

Fractionation and Concentration of Growth
Factors for Lactobacillus Bulgaricus
09 and Lactobacillus Lactis 8000.

by

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Thesis submitted to the Faculty of the Graduate School
of the University of Maryland in partial
fulfillment of the requirements for
the degree of Doctor of Philosophy

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8000.

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Introduction

Although many growth factors have been discovered within the last twenty years, research in this field of bacteriology and biochemistry is still in its infancy. A great many more growth factors will probably be discovered and many presently observed phenomena need to be clearly explained.

A growth factor required by a hemolytic streptococcus, X 40, was reported by Woolley (1939, 1940, 1941, 1944). This growth factor stimulated the growth of Lactobacillus casei and Streptococcus lactis. The factor was termed "streptogenin". The replacement of streptogenin by some chemically pure compounds was tried by several investigators (Wright and Skeggs 1944, Woolley 1946, Krehl and Fruton 1948, Rickes et al 1949, Woolley 1948, Peeler, et al 1949), but the chemical composition was not definitely established. An improved assay for streptogenin based on the essential nature of material for Lactobacillus bulgaricus 09, was suggested by Wright, et al (1950). A satisfactory assay of streptogenin using L. casei was reported by Kodicek, et al (1952). Woolley (1947, 1949), has done some studies on the structure of streptogenin recognizing it to have peptide properties. This is of interest since peptides have been recognized as growth factors for several other bacteria. Other growth stimulating factors of unidentified nature have also been reported for several bacterial species.

The growth factors for Lactobacillus lactis, Dorner strain, (ATCC 8000) consisting of multiple components, i.e. LLD (lactobacillus lactis Dorner factor), TJ (tomato juice factor), and factors synthesized by L. lactis Dorner, was reported by Shorb (1947). Vitamin B₁₂ is considered either wholly or partially responsible for the LLD growth activity (Shorb 1948). The TJ factor is replaced by pyridoxamine phosphate (Hendlin, et al 1950). Growth inhibitors have been reported repeatedly by Shorb (1947, 1949). An unidentified growth stimulating factor also produces a rapid rate of growth of L. lactis (Shorb 1947, 1949, Shorb and Briggs 1952). In this respect it is similar to streptogenin for L. casei. Since the chemical structure of streptogenin is undefined and it is the required factor for L. bulgaricus 09 and stimulates the growth of other organisms, including L. lactis 8000. The present investigation was planned to study these growth factors along the following lines:

Fractionation and concentration of the growth factors for L. bulgaricus 09 and L. lactis 8000; characterization of the growth factors; distribution of the growth factor; comparison of the relative activities of the source materials and of some fractions for micro-organisms; effect of the growth factors on other micro-biological assays such as vitamin B₁₂; chemical composition of the growth factor; and inhibitor studies.

Literature Review

In 1939 Woolley established the existence of several growth factors for hemolytic streptococci. Woolley and Hutching later (1940) reported that in a synthetic medium hemolytic streptococci require the addition of natural substances for growth. The effect of this new growth factor was reported by Woolley (1941) and confirmed by Grossowicz (1942). Later peptone, yeast and tomato juice were reported as growth factors for Lactobacillus casei, Streptococcus lactis, and Lactobacillus arabinosus respectively (Pollack, et al 1943, Smith 1943, Kuiken, et al 1943). The properties of this growth factor were studied by Sprince and Woolley (1944). It was amphoteric and occurred in partially hydrolyzed casein. It was insoluble in alcohol and not adsorbed by Morite. They suggested the name "streptogenin" and believed it was responsible for the stimulating effects noted with all of the organisms tested and was essential for hemolytic streptococci. Streptogenin stimulated fast growth in L. casei, so they recommended the use of L. casei as the assay organism. Wright and Skeggs (1944) reported that asparagine but not glutamine was effective in promoting growth of Streptococcus lactis, and trypsinized vitamin-free casein was effective in promoting growth of both Streptococcus lactis and Streptococcus fecalis. The activity of trypsinized casein was much greater than that of asparagine. Sprince and Woolley (1945) further studied the distribution

of strepogenin in various trypsinized proteins as well as methods to release strepogenin activity and made some studies of the chemical properties of strepogenin. They concluded that strepogenin was a peptide (or part of a protein molecule) rather than an amino acid. Crystalline insulin was reported to be the best source of Strepogenin while dialyzed egg white, salmine, and gelatin were very poor sources. Strepogenin was reported (Woolley, 1945) as a growth stimulating factor for mice, the growth stimulating activity being destroyed by acid hydrolysis. Scott, et al (1946) worked on L. casei and concluded that L. casei required, in addition to Strepogenin, a factor associated chiefly with animal products and glutathione. Lycomarasmin, a peptide from the tomato plant containing aspartic acid and causing wilting of the tomato plant was reported by Woolley (1946) to inhibit the growth of L. casei. This inhibition could be reversed by Strepogenin. Woolley (1946) synthesized some tripeptides containing aspartic acid and reported that synthetic seryl-glycyl-aspartic acid had one-sixth and glycyl-seryl-aspartic acid had one half to one-ninth the toxic activity of lycomarasmin. Because of the observed high percentage of glutamine in strepogenin and the prediction of similarity in structure of strepogenin and lycomarasmin, tripeptides containing glutamic acid were synthesized. Synthetic seryl-glycyl-glutamic acid had one tenth the activity of strepogenin. Therefore seryl-glycyl-glutamic acid was suggested to be either a fragment of, or closely related to strepogenin. The growth stimulating activity of strepogenin

on animals was confirmed by several investigators (Woolley and Collyer 1946, Womack and Rose 1946, Lecoq, et al 1949, Bourgeat 1948, Kollath 1950). The influence of strepogenin concentrate on the metabolism of glutamic acid by Streptococcus fecalis was reported by Tottler and King (1946). In addition to strepogenin, a factor from animal products along with glutathione stimulated the growth of L. casei according to Scott, et al (1946). By the dinitrofluorobenzene technique, Van Slyke's amino nitrogen determination and enzyme digestion, Woolley (1947) was able to arrive at the tentative conclusion that in insulin, strepogenin activity is at the end of the peptide chain and glycine is the amino end group of strepogenin. The reaction of insulin with dinitrofluorobenzene almost completely destroyed its strepogenin potency. If an enzymatic digestion was performed after Van Slyke's amino nitrogen determination, the resulting product had about 10 percent of the activity of strepogenin. Of 17 peptides prepared by Woolley (1946) only certain derivatives of glutamic acid containing a free amino group (serylglycylglutamic acid, glycylserylglycylglutamic acid, alanylglycylglutamic acid, glycylalanylglycylglutamic acid and glycylglutamic acid) were able to replace strepogenin as growth stimulators for L. casei. Glutamine and glutathione have a pseudostrepogenin activity, which can be distinguished from strepogenin by heat stability (Pollack and Linder 1943, Woolley 1946, 1948). Serylglycylaspartic acid is antagonistic toward strepogenin (Woolley 1948). Krehl and Fruton (1948) reported that a number of synthetic peptides containing gluta-

mic acid were either inactive or had a negligible streptogenin activity, but l-serylglycyl-l-glutamic acid was confirmed to be active for L. casei. Peptides from an enzymic digest of casein stimulating the growth of L. casei, were reported by Verdier, et al and Agren (1948, 1949). Riches, et al (1949) reported that when a rapid initial growth of L. Casei was obtained with a synthetic medium enriched by adding dl-serine, glutamic acid, asparagine, vitamin C and other vitamins, addition of streptogenin did not further increase the growth. Peeler, et al (1949) reported that the growth response of L. casei to the addition of sterile l-glutamine was identical with that obtained with a streptogenin concentrate, while l-glutamic acid had less activity, indicating its probable conversion to glutamine. Daniel, et al (1949) reported that L. casei required two factors in addition to glutamine, the factors exist in large amount in liver and whey. Kodicek and Histry (1949) reported that asparagine enhanced the effect of streptogenin preparations during the early incubation period. Using dinitrofluorobenzene and counter current techniques, Woolley (1949) has isolated four crystalline yellow peptides from pancreatin digests of trypsinogen. The amino acid compositions of these peptides are different. The chromophoric group was considered to be DNP-glycine. The activity of these dinitrofluoro peptides for L. casei was not reported, but earlier work (Woolley 1947) has shown that the dinitrofluoro derivative of streptogenin had no activity. Dunn and McClure (1950) suggested that the streptogenin activity for L. casei

may be due to an unspecific effect of peptides, since the organism may use an essential amino acid more rapidly in the bound than in the free form. Recently Kodicek and Mistry published the satisfactory assay of streptogenin activity with L. casei (1951, 1952) which further emphasized that insulin had the highest streptogenin activity. Glutamine, asparagine and aspartic acid showed a slight streptogenin activity. Glutamic acid and glutathione were inactive. None of these substances could replace streptogenin activity. Intact protein molecules showed little or no activity. They classified the biological materials into two groups. The first group showed a growth response curve lying, within experimental error, parallel to that of the streptogenin standard and for the second group a steeper dose response curve was obtained, indicating the presence of other factors. Wright, et al (1950) suggested the use of L. bulgaricus 09 as assay organism for streptogenin. For L. bulgaricus 09 streptogenin is an essential growth factor instead of a stimulating factor and therefore L. bulgaricus 09 is a better organism for fractionation studies.

Nutrition requirements of Tritrichomonas foetus with special reference to partially digested protein was reported by Weiss and Hall (1947). Agren (1948) reported the utilization of peptides, especially leucine peptides, by ten lactic acid bacteria (Streptococcus faecalis 9790 and 8043, L. dolbruckii, L. casei, L. dextranicum, L. citreovorax 8081 and 8082, L. arabinosus, L. fermenti and Leuconostoc mesenteroides) and show-

ed that growth promotive activity depended upon the position of leucin and the nature of the surrounding amino acid. This type of analysis may become useful for the establishment of the order of amino acid linkage in peptides. The growth stimulating action of l-leucine and l-phenylalanine peptides on Escherichia coli was reported by Simmon and Fruton (1949). L-leucylglycylglycine was the most active peptide studied. L. delbrückii, L. arabinosus and Leuconostoc citrovorum have been shown to utilize peptide-bound amino acids to a very high degree (Klungsoyr, et al, 1950). The utilization of phenylalanine and alanine peptides for reversal of the toxicity of β -2-thienyl-d-alanine toward E. coli was reported by Dunn, et al (1951). Naphthylalanine peptides could not be used by bacteria and the acylated derivatives were used only at high concentrations. The utilization of glutamine and asparagine peptides by L. arabinosus and Leuconostoc mesenteroides was reported by Miller, et al (1952).

The most common instances of peptides as growth inhibiting factors are the antibiotics (polymyxin, penicillin, streptomycin, chlormycetin, circulin etc.) which have been in use in medicine for about ten years. Synthetic pentapeptides related to Gramicidin S were reported by Harris (1948) to have growth inhibiting action on Staphylococcus aureus, Streptococcus hemolyticus and E. coli at a concentration of 1-2 mg in vitro. The inhibiting effect of synthetic lysine polypeptides on the growth of influenza virus in embryonated eggs was reported by Rubbini (1951). Synthetic β -2- and β -3-thienylalanine having growth inhibiting action on E. coli 9723 and Saccharomyces

cerevisiae was reported by Dunn (1951); this inhibition being reversed by phenylalanine peptides. The inhibition of E. coli by leucine peptides was reported by Simmonds (1951). The inhibiting action of 4-methyltryptophan to E. typhosus, S. faecalis R, Leuconostoc mesenteroides P60, L. arabinosus 7-5, Staphylococcus aureus, and E. coli was reported by Marshall and Woods (1952); this inhibition being reversed by tryptophan, phenylalanine and tyrosine. Four tryptophan dipeptides were synthesized but none of these synthetic dipeptides of tryptophan could stimulate the growth of the tryptophan-requiring organism better than tryptophan itself.

Slade (1951) prepared a peptide mixture from the non-dialyzable portion of pancreatic digests of vitamin-free casein, which had growth stimulating activity for a group "A" hemolytic streptococcus. This peptide mixture was shown to contain glutamic acid. No mention of streptogenin activity was made. Slade's preparation can be distinguished from streptogenin by the fact that the latter is dialyzable. Glutathione enhanced the growth stimulating activity of this peptide mixture (Slade 1951).

Shorb (1947) reported that L. lactis 8000 required two growth stimulating factors, ILD and TJ. In addition to these there is another factor which can be synthesized by L. lactis. Inhibitory substances present in the natural material also were reported. ILD factor was later found to be replaced partially or wholly by vitamin B₁₂ (Shorb 1948) and the TJ factor was proved to be identical with pyridoxamine phosphate

(Hendlin, et al 1950). Further study of inhibiting factors has shown a transitory inhibition with 4-amino-pteroyl glutamic acid, pteroyl- γ -glutanylglutamic acid, pteroylheptaglutamic acid, pteroylglutamic acid, pteroyl- γ -glutanyl-glutamic acid, pteroyl- γ -glutamyl- γ -glutanyl-glutamic acid; whereas pteronic acid, rhizopterin, xanthopterin and thymidine have little effect (Shorb 1949). Kodicek reported (1949) that carbon dioxide, LLD and vitamin B₁₂ are of the same importance in the nutrition of L. lactis and act as antioxidants. Air has the ability to reduce the growth of L. lactis. The growth can also be inhibited by oxidizing agents; such as, hydrogen peroxide, potassium permanganate, and potassium ferricyanide.

