fund of information on both the theoretical and practical aspects of this technique. The extension of the technique to the separation of sugars (62,63,64,65), flavines (66), purines (67), organic acids (68), keto acids (69), urea and uric acids (70), Keto-steroids (71), adrenaline (72), creatine and creatinine (73), the constituents of normal and pathological urines (74), composition

of gramicidin S. and components of crude penicillin (75) soon followed. The use of the technique in a step-wise procedure to identify the components of partial hydrolysates of wool (52) led to the hypothesis that the components of such complex mixtures as Liver Extract L., Yeast solubles and Trypsinized casein, might be similarly fractioned into growth-stimulating and inert components. It might, as well, indicate the number of such growth-stimulating fractions in each of these crude materials and indicate something of the essential similarity of the growth-stimulating materials in each source by the similarity of R_f value. The technique, too, might profitably be used to establish the peptide nature of such materials and to indicate the composition of these growth-stimulating peptides (if such) by identifying the constituent amino acids after hydrolysis.

Apparatus: Two chromatographic chambers 21" x 21" x 11" inside dimensions of plywood were constructed for elution with phenol and collidine. Both were fitted with rubber-sealed, tight-fitting lids clamped by screw clamps. The whole interior of the assembly was heavily coated with paraffin. Troughs 1" x 1" x 20" were constructed of sheet metal with provision for glass rods on each side of the trough for support of the paper during downward elutions. These troughs were heavily paraffined and cleaned up monthly with heating and benzol, for recoating. The paper was held in the trough by a 1/4" support rod of glass. The assembly is shown in Diagram 1. The two assemblies plus racks for equilibrated solutions were retained in an insulated constant temperature cabinet maintained at 21 ± 2°C. by means of an electric heater, fan and mercury thermoregulator. Conditions for maximal temperature control required that the relative

DIAGRAM I



Section through chromatographic chamber and trough

A - Lid
B - Rubber seal
C - Trough of galvanized metal - paraffin coated
D - Glass support rods
E - Elution Solvent
F - Filter paper
G - Equilibration solvent

humidity of the cabinet be maintained as high as possible which was accomplished by open evaporation of large surface of water in front of circulating fan. During runs temperature was recorded on a bimetallic recording thermometer.

Paper: The filter paper found most useful in this work was Schleicher and Schuel #595, a thin paper but sufficiently strong when wet to be handled without tearing. The paper gave much better resolution, less lateral dispersion and more consistent R_f values in a much shorter period of elution than the Whatman #1 paper recommended by Consden and Dent. It was obtained in 20" x 20" sheets, and cut to 10" x 17½" for one way chromatograms, 17½" x 17½" for 2-way studies. Care was taken to see that all sheets were cut in the same direction of the "grain" of the paper. For use a base line 2-1/4" from one edge was marked in soft lead pencil (HB) and marks for a fold at 1-3/4" made. Stocks of paper were maintained in a moist atmosphere and, if dry stock was used, 4-6 hours' saturation of the paper in the chamber allowed before run was commenced.

Solvents: British Drug House phenol, reagent grade, was found satisfactory for runs without any purification. Distillation of the phenol over zinc dust produced a product (m.p. 41.5°C.) which gave no better results than the commercial product. No serious staining of the S & S paper resulted if the phenol used was freshly prepared. For use 100 gms. phenol was placed in a mortar and ground with 15 ml. of water until one phase existed. This was then poured into a separating funnel, an equal volume of water added, shaken and placed in constant temperature cabinet to equilibrate. This proceeded for 48-72 hours with intermittent shaking. Saturated phenol was stored

in contact with its aqueous phase for use. Some tendency of the phenol to turn pink on long storage (3 weeks) was noted but seldom did the small quantities last longer than 1-2 weeks in which period no discoloration was noticed.

"Collidine" solution was prepared by shaking at constant temperature a mixture of 2,4,6 - collidine (1 vol.); 2,4-lutidine (1 vol.) with two volumes of water and allowing 48-72 hours for complete equilibration.