Experimental

I. Microbiological Methods of Study.

1. L. bulgaricus O9 Assay.

(A). Plate assay: The organism was carried in a milk medium containing 1 percent tryptose in 10 percent skin milk. The milk culture was incubated at 37 C for 24 hours and then stored in the refrigerator. The stock culture was transferred to new milk every two weeks. For an inoculum in each assay, 0.2 ml of milk culture was supplemented by 1 mg of trypsinized casein per ml of medium and incubated at 27 C for 24 hours. The culture was washed twice with sterile saline and then diluted with sterile saline to a concentration which gave a galvanometer reading of 50 on the Evelyn colorimeter with the 620 m μ filter. The agar assay medium consisted of 2 percent agar in the basal medium of L. bulgaricus O9 (Table I). The medium was sterilized in the autoclave at 121 C for 15 minutes. After the medium was cooled in a water bath to 47 C four ml of the above diluted inoculum culture was added to 100 ml of agar medium. Fifteen ml aliquots of seeded agar medium per plate were poured into dry sterile flat-bottom petri dishes. After solidification of the medium, four holes were cut symmetrically in the agar with an 8 mm cork borer. Plates prepared in this manner may be kept in the refrigerator for a week without any appreciable change in assay results.

Norite-treated trypsinized casein was chosen as a

Table I
Composition of Basal Medium¹ for L. bulgaricus 09

Casamino acids	5.00 mg
Glucose	20.00 mg
Orotic acid	0.02 mg
Sodium acetate	6.00 mg
dl-tryptophan	0.20 mg
l-cystine	0.10 mg
Adenine HCl	5.00 Y
Guanine HCl	5.00 Y
Uracil	5.00 Y
Xanthine	5.00 Y
Salts A ²	0.005 ml
Salts B ³	0.005 ml
Thiamin hydrochloride	1.00 Y
Calcium panthothenate	1.00 Y
Riboflavin	1.00 Y
Nicotinic acid	1.00 Y
Pyridoxine HCl	2.00 Y
Pyridoxal	0.25 Y
Folic acid	0.25 Y
p-aminobenzoic acid	0.50 Y
Biotin	0.005 Y
Tween 80	0.001 ml
Vitamin B ₁₂	0.0002 Y ⁴
pH	5.6-5.8

¹ Amount per ml of a single strength basal medium.

² Salts A: K₂HPO₄ 1 mg, KH₂PO₄ 1 mg, H₂O 1 ml.

³ Salts B: MgSO₄·7H₂O 0.4 mg, NaCl 0.02mg, FeSO₄·7H₂O 0.02 mg, MnSO₄·4H₂O 0.02 mg, H₂O 1 ml.

⁴ This amount was for tube assays, but 10 times this value was used for plate assays.

standard. The trypsinized casein was supplied by Merck and Co., Rahway, N.J.. For each assay a standard curve of trypsinized casein was prepared. The material to be assayed, 0.05 ml of four different dilutions (30 mg, 6 mg, 3 mg and 1.5 mg per ml), was placed in the four holes on duplicate plates. The plates were incubated at 37 C for 17 hours. The diameter of the growth zone was plotted against mg of trypsinized casein on semilogarithmic paper. A straight line relationship was obtained. All samples were likewise diluted into 4 different concentration and the activities were expressed in terms of mg of trypsinized casein. One unit was defined as the activity of 1 mg of trypsinized casein. A standard curve of trypsinized casein in a plate assay is shown in Figure 1.

(B). Tube assay (Wright 1950): The stock culture used in the tube assay was the same as in the plate assay. In each assay the inoculum was made by adding 2 drops of a fresh milk culture to 10 ml of sterile saline. Different amount of samples were diluted to 5 ml with water. Five ml aliquotes of a double strength L. bulgaricus O9 basal medium were added to each tube. The tubes were sterilized in autoclave at 121 C for 15 minutes, and then quickly cooled to room temperature in a water bath. One drop of the above mentioned inoculum culture per tube was used. The tubes were incubated at 37 C for 48 to 72 hours. Growth was determined turbidimetrically in an Evelyn Colorimeter using 620 m μ filter for each assay

a standard curve of trypsinized casein was made using 4 to 10 different levels. The galvanometer readings were plotted against mg of trypsinized casein on an ordinary graph paper. A standard curve of trypsinized casein in a tube assay was shown in Figure 2. All samples were likewise tested at several levels. The activities were expressed in terms of mg of trypsinized casein. One unit was defined as the activity exerted by 1 mg of trypsinized casein.

2. L. lactis 8000 assay.

(A). Plate assay (Silver 1950): The plate assay was similar to the plate assay of L. bulgaricus 09 with the following exceptions. The stock medium was Silver's tomato juice medium containing yeast extract, glucose and milk. The stock culture was incubated at 37 C for 17 hours and stored in the refrigerator. The stock culture was transferred to new milk every 2 weeks. In each assay 0.2 ml of a milk culture was transferred to 10 ml of difco Micro Inoculum broth and incubated at 37 C for 8 hours. The culture was washed twice with sterile saline and diluted to a galvanometer reading of 70, and 4 ml of inoculum per 100 ml of agar was used. The agar medium consisted of 2 percent agar in the basal medium of L. lactis 8000 (Table 2). A Norite eluate of phytone dialysate, which will be described later, was chosen as standard. One unit was defined as the activity exerted by 1 mg of solid of norite eluate of phytone dialysate (phytone eluate)

(B). Tube assay (Shorb and Briggs 1952): The Tube assay

Table II

Composition of Basal Medium¹ for L. lactis 8000

Amino acid mixture ²	3.4 mg
Cystine	0.2 mg
Sodium acetate	6.0 mg
Glucose	10.0 mg
Adenine HCl	10.0 γ
Guanine HCl	10.0 γ
Uracil	10.0 γ
Salts A ³	0.005 ml
CaCl ₂	100.0 γ
Salts B ⁴	0.005 ml
Thiamin hydrochloride	10.0 γ
Riboflavin	2.5 γ
Nicotinic acid	4.0 γ
Calcium pantothenate	2.0 γ
Biotin	5.0 γ
p-aminobenzoic acid	2.0 γ
Xanthine	10.0 γ
Tween 80	0.001 ml
pH	6.8
Vitamin B ₁₂	0.001 γ ⁵
Ethyl oxalacetate	0.5 mg
Folic acid	0.005 γ
Pyridoxal phosphate	1.0 γ
Asparagin	0.2 mg ⁶
L.B.F. (synthetic pantothenine)	0.5 unit ⁶
KCl	0.25 γ ⁶

¹Amount per ml of a single strength basal medium.

²Amino acid mixture contained 0.2 mg each of dl-alanine, l-arginine, dl-aspartic acid, l-leucine, l-lysine, dl-methionine, l-glutamic acid, glycine, dl-histidine, dl-isoleucine, dl-phenylalanine, dl-serine, dl-threonine, dl-tryptophan, l-tyrosine, dl-valine.

³Salts A: H₂HPO₄ 1 mg, KH₂PO₄ 1 mg, H₂O 1 ml,

⁴Salts B: MgSO₄·7H₂O 0.4 mg, NaCl 0.02 mg, FeSO₄·7H₂O 0.02 mg, MnSO₄·4H₂O 0.02 mg, H₂O 1.0 ml.

⁵This amount for tube assays, but 10 times this value was used for plate assays.

⁶Used in tube assays only.

was also similar to the assay of L. bulgaricus 09 with the following exceptions. The assay medium used was the basal medium for L. lactis 8000 (Table 2). The inoculum was the same as for the plate except that two drops of the diluted inoculum were added to 10 ml of sterile saline. Then one drop of this inoculum per tube was used. The incubation period was seventeen hours. The activity was determined turbidimetrically. A norite eluate of phytone dialysate was chosen as standard. One unit was defined as the activity exerted by 1 mg solids of Norite eluate of phytone dialysate.

II. Fractionation and concentration of growth factors from hemoglobin.

1. Preparation of globin from hemoglobin (Sahyun 1944).

One percent (weight / volume) of hemoglobin was suspended in water. Hydrochloric acid was added dropwise with continuous shaking until hemoglobin was completely dissolved. Acetone was added to this hemoglobin solution, until the globin hydrochloride was completely precipitated. The globin hydrochloride was washed free from heme with acetone and redissolved in water. Free globin was precipitated by adjusting the pH to the isoelectric point with sodium hydroxide solution.

2. Enzymatic digestion of globin.

A. Sprince and Woolley's method (1945): A water solution of globin (0.5 percent) was heated to boiling for three minutes. The solution was cooled and adjusted to pH 8.0

with NaOH solution. Difco trypsin was added to about one percent of the dry weight of globin. The mixture was incubated at 37 C under toluene, the pH of the mixture being adjusted to pH 8.0 occasionally. After 20 hours incubation the reaction mixture was acidified to pH 3.0 and heated to 100 C. After cooling , the mixture was neutralized to pH 7.0. The resulting solution was ready for further fractionation. The method is preferred to Roberts and Snell's method, because there was a lower concentration of salt and the Norite used in Roberts and Snell's method was capable of removing part of the active substance.

F. Roberts and Snell's method (1946): Globin was dissolved in 0.8 percent NaHCO_3 solution, and trypsin was added to about 0.3 percent of dry weight of globin. The mixture was incubated under toluene for 48 hours, followed by autoclaving with 15 lbs steam pressure for 20 to 30 minutes. After cooling the mixture was adjusted to pH 6.0 with glacial acetic acid. Norite was added to an amount one half that of globin, and the mixture stirred for 30 minutes and filtered. The filtrate was adjusted to pH 3.8 and Norite was added to about one fifth the weight of globin. The mixture was adjusted to pH 7.0. This solution was ready for further fractionation.

3. Fractionation.

On dropwise addition of 10 percent $\text{Pb}(\text{AcO})_2$ solution to any of the above solutions of globin, a large amount of flocculant precipitate was formed. The addition was continued until precipitation was completed. Hydrogen sulfide was used

to remove the lead both from the precipitate and from the filtrate. The active substance recovered from the precipitate was small compared with that from the filtrate and the material could not be reprecipitated with lead acetate. Therefore, further work was done only with the filtrate. The filtrate was concentrated under reduced pressure and the last traces of water were removed by azeotropic distillation with benzene and alcohol. After removal of the solvent and cooling, absolute alcohol was added to the mixture and a precipitate appeared. This precipitate had no activity. The resulting filtrate was dialyzed against water for 3 to 4 periods of 6 hours each. The residue in the dialyzing bag was concentrated under reduced pressure and then dried in the vacuum oven. The resulting substance was the most active fraction and called "fraction A" in this paper. Prolonged dialysis will cause the movement of all of the activity to the dialysate.

III. Chromatographic studies.

1. Paper chromatographic method (A.A. Consden, Gordon and Martin 1944, Sanger 1945, Dent 1947, 1948, Machly and Palcus 1950) and chromatography of growth stimulant and growth inhibitor (Nash and Smashly 1951, Harrison 1951).

Chromatographic chamber: For the ascending method a glass cylinder with rubber stopper was used for one dimensional chromatography and fish tanks with trays, hanging strings and tight covers were used for two dimensional chromatography. For the descending method, a well-sealed wood

box with glass window and a trough at the top was employed. All chambers were kept at room temperature or a constant temperature of 37 C in an insulated chamber.

Solvents: The solvents used for most studies were BuOH-H₂O-HAcO (Water-saturated butanol: HAcO=90:10); Water-saturated butanol, 60 percent cellosolve and 80 percent phenol, which was purified by the method of Draper and Pollard 1949. Because the present study was done with a microbiological assay, the choice of solvent was restricted to that having no bacteriostatic effects.

One dimensional chromatography:

Ascending method: Whatman No.1 filter paper was cut into strips 1 inch wide. A sample containing 0.01 ml was put on one end 1.5 inches from the end of the paper and dried in air. Then the strip was hung on the hook of the rubber stopper with a paper clip and suspended so that about 0.5 inch was in the solvent. The solvent front went up to within 0.5 inch of the paper clip usually in about 8 hours (varies with different solvents). The paper was removed and the solvent front was marked. The filter paper was dried in the air. If 80 percent phenol was used as the solvent, the strip was washed with ether until freed sufficiently from phenol for the microbiological assay.

Descending method: Different samples containing 1 mg in 0.01 ml were put on a line one inch apart on two sheets of

Whatman No. 1 filter paper. After the samples were dried in air, they were separated from each to other by removing a thin strip of paper between each sample strip, leaving only the 1.5 inches edge of the square intact. The two papers were put in the trough fixed with glass hooks and supported with a glass rod on each side of the trough. After the papers were properly situated in the box, the solvent was added to the trough and the box closed tightly. Usually 12-20 hours was needed to let the solvent front migrate to a point one inch from the end of strips. After marking the solvent fronts the papers were air dried.

Two dimensional chromatography:

The method was similar to that of one dimensional chromatography except that the paper was cut in a square. For a small paper (7.5 X 7.5 in.) the sample was placed in one corner one inch from each side and the fish tank was used. Three sheets were the most which could be run at one time. After accomplishing the one dimensional chromatogram, the paper was turned 90 degrees using the sample spot as the center of rotation and sample was rechromatographed in the same manner, but with a different solvent.

Amino acid determination: The chromatogram was sprayed with a solution of 0.1 percent ninhydrin in watersaturated butanol. After air-drying, the sheet was heated in an oven at 100 C for 3 minutes. Colored spots were recorded in term of

R_f values and compared with R_f values of known amino acids.

Bioautographic method: The chromatogram was cut in small suitable sections and laid on seeded agar plates for 10 minutes. The plate was incubated at 37 C for 17 hours for L. bulgaricus 09 and for 8 hours for L. lactis 8000. The R_f value of the growth zones were recorded.

Each one dimensional chromatogram was also cut into 10 equal sections and each section was leached with water. The leachings were used as samples in the tube assay. The R_f value of the growth factors could also be determined by the turbidimetric method.

2. Column chromatographic method.

Norite was able to absorb the active substance, but not completely. In addition the recovery from Norite was incomplete.

IV. Microbiological assay for the amino acid composition of "Fraction A" of trypsinized globin (Stokes 1944 and 1945, Lewis, et al 1945 and Lyman, et al 1945).

Sample: Fraction A of globin (150 mg) and 1.5 ml of 10 percent HCl was sealed in a pyrex tube. Fraction A of globin (25 mg) and 2.0 ml of 5 N NaOH was sealed in another tube. The two tubes were autoclaved at 121 C for 10 hours and then neutralized and diluted as samples for the amino acid determination.

Organism: Streptococcus fecalis R was used for all amino acid except phenylalanine, norleucine, proline, hydroxyproline and glutamic acid.

Lactobacillus arabinosus 17-5 (ATCC 8014) was used for

the determination of L (+)- glutamic acid. L. casei, ATCC 9595 was used for the determination of phenylalanine. Streptococcus fecalis R was carried in a stab medium containing 1 percent glucose, 0.5 percent Bacto peptone, 0.6 percent anhydrous sodium acetate, salts A and salts B at half the concentration given in Table 3, adjusted to pH 6.6. Then 1.5 percent agar was added. The cultures were stored in a refrigerator and subcultured each month. Inoculum for the assay was prepared by transferring a small amount of growth with a needle to a tube containing 10 ml of the same medium, but without agar. After incubation for 16 hours at 37 C, the culture was washed twice with sterile saline and suspended in 100 ml of sterile saline. One drop per tube was used in this assay. L. casei 9595 culture was treated the same as Streptococcus fecalis R. except that the final inoculum was suspended in 20 ml of sterile saline. The L. arabinosus culture was carried in a stab medium containing yeast extract and dextrose. The stock culture was transferred monthly. The inoculum was made by transferring two loops of stab culture into 10 ml of sterile saline and one drop of the diluted culture per 10 ml of basal medium of L. arabinosus containing 2 mg of glutamic acid (Table 3). After incubation for 24 hours at 37 C, the culture was washed twice with sterile saline and suspended in 10 ml of sterile saline. One ml of this diluted culture was again diluted to 8 ml with

sterile saline. One drop per tube was used in the assay.

Method of study:

The complete amino acid assay media are tabulated in Table 3. In each assay the amino acid being assayed was omitted from the medium. The known amino acid was added in 8 levels in tubes and the medium was added after which the mixture was sterilized and cooled. The organism was inoculated and the test tubes incubated at 37 C for 17 hours. The culture was steamed in the autoclave for 10 minutes and the growth stimulating activity was determined by the amount of 1 N NaOH needed to neutralize the acids produced. A standard curve for each amino acid was made by plotting the ml of 1 N NaOH used against mg of amino acid on ordinary graph paper. The hydrolyzed samples of Fraction A of globin were tested in ten levels and the amino acid content was determined from the standard curve. The basic hydrolysate was used in the assay of tryptophan and phenylalanine. For the glutamic acid assay, 0.25 mg of glutamine was added to each tube in order to get a normal growth curve (Lyman, 1945).

V. Preparation of source material:

1. Trypsinized proteins: The proteins from different sources were trypsinized according to Woolley's method (see II).

2. Acid hydrolyzed protein (Kodicek 1951, 1952): Protein (200 mg) and a six normal HCl (5 ml) were placed in a sealed tube which was then incubated at 37 C for 3 hours. The re-

sulting hydrolyzate was neutralized with NaOH and made up to volume. The assay methods were the same as stated before.

3. Preparation of phytone dialysate Horite eluate.

Phytone (40 gm) was dissolved in 60 ml of water. The solution was dialyzed against water. The water was changed every day for 10 days. The combined dialysate was concentrated under reduced pressure to 280 ml. Horite (14 gm) was added to the concentrate and the mixture was stirred for 15 minutes. The Horite was filtered off and the absorption was repeated three more times. The combined Horite residues were washed four times with 25 ml of distilled water. The Horite was eluted with 75 percent alcohol containing NH_4OH . The combined eluates were distilled under reduced pressure to remove alcohol and NH_4OH . The resulting solution was made to volume and used as standard for the L. lactis assay.

Table 3

Basal medium¹ for S. fecalis R and L. casei (ATCC9595) and
 basal medium for L. arabinosus 17-5 (ATCC8014)

Substance	Amount	
	<u>S. fecalis</u> R and <u>L. casei</u>	<u>L. arabinosus</u>
dl-Leucine	100 mg	4 mg
dl-Isoleucine	100 mg	2 mg
dl-Valine	100 mg	3 mg
l(-) Cystine	100 mg	2 mg
dl-Methionine	100 mg	1 mg
dl-Tryptophane	200 mg	3 mg
l(-)Tyrosine	100 mg	2 mg
dl-Phenylalanine	100 mg	2 mg
dl-Glutamic acid	100 mg	0 mg
dl-Threonine	100 mg	1 mg
dl-Alanine	100 mg	1 mg
dl-Aspartic acid	100 mg	2 mg
l(+)-Lysine	50 mg	3 mg
l(+)-Arginine	100 mg	2 mg
l(+)-Histidine	100 mg	2 mg
dl-Serine	100 mg	2 mg
l(-)Proline	100 mg	1 mg
l(-)-Hydroxylproline	100 mg	1 mg
dl-Norleucine	100 mg	2 mg
Glycine	100 mg	2 mg
Glucose	5 gm	100 mg
Sodium acetate(anhydrous)	3 gm	60 mg
Adenine	5 mg	0.2 mg
Guanine	5 mg	0.2 mg
Uracil	5 mg	0.2 mg
Pantothenic acid	100 γ	2 γ
Riboflavin	100 γ	2 γ
Thiamine HCl	100 γ	10 γ
Nicotinic acid	100 γ	5 γ
Pyridoxamine	200 γ	5 γ
p-aminobenzoic acid	20 γ	2 γ
Biotin	0.1 γ	0.1 γ
Folic acid	1 γ	0.01 γ
Salts A:		
K ₂ HPO ₄	250 mg	5 mg
NH ₂ PO ₄	250 mg	5 mg

Table 3(continue)

Basal medium¹ for S. faecalis R and L. casei(ATCC9595) and
 basal medium for L. arabinosus 17-5 (ATCC8014)

Substance	Amount	
	<u>S. faecalis</u> R and <u>L. casei</u>	<u>L. arabinosus</u>
Salts D:		
MgSO ₄ ·7H ₂ O	100 mg	2 mg
NaCl	5 mg	0.01 mg
FeSO ₄ ·7H ₂ O	5 mg	0.01 mg
MnSO ₄ ·4H ₂ O	5 mg	0.01 mg
adjust to pH 6.8		
add distilled water to	250 ml	10 ml

¹The amino acid being assayed was omitted from the medium.

Results

Distribution of the growth factors for L. bulgaricus 09 and L. lactis 3000 in the source materials was investigated to determine the best sources. After determining the best source material, it was subjected to fractionation and its growth stimulating activity on both organisms compared. The chemical properties of the most active fraction, Fraction A, were studied. The results are as follows:

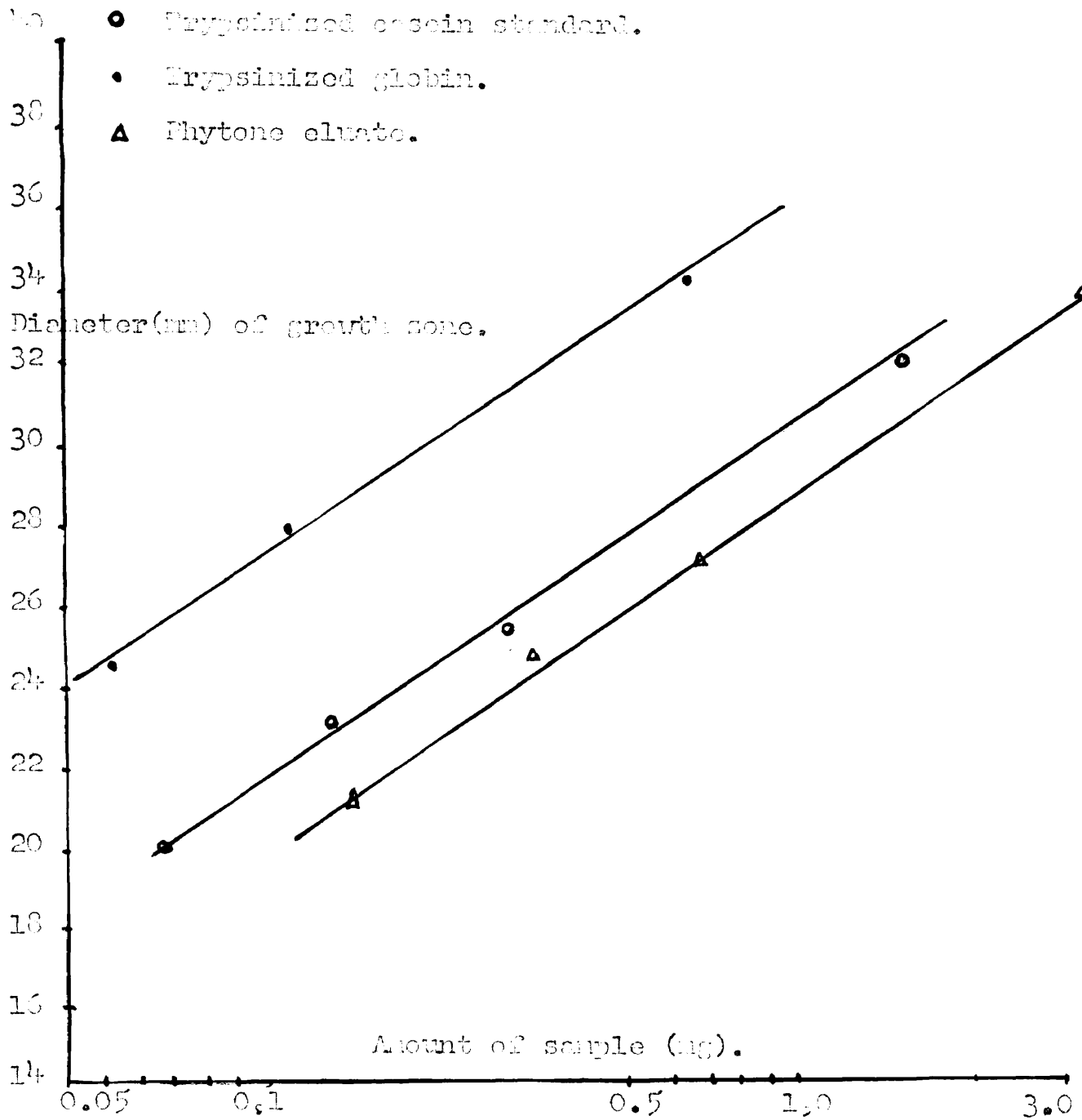
I. L. bulgaricus 09 plate and tube assays.

1. Standard curves.

A standard curve of trypsinized casein for the plate assay was first prepared and from this the activity in the source material could be estimated. In Figure 1 there are three curves, one for the standard and two for the samples. The lines for trypsinized globin and phytone dialysate Horite eluate are parallel to the standard curve and, therefore, indicate the presence of the same growth factor, although in different concentrations in each sample. Thus, the curve of trypsinized globin has a position above the standard curve and that of phytone dialysate below the standard curve. The trypsinized globin, therefore, has higher activity than the standard, and phytone dialysate Horite eluate has less activity than the standard.