Reagents: Ninhydrin reagent was prepared by dissolving 250 mgms. of Eastman Kodak tri-ketohydrindine hydrate in 100 ml. of water -saturated butanol (Merck). Only fresh samples of ninhydrin were used. Solution was sprayed on paper evenly but lightly to avoid flooding with a spray. No advantage was found in mixing the reagent in saturated butanol/brine (58) or in butanol/10% acetic acid (53).

General Technique: Five microlitres of amino acid or hydrolysate solution were placed on base line. Spots applied should not exceed a diameter of 5.0 mm. This spot was dried over a hot plate care being taken to avoid decomposition by too rapid heating. Concentration of the material under test could be increased by multiple applications of test solution with subsequent drying but in general the technique was avoided since it led to a decrease in resolution. In general, amino acids were run at 20-30 micrograms quantities although much less is adequate for detection. Hydrolysates, Liver Fraction L. and Yeast Solubles were all run in 200 microgram amounts.

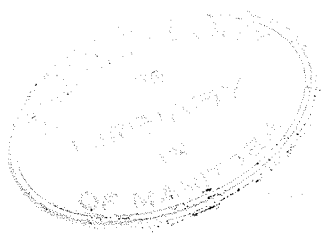
After drying, sheets were folded and placed in trough containing solvent, with the duplicate sheet occupying the opposing side of the same trough. Lid greased with vaseline clamped tight and elution allowed to proceed for 18-20 hours (phenol), 20-24 hours

(collidine). At the end of this period, sheets were removed, solvent front marked, and dried in a fume cupboard over a hot plate in a current of air. All handling of sheets was done with rubber gloves to avoid finger prints.

At the end of this time the sheets were sprayed with ninhydrin reagent and solvent evaporated over hot plate. The sheets were developed in an oven at 85°C. for 5-10 minutes. A longer period of development gave denser colours, but these colours faded rapidly.

R_f values were calculated by choosing the geometric centre (visually) of the elliptical spots developed, measuring the distance in centimeters from the base line and comparing with the distance of movement of solvent.

Standardization of Technique: A group of 20 amino acids were chosen to cover the full range of R_f values given by Consden et al. Aqueous solutions were prepared at 6.0 micrograms/microlitre. Only tyrosine was dissolved as the sodium salt, histidine as the hydrochloride. Chromatograms were set up in quadruplicate on S & S paper in both phenol and collidine with 5 ml. of each amino acid solution applied on the base line. Runs were made for 20 hours in phenol and 24 in collidine. A typical chromatogram is shown in Diagram 2. The results after spraying and developing are shown in Table VIII and compared with those obtained by Consden (52), Dent (74) and Pratt and Auclair (76). The general agreement of the results gave reasonable assurance of accurate separation. The discrepancies noted are likely due to variations in purity of solvents used. The determined values agree within $\pm$3% on quadruplicate runs made.



R_f VALUES

TABLE VIII

AMINO ACID	Average of 4 Runs		Consden et al		Dent et al		Pratt & Auclair	
	Phenol	Collidine	Phenol	Col.	Phenol	Col.	Phenol	Col.
l-cystine	0.09	0.21	0.13	0.14	0.08	0.40	0.10	0.43
dl-phenyl-alanine	0.86	0.63	0.86	0.59	0.82	0.49	0.90	0.67
Isoleucine	0.84	0.52	0.82	0.54	0.84	0.45	0.88	0.62
l-lysine HCl	0.17	0.10	0.50	0.14	0.82	0.12	0.14	0.14
dl-serine	0.30	0.34	0.33	0.28	0.35	0.29	0.37	0.37
Aspartic Acid	0.20	(0.18 (0.24	0.14	0.22	0.20	(0.20 (0.30	0.19	0.24
Methionine	0.75	0.51	0.82	0.57	0.81	0.42	0.85	0.61
l-hydroxy-proline	0.69	0.26	0.66	0.34	0.64	0.27	0.72	0.42
Asparagine	0.39	0.27			0.40	0.22	0.42	0.29
Glutamic Acid	0.26	0.22	0.24	0.25	(0.28 (0.31	(0.20 (0.30	0.32	0.26
Leucine	0.88	0.52	0.84	0.58	0.84	0.45	0.88	0.65
l-proline	0.90	0.30	0.87	0.35	0.89	0.27	0.90	0.41
Arginine	0.61	0.14	0.67	0.16	0.89	0.16	0.66	0.14
Tryptophan	0.82	0.50	0.76	0.62	0.78	0.50	0.79	0.66
Alanine	0.65	0.36	0.57	0.32	0.60	0.26	0.63	0.41
Histidine	0.87	0.34	0.72	0.28	0.70	0.25	0.77	0.34
Threonine	0.56	0.37	0.50	0.32	0.50	0.31	0.53	0.43
Tyrosine	0.75	0.50	0.59	0.64	0.60	0.51	0.82	0.63
Glycine	0.41	0.33	0.40	0.25	0.42	0.23	0.42	0.33
Valine	0.76	0.50	0.78	0.45	0.78	0.36	0.82	0.62
Glutamine	0.42	0.21			0.58	0.10	0.62	0.32