The standard curve (Figure 2) for the tube assay differs from that for the plate assay. Ordinary graph paper was used

Figure 1

Growth curves of *L. vulgaris* 09 in the plate assay.

and curves, instead of straight line relationships, were obtained. In Figure 2, a standard and a sample of phytone dialysate were plotted. The curve for the phytone dialysate was below the standard curve of casein hydrolysate and therefore phytone dialysate has much lower activity than the standard.

From Figure 1 and Figure 2, it can be observed that the plate assay has greater sensitivity than the tube assay. Dosages required for growth in the tube assay are ten times those for the plate assay. This difference suggested that agar contained other required or growth stimulatory factors. However, the more active substance appeared to be measured by the plate method and this was the method of choice for most of the study on the distribution of the growth factor.

2. Distribution of the growth factors for L. bulgaricus 09 is summarized in Table 4. Some samples were used as obtained, while other were digested by Woolley's method. According to plate assay results, the growth factor existed mainly in liver, enzymes and enzymic digests of protein. In general, liver had the lowest activity, enzymes higher activity and enzymic digests of protein the highest. Among proteins, hemoglobin was the best source. Through observation of occasional inhibition zones within the growth zone and a study of the inhibitor, it was discovered that globin had more activity as a source material than hemoglobin.

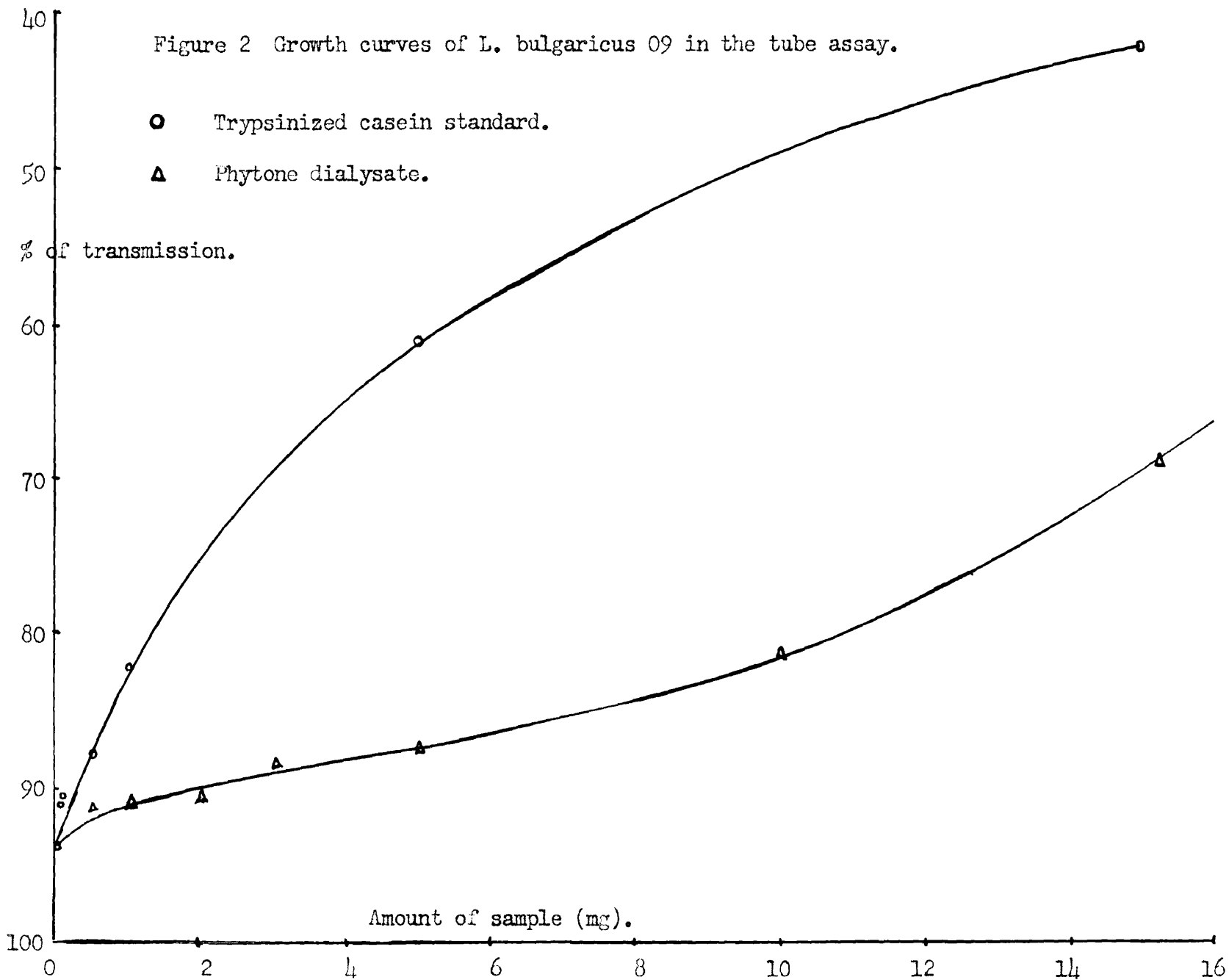
All samples tested by the tube method had a lower activity than the standard. Phytone eluate had a very low ac-

Figure 2 Growth curves of *L. bulgaricus* 09 in the tube assay.

○ Trypsinized casein standard.

△ Phytone dialysate.

% of transmission.



I. case, this would suggest that the putrescine factor is not essential in the highest activity to stimulate the growth of I. putrescens O9. However, they are different types. Therefore Zn-insulin does not stimulate the normal growth of I. putrescens O9. However,

Growth zone stimulated by homoglobin
 Growth zone stimulated by Zn-insulin



are denser. The growth zones are illustrated as follows:
 are lighter, whereas the colonies stimulated by homoglobin
 The colonies in the growth zones stimulated by Zn-insulin
 second place. In contrast Zn-insulin has a weak stimulation.
 homoglobin has the highest activity. Good albumin is in the
 hydrolyzed proteins is shown in Table 5. In the plate assay
 growth required factors for I. putrescens O9 in the acid
 by their acid hydrolysis method. The distribution of the
 may consist of two or more peptides, samples were prepared
 theory and Haddock (1951) have indicated that streptococci
I. putrescens O9 must have two or more components. Since
 growth of I. putrescens O9. Thus the growth factor affecting
 appears therefore that some factors in agar is affecting the
 lighter, but with pyruvate the tube value is lower. It
 pyruvate dextrose and glucose peptide, the tube value are
 and the plate methods compared, they are not identical. With
 activity. When the tube value of samples obtained by the tube

Table 4

Distribution of growth factors for L. bulgaricus 09.

Source material	Unit value ¹	
	Plate	Tube
Wilson's liver L	0.08	
Phytone ²	0.19	
Papain	0.25	
Pancreatin	0.27	
Edamin ³	0.34	
Trypsinized Cytochrom C	0.38	
Slade's peptide	0.39	0.56
Pepsin	0.43	
Trypsinized Zn-insulinate	0.55	
H-Z-case ³	0.57	
Phytone dialysate	0.61	0.88
Phytone ₂ eluate	0.64	0.21
Soytone ²	0.79	
Trypsinized casein(Merck 8R3051)	1.00	1.00
Pancreatin digested hemoglobin	1.18	
Trypsin	2.10	
Trypsinized hemoglobin	2.26	
Trypsinized globin	4.16	

¹One unit is defined as the activity equivalent to 1.0 mg of trypsinized casein(Merck 8R3051).

²Products of enzymic digests of soybean.

³Products of enzymic digests of casein.

Table 5

Growth stimulating activity of acid hydrolyzed protein*
for L. bulgaricus 09.

Samples	Plate assay
Trypsinized casein(Merck-8R3051)	1.00
Dialyzed agar	0.00
Takadiastase	**
Zn-insulinate	**
Pancreatin	**
Polidase-S	**
Mylase-P	**
Gelatin	0.33
Casein NBC	0.33
Alpha-protein	0.33
Yeast	0.52
Pepsin	0.52
Lactamin	0.58
Trypsin	0.76
Zein	0.86
Blood albumin	1.10
Hemoglobin	1.24

* Mistry and Kodecek's method(1951, 1952) was used to hydrolyze the proteins.

** light growth zone(small colonies).

tirely identical with the L. casei factor.

Compounds lacking activity for L. bulgaricus 09 are: L.B.F. (Lactobacillus bulgaricus factor), vitamin B_T (carnitine), asparagine, yeast nucleic acid, biocytin, citrovorum factor (folinic acid), 2,4-dimethyl-6,7-dipteridine, d-ribazole, 5,6-dimethylbenzimidazole and vitamin B₁₂.

Native proteins and proteins completely hydrolyzed by acids or base, individual amino acids or mixture of amino acids are lacking in growth stimulating activity for L. bulgaricus 09. A nondialyzable portion of trypsinized egg albumin, gelatin and beef serum prepared by Slade also have no growth stimulating activity for L. bulgaricus 09.

The assays with L. bulgaricus indicated that the growth factor had two or more parts, that it was not identical in activity with streptogenin for L. casei, and that hemoglobin was the best source. It was of interest to learn whether L. lactis would respond to the same source materials and fractions which were required for growth for L. bulgaricus 09.

II. L. lactis 8000 Plate and Tube Assays.

1. Standard curves.

Preliminary work had shown that phytone (Baltimore Biological Laboratory) had good growth stimulating properties for L. lactis 8000. A Horite eluate of phytone dialysate, described previously was chosen for a standard. A standard curve of the phytone eluate was prepared for the plate assay from which the activity of the source material could be estimated.

In Figure 3, the standard curve and curves of two samples are shown. These three curves are parallel. This parallelism indicate the similarity in the properties of these substances. Trypsinized globin had a higher activity than the standard and is located at a higher position in the figure; Wilson's liver L had a lower activity than the standard and is located at a lower position.

The phytone eluate standard curve for the tube assay is showed in Figure 4. Trypsinized casein had higher activity than the standard, while the curve for hemoglobin was not identical in shape with that of the standard curve. This would indicate that the distribution or concentration of factors in the samples was not entirely identical.

In comparison of Figure 3 and 4, it is found that the sensitivity of the plate assay is about ten times that of the tube assay, in that ten times the dosage is required for growth in the tube. It is identical with the result of L. bulgaricus in this respect.

2. The distribution of the growth factors for L. lactis 8000 are summarized in Table 6. In the plate assay, the growth factor existed mainly in liver, enzymes and enzymic digests. Asparagine had weak growth stimulating activity for L. lactis. Asparagine stimulated a diffused growth zone, which is different from the normal sharp growth zone observed. Among trypsinized proteins, trypsinized globin was again the best source material.

In the tube assay only Wilson's liver L had higher act-

Figure 3

Growth curves of L. lactis 8000 in the plate assay.

- Phytone eluate standard.
- Trypsinized chicken globin.
- ▲ Wilson liver L.

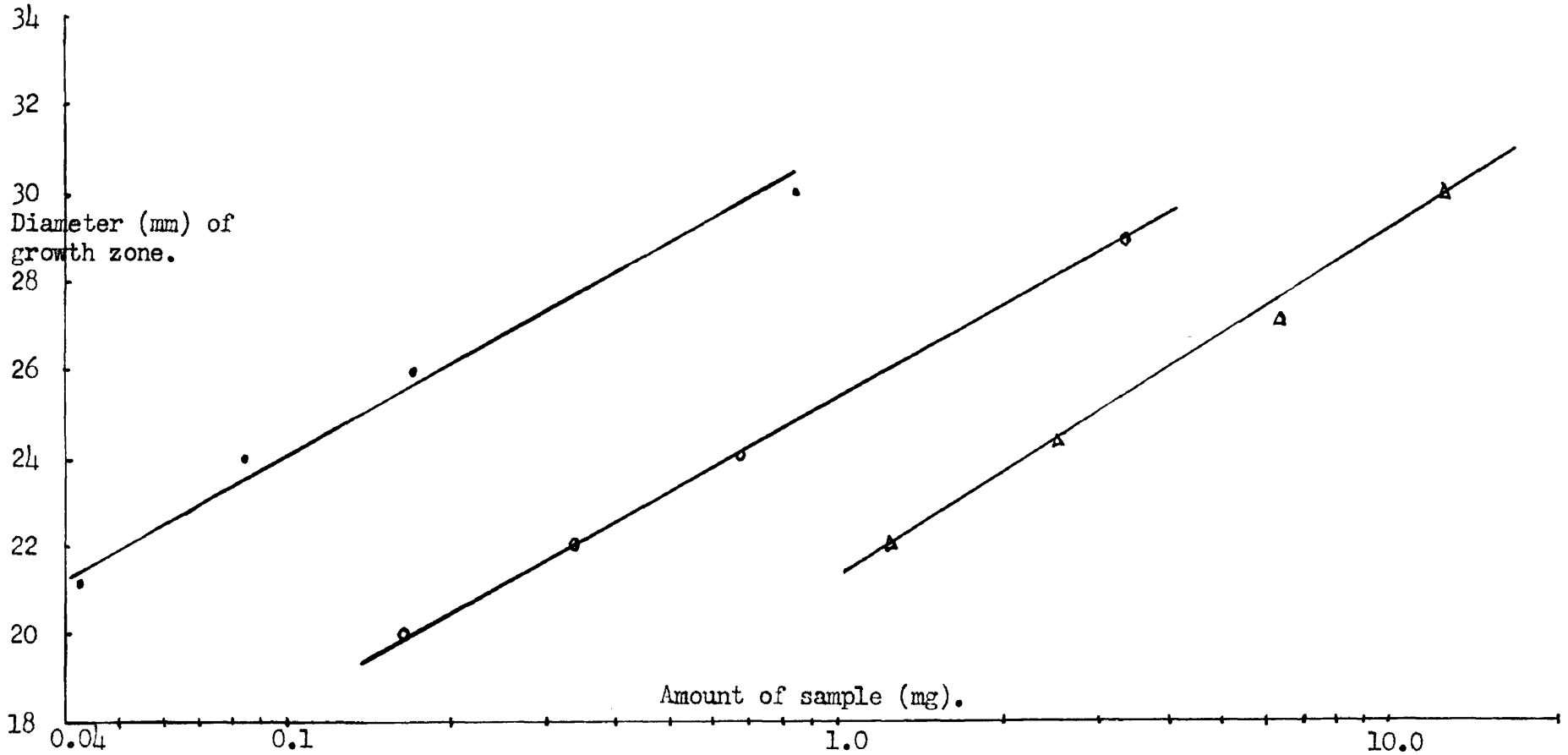


Figure 4

Growth curves of L. lactis 8000 in the
tube assay.