MODIFIED TECHNIQUE FOR CHROMATOGRAPHY OF STREPOGENIN

Phenol has been already described by Woolley (31) as a solvent for strepogenin and hence it was chosen as the preliminary separating solvent. On a 10" x 17½" sheet of S & S paper, a solution containing 80 mg./ml. of Liver Fraction L. Yeast solubles, or casein hydrolysate, was applied in a streak 5 mm. wide along the base line. After drying, the chromatogram was run for 20-24 hours in phenol, and dried. A test strip 1½" wide was cut longitudinally and sprayed with ninhydrin and developed in the usual fashion. This indicated the sharpness of the resolution. If satisfactory, the main sheet was transversely sectioned into strips parallel with the base line and 0.5 R_f units in width (about 1.0 - 2.0 cm.). These strips were rolled loosely and placed in a modified Soxhlet extractor and extracted with a 1:1 by volume mixture of acetone-ether to remove phenol. Completeness of removal of phenol was indicated by Folin (81) reagent. At the completion of extraction, ether/acetone mixture was flashed off the paper by heating over a water bath. The strips were then separated and placed in numbered Evelyn tubes and 5.0 m.l of dilute media added. About 1 hour soaking was allowed to wash out strips which were then removed, tubes plugged and sterilized by steaming.

The cooled tubes were innoculated with L. casei in sterile saline in usual assay procedure, and tubes read at the end of 20 hours' incubation. Results were plotted on graphs and R_f of zones causing maximal optical density indicative of highest concentration of strepogenin were noted and marked on test strip. Duplicate sheets were simultaneously run and evidence of the nature of the

growth-stimulating material obtained by later sectioning at the R_f zone of this sheet, eluting with water, and chromatographing in collidine to purify the zone. After secondary separation, the material was hydrolysed in 5N hydrochloric acid and again chromatographed by two-way chromatogram in phenol and collidine. Identification of the amino acids involved was done by one-way chromatography in collidine with purified amino acids as standards.

CHROMATOGRAPHIC SEPARATION OF YEAST SOLUBLES

Preparation of Chromatogram

A solution containing 80 mg./ml. of Difco Dried Yeast Solubles was prepared without any attempt at preliminary purification. Duplicate sheets of S & S No. 595 paper 10" x 17½" were ruled for base line and folded. The solution was applied along the base line in a smooth streak, 5 mm. wide across the sheet and dried slowly over an electric hot plate. The sheets were then placed in phenol chamber for 4 hours before elution to become saturated with water-phenol phase, then elution commenced with phenol and allowed to continue for 20 hours. At this time the sheets were removed and dried thoroughly over a hot plate in a current of air. A test strip 2" wide was cut longitudinally, sprayed and developed with ninhydrin and filed away with the duplicate if the resolution was adequate.

Sectioning of Chromatogram

In the first technique used, sectioning of the paper was done following the pattern of the zones on the test strip but this was later abandoned in favour of simple sectioning in 0.5 R_f units. Here the main sheet and test strip were marked out similarly in 0.5 R_f units and each strip numbered in pencil (numbered increasing

from base line). Only sheets showing solvent fronts nearly parallel to base line were used and hence sectioning was always parallel to base line. These sections were collected and loosely rolled for extraction of solvent.