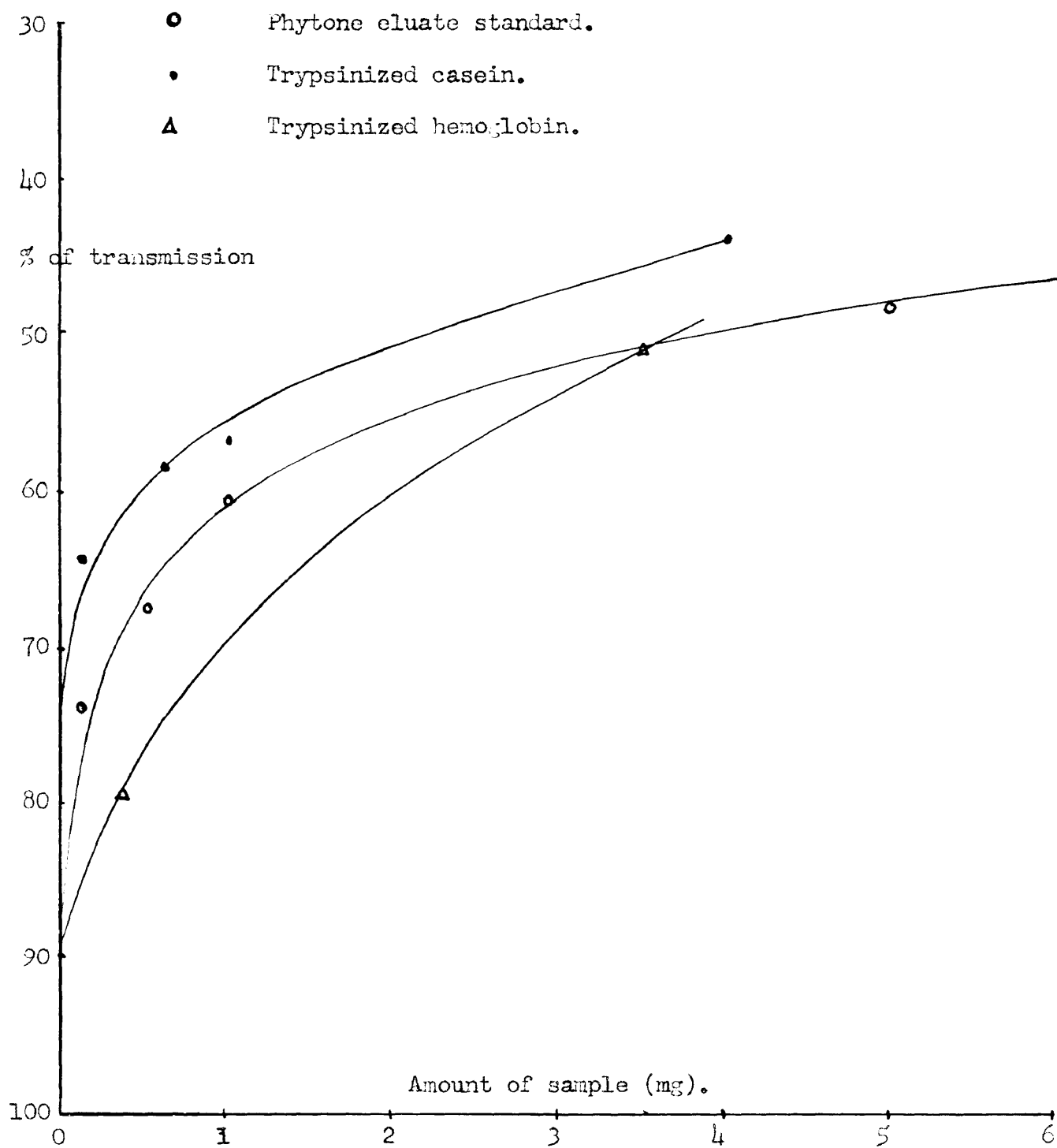


Table 6

Distribution of growth factor for L. lactis 8000.

Source Material	Unit value ¹	
	Plate	Tube
Tischkoff's peptide #1 ²	0.00	
Tischkoff's peptide #2 ²	0.00	
Peptone	0.11	
Wilson's liver L	0.12	1.02
Edamin ³	0.68	
Phytone eluate	1.00	1.00
Trypsinized Zn-insulinate	1.10	
N-Z-amine ³	1.04	0.46
Phytone dialysate	1.47	
Trypsinized cytochrom C	1.63	
Trypsinized blood albumin	2.35	
Trypsinized casein (Merck 8R3051)	2.68	2.11
Trypsinized blood fibrin	6.28	
Trypsinized hemoglobin	11.40	0.90
Trypsinized globin	14.17	

¹One unit is defined as the activity equivalent to 1.0 ng of solid of phytone eluate.

²Peptides isolated from liver.

³Products of enzymic digests of casein.

ivity than in the plate assay, while H-Z-amine, trypsinized casein and trypsinized hemoglobin had lower activity than in the plate assay. This discrepancy in values between plate and tube assay is similar to the findings in the L. bulgaricus assay.

Since asparagine and crude L.B.F. had weak growth stimulating activity for L. lactis 8000, an experiment was performed to supplement L.B.F. and asparagine in the tube medium and to determine the effect on the growth stimulating activity of phytone dialysate. The effect of the addition of synthetic L.B.F. (pantothen) and of asparagine was shown in Table 7. Increased growth was obtained by the supplementing in each tube to an extent of 4 to 9 points on the galvanometer scale. These supplements have been included in the L. lactis tube assay in all subsequent work.

The distribution of the growth factors of L. lactis 8000 in the acid hydrolyzed proteins was shown in Table 8. In the plate assays lactamin had the highest activity, while hemoglobin and trypsin were in second place. Pepsin, pancreatin and povidase-S had weak activity. Takadiastase, mylase-P, gelatin and insulin hardly showed growth stimulation.

Growth factors that lack activity for L. lactis 8000 are orotic acid, vitamin B₁₂, vitamin B_{12a}, vitamin B_{12b}, inositol, calcium and iron phytate, phytic acid, 5,6-dimethylbenzimidazole, biocytin, d-ribozole, pteroylamino acid (with the exception of pteroyl-dl-alanine, pteroyl-dl-methion-

Table 7

The improvement of L. lactis tube assay by the supplement of L.B.F. and asparagine.

Sample	amount (mg/tube)	Galvanometer Reading	
		Normal Medium	Normal Medium + L.B.F. (10 U./ tube) and L- asparagine (2mg / tube)
Phytone dialysate	0.0	87	73
	0.4	73	64
	0.8	70	60
	2.0	63	54
	6.0	56	51
	10.0	48	44

Table 8

Growth stimulating activity of acid hydrolyzed protein* for L. lactis 8000.

Sample	Plate assay
Phytone eluate	1.00
Mahadiastase	-
Mylase-P	+
Zn-insulinate	+
Gelatin	-
Pepsin	+
Pancreatin	+
Polidase-S	+
Yeast	0.22
Cascin NBC	0.26
Alpha-protein	0.31
Zein	0.31
Flood albumin	0.50
Trypsin	1.33
Hemoglobin	1.33
Lactamin	1.60

* Hirsty and Kodocek's method (1951, 1952) was used to hydrolyze the proteins.

ine, pteroyl-dl-valine and pteroyl-dl-alanine).

Protein, acid and base hydrolyzed protein, amino acids and a mixture of amino acids did not stimulate the growth of L. lactis 8000.

In the plate assay there were two types of growth zones. One was a zone with a sharp edge and the other was a zone with a diffuse edge. In L. lactis assay, asparagine, Wilson liver L and Lederle Liver extract had zones with a diffuse edge and enzymic digests had zones with a sharp edge. In the L. bulgaricus assay all samples had sharp edges. In both assays zones with diffuse edge were observed when samples of very low concentration were put in the cups.

The results of the L. bulgaricus assays and the L. lactis assays showed that the growth factors for both organisms consisted of two factors, i.e., the plate factor and the agar factor. The plate assay required only one tenth the amount of sample required for the tube assays, therefore, the plate assay was used generally for the determination of activity in the following fractionation program.

III. Fractionation of the growth stimulating factors for L. bulgaricus 09 and L. lactis 8000.

Since the distributions of the growth factors for L. bulgaricus 09 and L. lactis 8000 were similar, fractions were tested with both organisms. The activities of the fractions of trypsinized hemoglobin and trypsinized globin as a required growth factor for L. bulgaricus 09 are shown in Table 9. In trypsinized protein some inactive substance

Table 9

Activity of growth required factor of L. bulgaricus O9 in the fractions of trypsinized hemoglobin and trypsinized globin.

Fractions	Unit value*	
	Plate	Tube
Trypsinized casein (Merck 8R3051)	1.00	1.00
Trypsinized hemoglobin	3.26	
Lead acetate filtrate	1.27	
Precipitate after concentration of the filtrate	0.00	
Fraction A of hemoglobin	0.92	0.44
Trypsinized globin	4.16	
Fraction A of trypsinized globin	1.28	0.53

* One unit is defined as the activity equivalent to 1.0 mg of trypsinized casein (Merck 8R3051).

Could be precipitated by lead acetate, and some inactive substance could be precipitated by alcohol. The filtrate after removal of the inactive substance was termed fraction A of hemoglobin. Fraction A of trypsinized hemoglobin had less activity than the lead acetate filtrate, because of decomposition of peptide during manipulation. Neither fraction A nor the filtrate after the removal of the inactive substances had higher activity than the original trypsinized protein in cases of both hemoglobin and globin. The nature of the growth factors is seen to be complex. Fraction A of trypsinized globin had more activity than fraction A of trypsinized hemoglobin. In the tube assay the fractions stimulated weak growth. It was apparent that L. bulgaricus must require an additional factor in the tube, this factor being supplied by agar.

The activities of the same fractions of trypsinized hemoglobin and trypsinized globin for L. lactis 8000 is shown in Table 10. The results are similar to those for L. bulgaricus 09. In the plate assay all fractions had less activity than the original trypsinized protein. All fractions of trypsinized globin had higher activity than that of the corresponding fractions of trypsinized hemoglobin. In the tube assay all samples had relatively lower activity than that of the plate assay. Although no concentration was effected in the lead acetate precipitation. It appeared that separation of factor had occurred and the one measured in

Table 10

Activities of growth stimulating factors of L. lactis 3000
in the fractions of trypsinized hemoglobin and
trypsinized globin.

Fractions	Unit value*	
	Plate	Tube
Phytone eluate	1.00	1.00
Trypsinized hemoglobin	11.40	0.90
Lead acetate filtrate	2.31	
Precipitate after concentration of the filtrate	0.00	
Fraction A of hemoglobin	6.82	
Trypsinized globin	14.17	
Fraction A of trypsinized globin	8.39	

* One unit is defined as the activity equivalent to 1.0 mg
of solid phytone eluate.

the plate method could be most readily detected. It was of interest to study some properties of the plate growth factor in the different fractions and under varying conditions.

IV. Properties of the growth factor for L. bulgaricus 09 and L. lactis 8000 measured by the plate assay.

Phytone (papain digested soybean), trypsinized hemoglobin and fraction A of trypsinized globin were used to study the properties. The factor was dialyzable through a cellophane bag. The factor was stable to boiling for five minutes or to autoclaving at 121 C for fifteen minutes. The factor in phytone was stable to sun light for 20 days. The factor was destroyed by autoclaving in 1 N HCl or 1 N NaOH at 121 C for 20 minutes. The factor gave no reducing test, no xanthine test and no pyrimidine test. The factor gave positive ninhydrin test, positive biuret test but no heat coagulation. The factor could be very slightly precipitated by a large amount of alcohol, saturated picric acid, saturated ammonium sulfate, heavy metal salt or trichloroacetic acid. Concentrated crude source material could be precipitated by a mixture of methanol and ether or a mixture of methanol and n-hexane. The factor was hygroscopic. It was soluble in acids and bases. It was not soluble in organic solvents, such as ether, hexane, petroleum ether or ethyl laurate. The factor was partially adsorbed by Norite or Darco, but not adsorbed by MgCO₃, supercel, AL₂O₃ or Fuller's earth. These properties showed that the plate growth factor was peptide in nature.

The growth stimulating curves of fraction A of hemo-

globin and fraction A of globin in both L. bulgaricus 09 and L. lactis 8000 assay are shown in Figure 5. Fraction A of hemoglobin had lower activity than fraction A of globin, the curve of the former was below the curve of the latter. All curves are parallel to each other, which indicates that similar growth factors are measured.

Since the inhibiting zones were occasionally observed within the growth zones, which caused drifts in unit values calculated from different dilutions, it was of interest to study the nature of the inhibitor.

V. Inhibitor study.

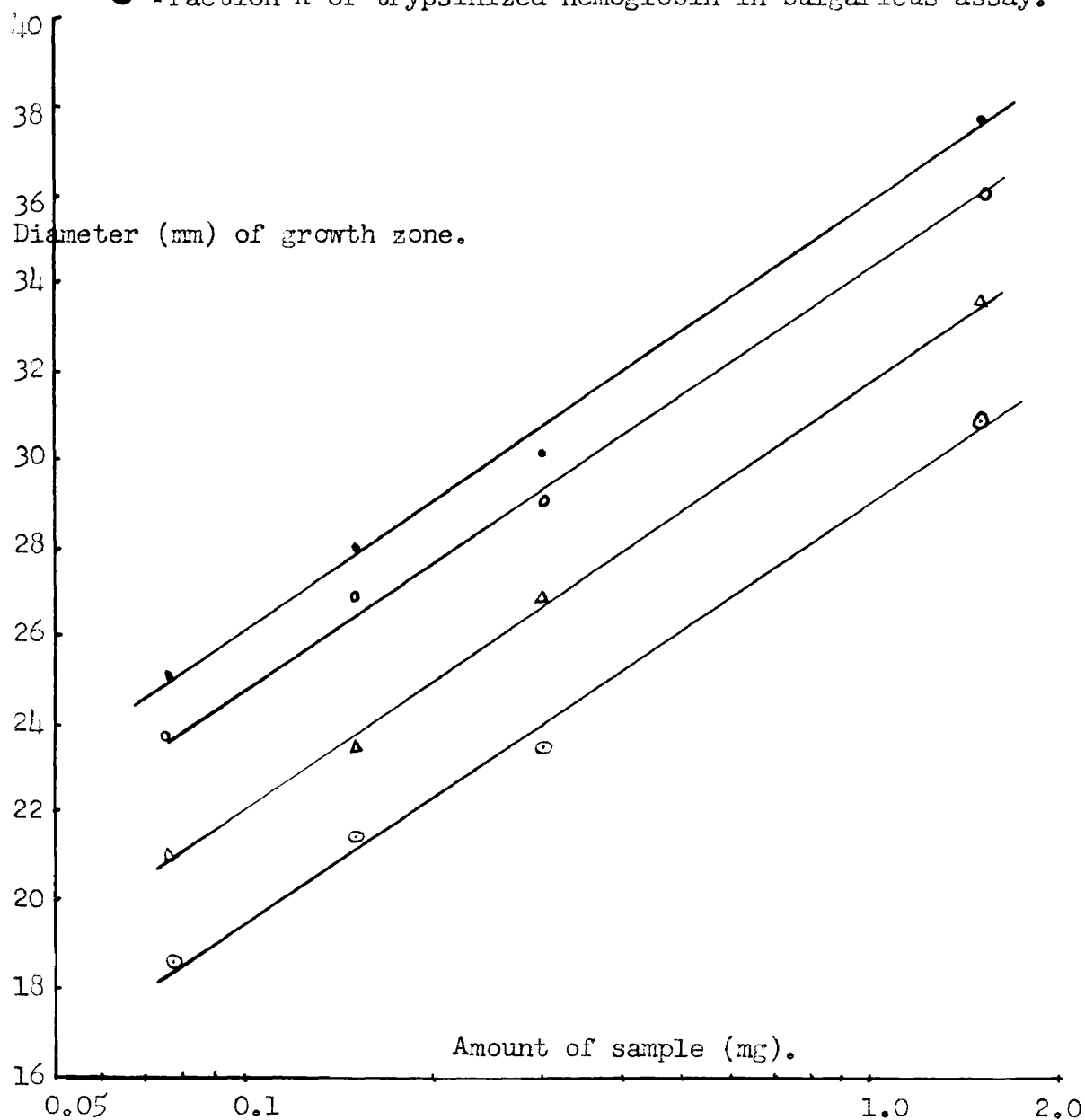
1. Inhibitor for L. bulgaricus 09.

The inhibitor was separated from the trypsinized hemoglobin solution by adjusting the pH of the solution to 3.0 and passing it through a column of filter paper and filtercel (1:2). The inhibitor was held on the top of the column. The column was washed with water having a pH of 3.0. The inhibitor was eluted from the column with water of pH 7.0. Characteristics of the inhibitor isolated from trypsinized hemoglobin were as follows: It was organic in nature. It was nondialyzable. A negative biuret test showed that it was neither a peptide nor protein. Iron was the constituted element of the inhibitor. The ash of the inhibitor gave red coloration upon the treatment with thiocyanide and blue coloration upon the treatment with ferrocyanide. The ash caused no inhibition in the microbiological assay. It suggested that heme part of hemoglobin was perhaps the inhibitor. This was proved to be

Figure 5

Growth curves of fraction A of trypsinized globin and trypsinized hemoglobin in the plate assay of L. bulgaricus 09 and L. lactis 8000.

- Fraction A of trypsinized globin in lactis assay.
- Fraction A of trypsinized hemoglobin in lactis assay.
- △ Fraction A of trypsinized globin in bulgaricus assay.
- ⊙ Fraction A of trypsinized hemoglobin in bulgaricus assay.



true by using heme in the L. bulgaricus assay. An inhibition curve was obtained (Figure 6) by adding fraction A of trypsinized hemoglobin to the medium (1 mg/ml of medium) to produce growth and different dilutions of heme in cups. The diameter of the inhibiting zone was plotted against the amount of heme on semi-logarithmic paper. A straight line relationship was obtained (Figure 6).

With fraction A of trypsinized hemoglobin (1 mg/ml) in medium the curve of heme had a lower slope, while the inorganic salt inhibitors, such as CaCl_2 had a steep line. Similar results were obtained when trypsinized casein (1 mg/ml) was added in the medium. The inhibiting action of heme was not as demonstrable in the tube assay as in the plate assay.

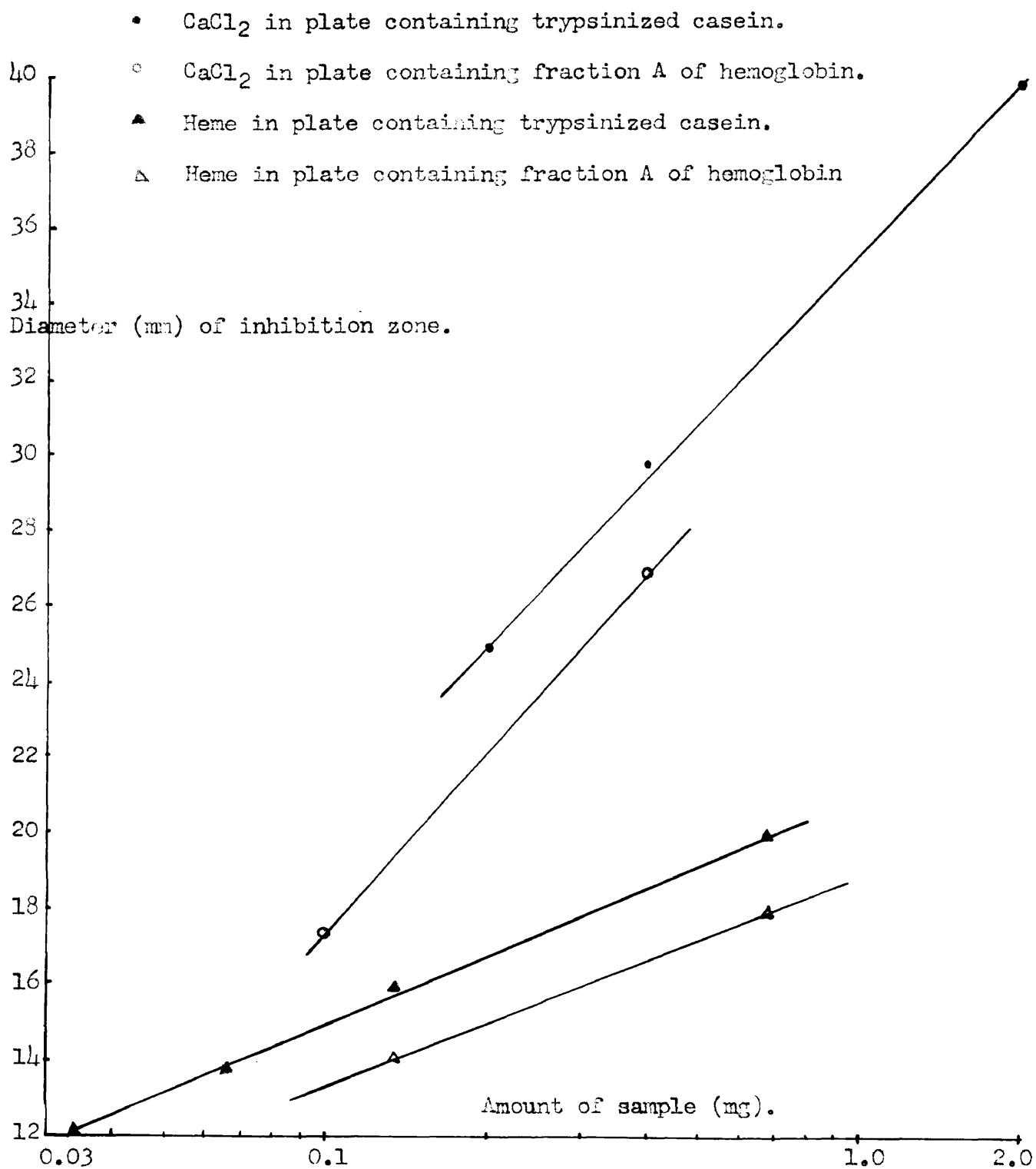
The effect of inorganic salt on the growth of L. bulgaricus 09 was also studied. $\text{pb}(\text{AcO})_2$ and AgNO_3 (500 γ) caused precipitation of the medium. Inorganic salts (500 γ) having inhibiting effect are listed as follows in decreasing activity: CoSO_4 , FeSO_4 , CuSO_4 , MnSO_4 , KCN, ZnCl_2 , Inorganic salts (1000 γ) having inhibiting action are: MgCl_2 and MgSO_4 . $\text{Ca}(\text{NO}_3)_2$ at 5 mg has inhibitory action. Other calcium salts, such as $\text{Ca}_3(\text{PO}_4)_2$ and CaSO_4 , are very slightly soluble and do not have growth inhibiting action.

2. Inhibitor of L. lactis 8000 in plate assay.

Potassium cyanide was reported (Cooperman, et al 1951, Soars and Hendlin 1951) to have a protective function for vitamin B_{12} in the L. lactis vitamin B_{12} tube assay, but when

Figure 6

Inhibition curves of heme and calcium chloride
in the bulgaricus assay.



KCN (0.25 μ /ml of medium) was used in the L. lactis plate stimulatory assay, no growth could be observed with the growth factor in the cup. The following table (Table 11) shows the effect of the addition of KCN to the plate medium. Products from liver and heme have the ability to reverse the growth inhibiting action of KCN on L. lactis 8000 in the plate assay, while the growth stimulator, fraction A, as well as trypsinized hemoglobin and casein fail to reverse KCN inhibition. In contrast, KCN (0.25 μ /ml) did not have a growth inhibiting effect on L. bulgaricus 09 in the plate assay.

The zone of stimulated growth in the L. lactis plate assay is observed only on early hour, normally after 8-10 hours of incubation. After this time growth occurs slowly over the entire plate. When KCN is in the medium, growth in the plate is inhibited even on prolonged incubation.