Removal of Phenol

The roll of sections was placed in an off-set Soxhlet extractor. A mixture of 1:1 by volume of acetone (Merck) and diethylether was placed in boiler and extraction allowed to proceed over a hot plate for 4 hours. At frequent intervals of extraction, completeness of phenol removal was tested by removing a small strip of paper from roll and testing with Folin phenol reagent (81). Extraction was allowed to proceed until the usual blue colour developed with these reagents and free phenol was not seen. At the conclusion of extraction the ether-soaked paper was placed in a beaker and heated on a water bath until ether was removed. About 3/4 hour was required.

Elution of Strips

Basal media was prepared in usual manner and diluted to assay strength with an equal volume of glass distilled water. 5.0 ml. was dispensed into clean, dry Evelyn tubes. The tubes were numbered as the strips and strips individually placed in each tube. All operations were performed using forceps to avoid contamination. The strips were extracted by shaking each of 20 tubes intermittently for 2 hours at the conclusion of which paper strips were removed with a sterile nickel hook with excess media squeezed out. Strips were discarded.

Innoculation

The Evelyn tubes were stoppered with cotton plugs sterilized by steaming for 15minutes, and cooled to room temperature. Innoculum was prepared the previous day, 20 hour, L. casei cells were washed in saline and suspended in saline until a galvanometer reading of 90 was obtained in the colorimeter. Innoculation was performed with one drop of cell suspension placed in each tube. Control tubes of basal mediaalone were similarly run to ensure that growth was not due to bacterial contamination along with a 5-tube standard series for growth comparison.

Growth Measurement

Optical density of tubes was measured in Evelyn Macro Colorimeter set at 5 cm. depth with basal media alone as blank. Readings made on 620 mu. filter. Growth measurements by acid production were also performed by titration with 0.01 N sodium hydroxide potentiometrically to a pH of 6.8 using the Beckman pH meter or with Bromthymolblue indicator.

CHROMATOGRAPHIC SEPARATIONS IN PHENOL

Yeast Solubles: The results of three assays of yeast solubles are plotted on Graph 4. These indicate a single growth-stimulating material which developed a faint blue colour with ninhydrin in most test strips but which faded very quickly. The potency of this material must be very high since only a total of 50 microlitres containing a total of 2400 micrograms were used. At least 20 fractions could be detected on strips with glycine, aspartic, glutamic in very high concentrations. Assuming equal concentrations of each component this will give a maximum concentration of 100

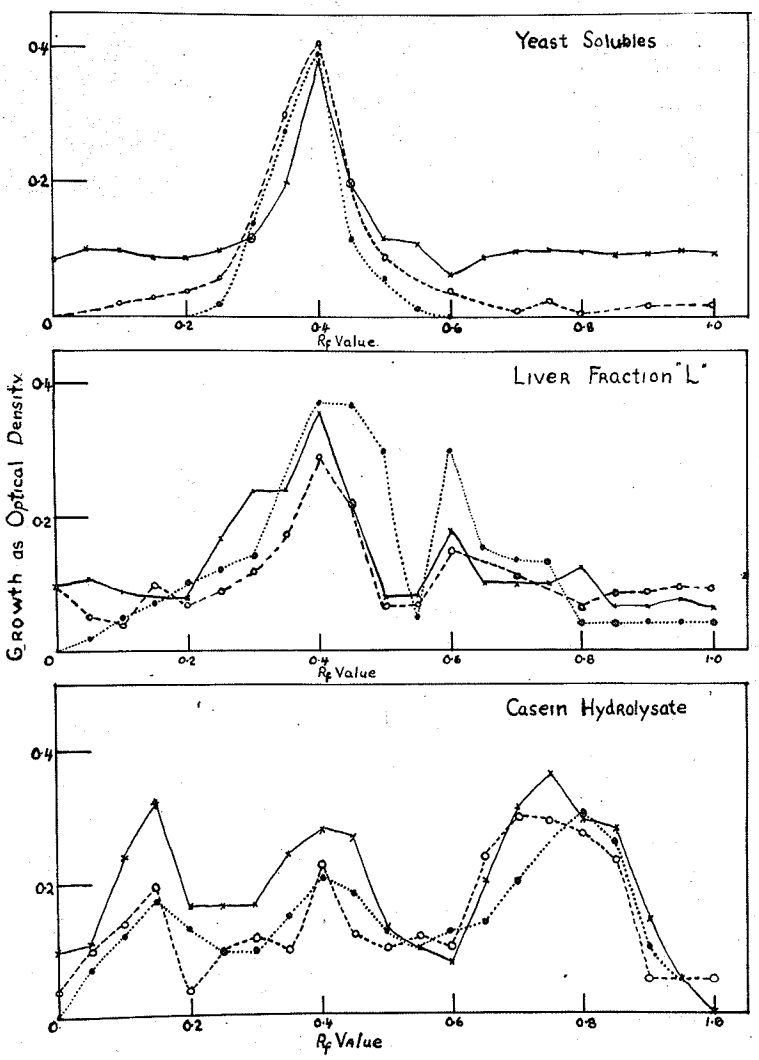
micrograms of active principle. Thus, if losses in the process are assumed to be of order of 50%, would leave 50 micrograms. This allows excellent growth-stimulating properties at a level of 10 micrograms per millilitre of media. For homogenity of this zone, see elution in collidine. Consideration of the presence of glutamine which has precisely the same R_f value in phenol led to running parallel chromatographs which confirmed the R_f values in phenol but no conclusions as to the similarity of the growth stimulation between the yeast compound could be made until similar runs were made in collidine. See Collidine runs for evidence that material in yeast having an R_f in phenol of 0.42 is not glutamine alone but could be the result of a mixture of glutamine plus another growth-stimulating material.