Table 11 shows that in the plate containing KCN, heme has growth stimulating activity.

In the previous section, it was shown that the distribution of growth factors for L. bulgaricus 09 and L. lactis 8000 is similar in the plate assay, being in highest concentration in trypsinized globin. It has also been shown that there are probably two factors for each organism. Since a ten fold concentration of samples is required for the tube assay as compared to the plate assay, the second factor may be supplied by agar and may also be found in combination with the plate factor in samples such as Wilson liver L and

Table 11

The effect of the addition of KCN (0.25 γ /ml)
to the plate medium of L. lactis 8000.

Samples	Amount (mg)	Diameter of growth zone(mm)	
		Normal medium	KCN added medium
Tischkoff's peptide #1 ¹	1.0	17	18
Tischkoff's peptide #2 ¹	1.0	16.5	16
Wilson liver L	12.5	27.5	26
Phytone eluate	3.3	29	?
Slade's peptide ²	1.0	25	-
Trypsinized casein	1.5	32	-
Fraction A of trypsinized hemoglobin	0.5	27	-
Heme	0.89	-	20

¹Peptides isolated from liver.

²Peptide obtained from non-dialyzable portion of trypsinized casein.

liver extract. The nature of the inhibitors for both organisms has been clarified. The presence of more than one growth factor usually can be demonstrated by the application of chromatographic techniques, therefore a chromatographic study was used on crude material and on the purified fractions.

VI. Chromatographic study of the plate growth factor for L. bulgaricus 09 and L. lactis 8000. The results of the chromatographic study are summarized in Table 12, 13, 14 and 15. In the L. bulgaricus plate assay, when water-saturated n-butanol was used as solvent, the R_f values of the most obvious zone of growth of the source materials were between 0.0-0.07 (Table 12). The R_f values of hemoglobin fractions were also between 0.01-0.08 (Table 13). Sometimes, as in phytone and trypsinized hemoglobin, one or two other weak growth zones were also observed, which indicated the complexity of the samples. When 60 percent cellosolve was used as solvent, the R_f values of the main growth zone was about 0.7-0.8 and the same R_f value was obtained from the fractions as from the crude materials.

In the L. lactis assay (Table 14-15), when water-saturated n-butanol was used as solvent, the R_f value of the plate growth factor was 0.0-0.01. There were weak growth zones with trypsinized hemoglobin $Pb(AcO)_2$ filtrate. When 60 percent cellosolve was used as solvent, the R_f value of the growth factor was about 0.8. When 80 percent phenol was used

Table 12

R_f values of the growth factors in the source materials for L. bulgaricus 09.

Source Material	R _f values		
	Tube Water-sate'd <u>n</u> -butanol	Water-sate'd <u>n</u> -butanol	Plate 60% Cel- losolve
Trypsinized casein 8R3051	0.01	0.01	0.83
Streptogenin (Trypsinized casein)		0.02 0.12*	
Wilson liver L		0.0	0.0-0.73
Phytone dialysate		0.05 0.125* -0.485	
Phytone eluate	0.01	0.01	0.80
Soytone		0.0	0.86
Yeast extract		0.0	
Slade's peptide		0.01	0.83
Tischkoff's peptide #1			0.89
Tischkoff's peptide #2			0.92
Trypsinized hemoglobin		0.07 0.27* 0.46* 0.67*	0.83

* Weak growth zone.

Table 13

R_F values of the growth factors in the fractions of trypsinized hemoglobin and trypsinized globin for L. bulgaricus O9.

Fractions	R _F values in plate assay	
	Water-sat'd n-butanol	60% cellosolve
Trypsinized hemoglobin	0.07 0.27* 0.46* 0.67*	0.84
Lead acetate filtrate	0.08	
Fraction A of trypsinized hemoglobin	0.01	0.81
Trypsinized globin		0.80
Fraction A of trypsinized globin	0.05	0.73

* weak growth zone.

Table 14 R_f values of the growth factors in the source materials for L. lactis 8000.

Source Material	R _f Value						
	Tube			Plate			
	Water-Sat'd n-BuOH:HAcO (1:1)	80% Phenol	Water-Sat'd n-BuOH	Water-Sat'd N-BuOH:HAcO (1:1)	80% Phenol	Water-Sat'd n-BuOH	60% Cellosolve
Trypsinized hemoglobin	0.08 0.2 0.4						0.39* 0.81
Peptone				0.0 0.22*			
H ₂ SO ₄ -hydrolyzed casein	-			-			-
Asparagine	0.0-0.4		-				
L.B.F.	0.05						
L.B.F. & Asparagine	0.0-0.4						
Yeast extract	0.03					0.0	
Lederle liver extract			0.05-0.15			0.0	
Phytone dialysate	0.03 0.2 0.5-1.0	0.03 0.1 0.17 0.2		0.0 0.07(I) 0.18* 0.24*	0.0 0.07(I) 0.42* 0.61* 0.80*		
Wilson liver L	0.02 0.1					0.0	-
Phytone eluate	0.05 0.15 0.30					0.0	0.77

Table 14 R_f values of the growth factors in the source materials for L. lactis 8000 (continue).

Source Material	R _f Value							
	Tube				Plate			
	Water-Sat'd n-BuOH:HAcO (1:1)	80% Phenol	Water-Sat'd n-BuOH	Water-Sat'd n-BuOH:HAcO (1:1)	80% Phenol	Water-Sat'd n-BuOH	60% Cellosolve	
Trypsinized casein	0.03 0.09		0.05 0.10 0.1-0.3		0.04(G&I) 0.16* 0.28* 0.43* 0.56* 0.78*			
Slade's peptide			-		0.0	0.0		-
Tischkoff's peptide#1								-
Tischkoff's peptide#								-

* Weak growth zone.

- Negative result.

I Inhibition.

G & I Growth and inhibition.

Table 15

R_f value of the growth stimulating factors for L. lactis 8000 in the fractions of trypsinized hemoglobin and trypsinized globin.

Fractions	R _f value		Plate 60% cel- losolve
	Tube Water-sat'd <u>n</u> -butanol	Water-sat'd <u>n</u> -butanol	
Trypsinized hemoglobin	0.02	0.01	0.81
Lead acetate filtrate	0.03	0.00	
	0.1*	0.23*	
	0.25*		
Fraction A of trypsinized hemoglobin			0.87
Trypsinized globin			0.86
Fraction A of trypsinized globin			0.95

* Weak growth zone.

as solvent, the activity of phytone dialysate could be separated by observation of several growth zones. When water-saturated n-butanol-HAcO (90:10) was used as solvent, the R_f value of the main growth factor was between 0.0-0.05. To compare the R_f values in these four tables, we find that the R_f values of the main growth factor for L. bulgaricus 09 were the same as those of L. lactis 8000 in both water-saturated butanol and in 60 percent cellosolve. These results suggested that the factors for the two organisms may be identical. The amount of sample used in the chromatogram is 0.4-2.0 mg. Since fraction A was the most active fraction of the tryptic digested hemoglobin and globin, a study of the fraction A was made.

VII. Studies on fraction A.

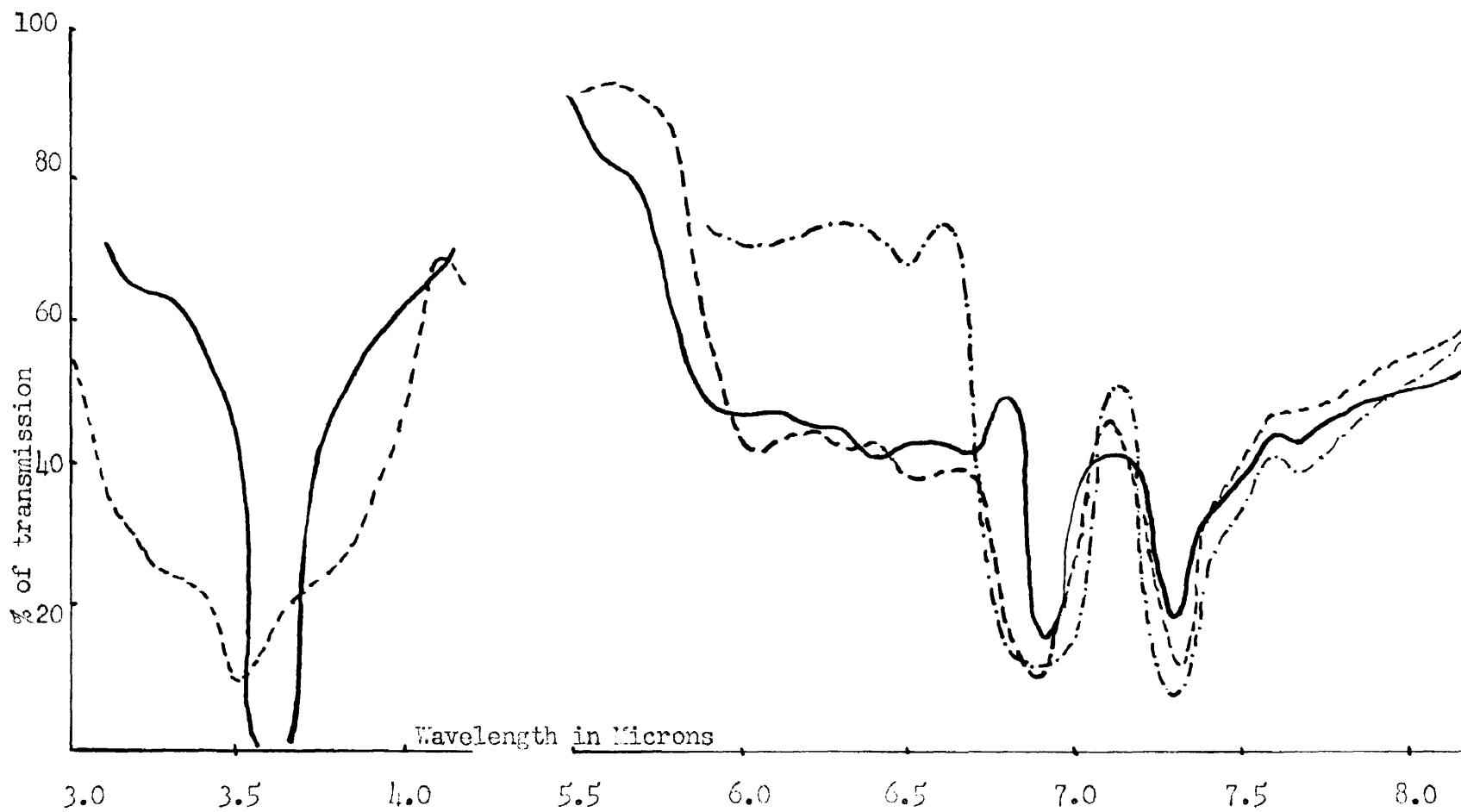
1. Infrared spectrum.

An attempt has been made to compare the effects of enzymic digestion on globin and hemoglobin by means of infrared spectroscopic study. The spectra shown in Figure 6 were obtained from Fujol null, i.e., suspension in paraffin oil, with a NaCl prism in a Perkin-Elmer Model 12C spectrometer. The spectra of fraction A of globin and of hemoglobin are quite similar, except for some minor differences in the medium and weak bands. Both fraction A of globin and of hemoglobin give strong peaks at around 3.5μ which is most probably the C-H frequency, and other two strong peaks (6.90 and 7.30μ), which are likely the CO-NH frequency.

2. Chromatographic study.

Figure 7 Infrared Spectra.

- Fraction A of trypsinized globin.
- - - - - Fraction A of trypsinized hemoglobin.
- - Lactoalbumin.