With Liver Fraction L.

The technique developed with Yeast Solubles was applied to Wilson Liver Fraction L., Antipernicous Anemia Extract (Lilly) and Trypsinized Casein. The last material used was the solid obtained after tannic acid precipitation of 20-hour digests of casein by trypsin. These materials were all run at 80 micrograms per microlitre in duplicate. Good separation of components was obtained with Liver Fraction L. and casein digests but no attempt to fractionate A.P.A. extract was successful in either phenol or collidine. All runs in this material tended to show striation from base line to solvent front with no zones of resolution evident. Hence, no growth studies were made on this material.

The growth zone curves for chromatograms of Liver Fraction L. and casein hydrolysate are shown in Graph 4. Liver L. demonstrated two major zones of growth stimulation at R_f

GRAPH NO. 4



Responses of L. casei to Yeast, Liver Fraction L. and Casein Hydrolysate after chromatographic separation in phenol. The results of these separate fractionations are plotted for each material as solid, intermittent and dotted lines.

values of 0.40 and 0.60. With casein hydrolysate neither the test strip nor growth zones indicate as sharp resolution as with yeast and Liver Fraction L. The mean values of the R_f indicating zone of maximal stimulation occurs at 0.17 with minor zones occurring at 0.43 and 0.72.

CHROMATOGRAPHIC SEPARATION IN COLLIDINE

Yeast Solubles, Liver Fraction L. and casein hydrolysate were set up and assayed in the same manner as described for yeast in phenol. Yeast solubles and trypsinized casein show only one zone of stimulation in this solvent, the former occurring at an R_f of 0.20, the latter at an R_f of 0.25. With Liver, one broad zone of stimulation occurred from an R_f of 0.30 to 0.45. Fractionation in collidine has been attempted only once and hence little emphasis can be placed on this study. In addition, growth records for this assay are much less dense (.23 vs .44) indicating some inhibition of L. casei perhaps due to incomplete removal of collidine by ether/acetone extraction.

Discussion of Chromatographic Separations

In a partition chromatogram, the movement of a compound from the base line is dependent upon two factors: (1) the movement of the solvent along the paper and (2) the solubility of the compound in the mobile and immobile solvents. The former is an easily determined variable of one chromatogram; the latter dependent upon a fixed property of the compound. This property is defined as the ratio:

$$\frac{\text{Solubility in solvent A}}{\text{Solubility in solvent B}} = \text{Partition Coefficient}$$

Thus, if a compound is allowed to equilibrate between two immiscible solvents, the ratio of its solubility in those two solvents, the partition coefficient, is a fixed property of that compound as rigorous

as its melting point, boiling point or refractive index (82). Since the R_f value of a compound in a chromatogram is directly dependent upon its partition coefficient between the solvents used to prepare that chromatogram, an equal interpretive weight should be permissible on the R_f value as upon the partition coefficient itself (51). Therefore, in a chromatogram of a mixture, if 2 different R_f values be obtained, these must represent compounds of differing chemical natures; so then in the chromatography of Liver Fraction L or casein hydrolysate, if two positions of growth stimulation be obtained at significantly different R_f these must represent compound of differing chemical constitution.

Interpretation of the curves (see graph No. 4) relating growth stimulation to the R_f value of the eluted zone in the chromatogram can be made by application of the criteria advanced by Consden (50) and Craig (82). Both these authors demonstrate mathematically and experimentally that in a counter-current separation of a very high order of theoretical plates, and distribution of a substance of known partition coefficient throughout those plates is one of a Normal Frequency Distribution; i.e. a symmetrical distribution about one maximum value, the median. The position in the sequence of plates of this median value is determined by the numerical order of the partition coefficient. Thus a compound having a partition coefficient of 0.2 in the solvents used will be distributed earlier in the sequence of extractions than will another having a partition coefficient of 0.8 and each distribution will be such that the median value of the distribution will be a function of 0.2 and 0.8 respectively. In a simple chromatogram

of amino acids, these distributions may be extended over a large number of 'plates' such as to occupy an area of several square centimeters. With Liver Fraction L. and Casein hydrolysate, the positions of growth stimulants are likely similarly dispersed so that growth zones rather than isolated sharp lines of growth stimulation. This makes interpretation of the growth versus R_f value graphs a matter of consideration of the "Peaks" of these curves which by analogy with the simple chromatogram should represent the median value of one particular growth stimulating substance.

With these considerations in mind, the following conclusions can be made on the evidence shown in Graph No. 4:-

(1) Yeast Solubles contains one growth stimulating compound (at least) which occurs at an R_f value of 0.42.

(2) Liver Fraction L contains at least two growth stimulation substances occurring at R_f values of 0.40 and 0.60 respectively whose compositions must be chemically distinct.

(3) Casein hydrolysate contains at least three growth-stimulating compounds distinct in compositions occurring at 0.17, 0.43 and 0.72 respectively.

If these conclusions are valid, the conception of the growth stimulation due to these mixtures on Lactobacillus casei as the result of concentration of one stimulating material in all is not a valid one. The implication of the term "strepogenin" must by this evidence be restricted to apply only to the composite growth stimulating effect, the result of all active components in the mixture. Thus the compilation of strepogenin content in naturally occurring materials shown in Table 1 and taken from the papers of Woolley and Scott et al (see previous references) cannot be interpreted as reflecting variations in concentration of a

single growth-stimulating effect as a result of a number of components. The use of Liver Fraction L. as a standard of growth comparisons is also questioned by these results since with this material the growth stimulation is demonstrably due to at least two components.

By the same token, dried yeast solubles since it presents but one growth stimulant should be a more valid standard reference material. But even with this material, use of this standard should be restricted to those materials which exhibit evidence of containing the same stimulant present in the yeast.

The multiplicity of stimulants in Liver Fraction L. and casein makes interpretation of the chemical properties of Strepogenin (See Section - Chemical Properties P. 12) very difficult. The chemical properties listed are now obvious properties of separate factors or of combinations of separate factors and hence of little value in deducing possible formulations of structure of any one.

The multiplicity of growth stimulants in Liver and casein is, in the main, in accord with the concepts of Peeler et al (23) who first demonstrated the multiplicity and concluded that the growth stimulating effect of Liver Fraction L for L. casei was due to the presence of glutamine and glutamic acid and at least two other "Animal Protein Factors". However, their conclusion (quote) "Lactobacillus casei requires glutamine and glutamic acid in addition to the unidentified factors in refined liver extracts" implying a synergistic action of these separate factors is not supported by this evidence. The nature of the chromatographic procedure does not allow synergisms to be demon-

strated except under the special case where all the synergistic factors have the same R_f value or where there is sufficient similarity of R_f value of the factors to allow overlapping to produce the combination necessary for growth stimulation. However in either case, only one growth zone should be shown in the chromatograms of each material. This is not the case. The common value of 0.40 of growth factors in Yeast, liver and casein might be assumed to represent a commonly occurring growth factor in each of these sources. Indeed this may be so but the conclusion has very little evidence to justify it. Compounds of quite different chemical composition may occupy the same R_f position in a chromatogram as can be seen from an examination of Table VIII, P. 42 where valine, tyrosine and methionine occupy the same R_f position in phenol elution. Such may equally be the case for the stimulating fraction occupying 0.40 position in the three source materials and hence no weight is placed on this similarity until further evidence is obtained.

Partial Identification of Growth-Stimulating Material of Yeast

Duplicate chromatograms of dried yeast solubles used to obtain the growth zone studies shown in Graph 4 were sectioned to remove the zone from R_f 0.40 to 0.45. This strip was macerated in 5.0 ml. water and the filter paper removed by filtration through a sintered glass funnel. The solution was reduced to dryness and remaining solids taken up in 50 microlitres of water. 10 microlitres of this solution were then chromatographed in collidine to determine the complexity of the zone. To the remaining solution was then added 2.0 ml. of 5N HCl and

the whole washed into a Pyrex test-tube and sealed. The assembly was then placed in an oven at 100°C. for 24 hours, used and then replaced for an additional 48 hours. At the conclusion of the hydrolysis periods, the hydrochloric acid was removed by distillation under reduced pressure, tubes washed with 2.0 m. water and again evaporated to dryness. The resulting solids were then taken up in 40 µl. water and transferred to a chromatogram sheet for fractionation in collidine. Amino acid standards were run simultaneously.

R_f VALUES OF COMPONENTS — TABLE IX

Before Hydrolysis	0.06				0.25		
24 hours Hydrolysis	0.06	0.128	0.17	0.20			0.57
48 hours Hydrolysis	0.06		0.17	0.22		0.33	0.57
Amino Acids	None	-	Aspartic	Glutamic	-	Glycine	Leucine

The chromatogram before hydrolysis showed two zones at 0.06 and 0.25 after development with ninhydrin. However, it had been previously shown that only the 0.25 zone was active. (See assays of yeast solubles after chromatography in collidine.) After 24 hours hydrolysis four zones were detectable as shown in Table IX in addition to the retained inactive zone at R_f 0.06. After 72 hours the zone at R_f 0.13 disappeared and a new zone at R_f 0.33 appeared. The four zones left showed R_f values similar to those of aspartic, glutamic, glycine and leucine, respectively and occupied the same positions on the chromatograms as these amino acids run simultaneously as standards.

From this data the following inference are possible but by no means conclusive:-

(1) The growth-stimulating material occupying the R_f position of 0.25 in collidine separation is a complex, fissionable by hydrolysis in strong acid and likely a polypeptide.

(2) That this complex yields on 72-hour hydrolysis four compounds which are indistinguishable from aspartic, glutamic, glycine, and leucine amino acids in the technique used.

(3) That this material is somewhat resistant to hydrolysis since on 24-hour hydrolysis a zone was distinguishable which on 72-hour hydrolysis completely disappeared. Coincident with the disappearance of the zone, a new zone appeared at an R_f similar to that of glycine which would lead to the conclusion that the intermediate was a glycine containing compound.

DISCUSSION OF PEPTIDE FACTOR OF YEAST:

The growth-stimulating properties of glutamine alone and its involvement in the growth stimulation of Liver fraction for Lactobacillus Casei has been demonstrated by various authors (15, 23, 24). The evidence presented here indicating the peptide nature of the growth-stimulating fraction of Difco Dried Yeast Solubles does not remove the possibility of glutamine playing a significant role. Glutamine, if present, would on acid hydrolysis yield glutamic acid but the evidence: (1) of the homogeneity of the zone after phenol and collidine elutions would indicate the presence of but one compound. Free glutamine has an R_f value in phenol of 0.42 equal to the value of the growth-stimulation of this complex mixture with the zone

contaminated with an inert peptide having the same R_f value. It seems unlikely, however, that this peptide contaminant would have the same R_f value in collidine as glutamine and simultaneously occur as a non-reactive contaminant when this solvent is used. Since the acid hydrolysis of the active zone after separation in phenol and purification in collidine could still demonstrate the presence of three amino acids other than glutamic, it would seem more likely that the growth-stimulation was due to a peptide than to a material which would yield only one amino acid. But it does not remove the possibility that glutamine is present in this peptide in a substituted form. This concept would satisfy the requirements of the amino acid composition and the known stimulatory effects of glutamine simultaneously.

Evidence indicating the peptide nature of the factor present in yeast is in agreement with the postulation of the peptide nature of 'strepogenin' advanced by Woolley (2) but the peptide involved can bear little resemblance to those synthesized by this author (serylglyclglutamic and glycylserylglutamic) which had stimulatory properties. No evidence could be obtained from the acid hydrolysate of the yeast factor which would indicate the presence of serine shown by this same author (19) to be necessary for positive action. The involvement of aspartic acid in the yeast peptide is likely signifcant but incapable of interpretation at the moment since all peptides synthesized by Woolley having aspartic acid as the substituted terminal acid in the peptide chain showed a definite growth inhibition for L. casei (19). It might then be inferred that the aspartic acid residue of the yeast peptide does not occur as the terminal amino acid but as a doubly substituted residue in a midposition along the peptide chain.

General Remarks on the Chromatographic Technique in This Problem:

The chief restriction to the usefulness of chromatographic procedures in analytical separations lies in the minute quantities of active product separated, perhaps of the order of 50 micrograms. This amount, while it is useful in ascertaining certain qualitative data on the nature of the reactive material, e.g., following hydrolysis, must be increased to at least the milligram level before attempts at classical chemical identification can be made. The alteration of filter paper chromatography to column partition chromatography has already been accomplished by Moore and Stein (77) with good separations reported following solvent front analysis from starch columns.

It would appear profitable in this study to modify the amino acid technique of these authors and attempt the separation of naturally occurring mixtures containing the L. casei stimulants. Here milligram separations would be feasible. It might also be useful to use some preliminary purification technique. Dent has shown that resolution of peptides is much improved by the desalting procedure of Consden (78). Agren has reported the fractionation of Liver to be greatly improved by preliminary separation by electrodialysis (79). If it can be demonstrated then these techniques have no effect on the overall potency of stimulation, application of them might result in a much sharper fractionation than was obtained in this work.

SUMMARY

1. The method of assay of strepogenin using Lactobacillus casei has been tested, and the effects of alteration in the washing of

innoculation cells, the dilution of innoculum and the results of increase in basal media components investigated.

2. Partition chromatography has been successfully applied to the problem of separating growth stimulating materials of Yeast Solubles, Liver Fraction L. and Tryptic digest of casein.

3. A method of microbiological assay has been devised and applied to the chromatograms of these materials relating the Growth-Stimulating effect and R_f value.

4. Evidence has been obtained indicating that Difco Dried Yeast Solubles contains one growth stimulating material occurring at an R_f of 0.40 in phenol, 0.20 in collidine.

5. Evidence has been obtained from the chromatograms of Liver Fraction L. at R_f of 0.43 and 0.60 in phenol that at least 2 growth stimulating zones are present.

6. Evidence has been obtained from the chromatogram of casein hydrolysate that 3 growth stimulating zones occur at R_f of 0.17, 0.43 and 0.72 in phenol elution.

7. It has been inferred from this data that Difco Dried Yeast Solubles contains but one growth stimulating factor for L. casei; that Liver Fraction L. contains at least two growth stimulating factors for L. casei; and that casein hydrolysate contains three growth stimulating materials for L. casei.

8. The possibility of the same growth-stimulating material occurring in all these materials has been discussed.

9. Evidence has been obtained of the peptide nature of the single growth-stimulating fraction of yeast which on acid hydrolysis yields aspartic, glutamic, glycine and leucine.

10. Some postulations on the constitution of this peptide have been discussed.

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