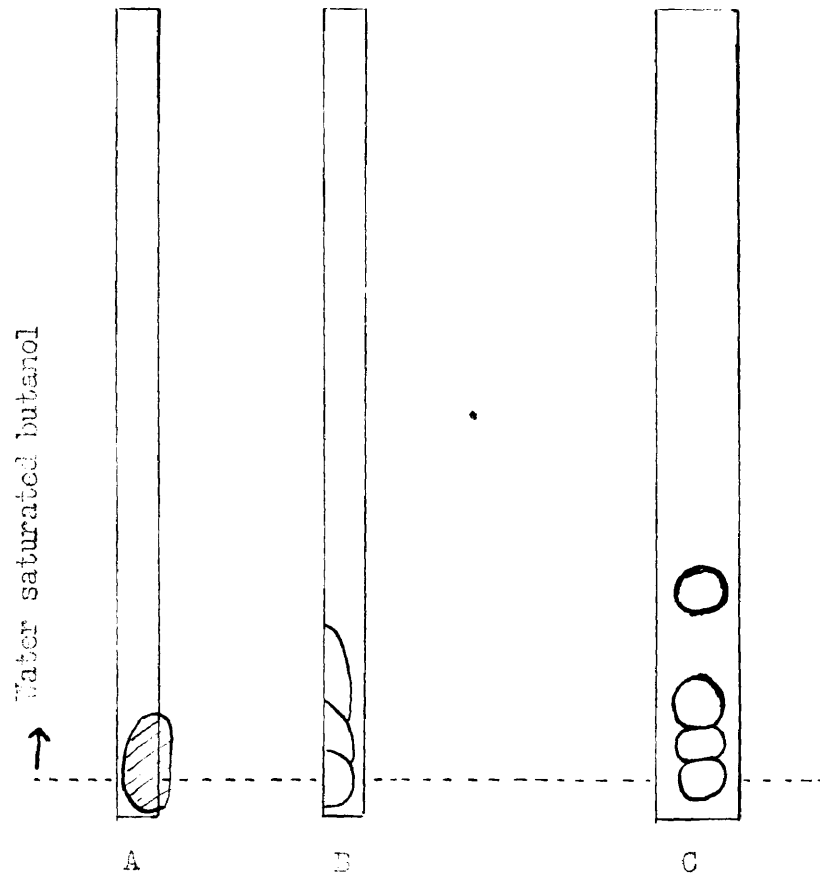


A one dimensional chromatogram of unhydrolyzed fraction A of hemoglobin using water-saturated butanol as solvent is shown in Figure 8. Strip A was the left half of the chromatogram. Water-saturated n-butanol was used as solvent and L. bulgaricus 09 was used as assay organism in the bioautographic study. The R_f value of the growth zone was 0.02. Strip B is the ninhydrin sprayed right half of the same chromatogram. There were three spots, which were not sharply separated. Therefore this fraction contained some free amino acids. Strip C is the ninhydrin-sprayed chromatogram of HCl-hydrolyzed fraction A of hemoglobin. The chromatogram was developed by water-saturated butanol. There were four spots.

A two dimensional chromatogram using 80 percent phenol as solvent for first dimension and water-saturated n-butanol as second dimension is shown in Figure 9. The chromatogram was ninhydrin-sprayed. Seven to eight spots were observed. The amino acids tentatively predicted were: Glutamic acid, aspartic acid, serine, glycine histidine, threonine, valine and leucine.

3. Since fraction A of trypsinized globin was more active than fraction A of hemoglobin, quantitative determination of the amino acid composition of fraction A of trypsinized globin was made by microbiological assay. The percentage composition of amino acids is leucine 23.5, valine 19.32, glutamic acid 11.0 serine 10.74, threonine 9.56, glycine 8.20, histidine 5.44, arginine 2.04, methionine 2.07, tryptophan 1.05 and tyrosine 1.03.

Figure 8 Bioautographic Assay and Ninhydrin Reaction of Chromatograms of Fraction (A) of Trypsinized Hemoglobin



A. Bioautographic assay (*L. bulgaricus*). Left half strip (1.45mg), unhydrolyzed sample.

B. Ninhydrin reaction. Right half strip (1.45mg), unhydrolyzed sample.

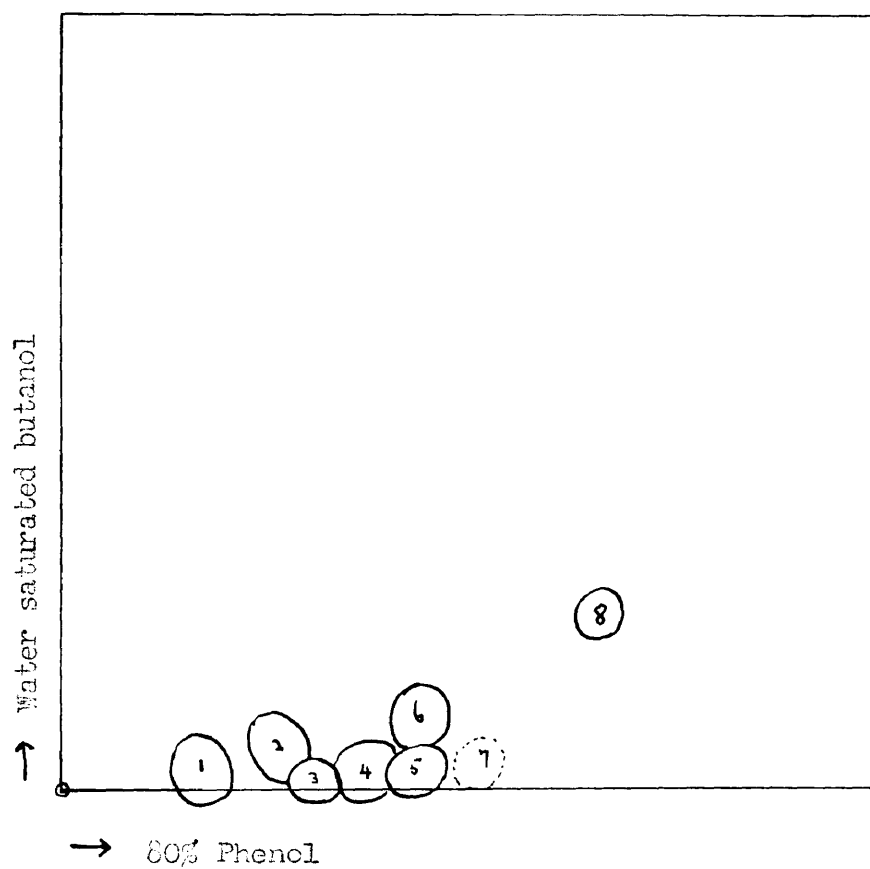
C. Ninhydrin reaction. Whole strip, HCl-hydrolyzed sample.

⊗ Growth zone

○ Ninhydrin spot

Figure 9

Two-dimensional Chromatogram of HCl-hydrolysate of
Fraction (A) of Trypsinized Hemoglobin.
Ninhydrin Reaction.



Amino acids predicted:

1. Glutamic acid
2. Serine
3. ?
4. Glycine
5. Histidine
6. Threonine
7. ?
8. Valine, leucine

It is of interest to compare the activity and the composition of fraction A of trypsinized globin with other peptides of known composition. The comparison is shown in Table 16. Slade's peptide consists of only six amino acids. Tischkoff's peptide consist of large number of amino acids. These two peptides have similar amino acids. By the microbiological assay fraction A consists of no aspartic acid, alanine, lysine and cystine, but much leucine and valine. Fraction A contains also tryptophan and methionine. Slade's peptide has one third of the activity of fraction A in L. bulgaricus assay and Tischkoff's peptides have only one six of the activity of fraction A in L. bulgaricus assay. In the L. lactis assay Slade's peptide has one twentieth the activity of fraction A, but Tischkoff's peptides had no activity. This table shows that glutamic acid, leucine, valine, serine and threonine peptide might be replaceable for the growth factor for L. bulgaricus 09 and L. lactis 8000.

VIII. The effect of the peptide growth factor on vitamin B₁₂ assay with L. lactis 8000.

The improvement of the vitamin B₁₂ assay by the addition of fraction A of globin to the medium of the tube and the plate assay are shown in Table 17 and 18. In B₁₂ tube assay (Table 17), the addition of 10 ng of fraction A of trypsinized globin to 0.1V of vitamin B₁₂ increased the galvanometer reading from 48 to 35. In vitamin B₁₂ plate assay (Table 18), the plate with fraction A gave smaller growth zones, which

Table 16

Amino acid content of different peptides and fraction A of trypsinized globin.

Amino acid	Slade's peptide	Fraction A of trypsin. globin(%)	Lischkoff's peptide*	
			#1	#2
Aspartic acid		0.0	7-8	10
Glutamic acid	+	11.0	8-9	9
serine	+	10.74	5-6	7-8
Threonine	+	9.56	3-0	5
Alanine	+	0.0	10	9-10
Valine	+	19.32	7	9
Leucine	+	23.5	9-10	10
Isoleucine	+	0.0		
Phenylalanine		0.0	1	3-4
Tyrosine		1.03	1	2
Arginine		2.04	3-4	3-4
Lysine		0.0	3-4	3-4
Proline			4	4
Hydroxyproline			4	1
Histidine		5.44	1-2	2
Glycine		8.20	10	10
Cystine		0.0	1	
methionine		2.07		
Tryptophan		1.05		
Unknown component 1			2	
Unknown component 2			1	

Activity by plate assay for:

<u>L. bulgaricus</u> 09	0.39	1.28	0.31	0.26
<u>L. lactis</u> 0000	0.43	8.39	0.00	0.00

* relative concentration.

Table 17

The improvement of vitamin B₁₂ tube assay by the addition of fraction A of trypsinized globin.

Samples	Galvanometer Reading at 20 hours
None	99
10 mg of fraction A of trypsinized globin	99
0.1 γ vitamin B ₁₂	48
0.1 γ vitamin B ₁₂ +10 mg of fraction A of trypsinized globin	35

Table 18

The improvement of vitamin B₁₂ plate assay by the addition of fraction A of trypsinized globin.

Samples	Diameter of growth zone(mm)	
	Normal plate	Fraction A added plate
Vitamin B ₁₂ 0.0025 γ	17s.	16.5s.d.
0.005 γ	20s.	18.0s.d.
0.01 γ	22.5s.	20.2s.d.
0.1 γ	27.2s.	26.5s.d.

s. sharp edge.
s.d. sharp and dense edge.

had a dense margin and facilitated the measurement of the diameter of the growth zones. Both tube and plate assay revealed that fraction A of trypsinized globin improved the microbiological assay of vitamin B₁₂.

The difference in the dosages of samples required for growth in the plate and in the tube assay led to the study of the growth factor in agar.

IX. Growth factor in agar.

Agar dialysate was added to the tube assay medium of L. bulgaricus and L. lactis at three levels, and agar dialysate was added to medium of tube assay of L. bulgaricus and L. lactis containing 10 mg of fraction A of globin at three levels, the effect of agar dialysate to the tube assay is shown in Table 19. The result shows that there is no definite increase of galvanometer readings in the L. lactis assay by the addition of agar dialysate, but definite increasing of growth can be observed in the L. bulgaricus assay by the addition of agar dialysate. The increase of growth can be shown by the increase of the turbidity, by the decrease of pH and by the increase of the amount of KOH used for neutralization. This result shows that agar does contain a growth factor for L. bulgaricus in the tube assay, but it is still not possible to explain the whole difference of the tube assay from the plate assay. There might be some other factor influencing the tube assay.

Table 19

Effect of agar dialysate on the tube assay of L. bulgaricus 09 and L. lactis 8000.

	<u>L. lactis</u> Galvonometer reading	Galvonometer reading	<u>L. bulgaricus</u> pH	ml of 0.477N KOH used
None	73.5	96.5	5.52	0.40
Agar dialysate 0.25ml	72.5			
2.50ml	65.0	96.0		
4.00ml	64.0	92.0	5.52	0.40
Fraction A of trypsinized globin 10mg	48.8	80.0	4.83	1.10
Fraction A of trypsinized globin 10mg + agar dialysate 0.25ml	49.0	82.0		
Fraction A of trypsinized globin 10mg + agar dialysate 2.5ml	46.5	83.0		
Fraction A of trypsinized globin 10mg + agar dialysate 4.0ml	46.0	63.5	4.61	1.65

Discussion

I. Comparison with strepogenin.

1. Distribution of growth factor for L. bulgaricus 09 and L. lactis 8000 and of strepogenin. In Table 4 and 6 the similarity in distribution of the growth factors for L. bulgaricus 09 and L. lactis 8000 was noted, except that liver sample had more activity for L. lactis 8000, crude L.B.F. concentrate and asparagine could stimulate only the growth of L. lactis 8000. The growth stimulating factor for L. bulgaricus 09 and L. lactis 8000 has a distribution similar to strepogenin in enzymic digests of protein (Table 20), except for insulin and crystallized trypsin for L. lactis. Insulin had high growth stimulating activity for L. casei, and low activity for L. bulgaricus 09 and L. lactis 8000. In Tables 5 and 8 the comparison of the distribution of strepogenin with the distribution of L. bulgaricus 09 and L. lactis 8000 in acid hydrolyzed protein also show that insulin was the best source for strepogenin and hemoglobin was the best source for L. bulgaricus 09 and L. lactis 8000.

2. R_f value. No reference can be found for the R_f value of strepogenin. In Tables 12, 13, 14, and 15 the growth factor for L. bulgaricus 09 and L. lactis 8000 had a R_f of about zero for water-saturated n-butanol and about 0.7-0.8 for 60 percent cellosolve. Since the growth factors had the same R_f value with different solvents for both organisms, it suggested the similarity or identity of these two growth factors.

Table 20

Comparison of the distribution of the growth factors for L. bulgaricus 09 and L. lactis 3000 with the distribution of strepogenin in enzymic digests of proteins.

Samples	Unit values per mg of solid Strepogenin			Growth factors for <u>L. bulg.</u> & <u>L. lactis</u>	
	<u>L. casei</u> Sprince, <u>et al</u> * 1945	<u>L. casei</u> Wright, <u>et al</u> 1950	<u>L. bulg.</u> Wright, <u>et al</u> 1950	<u>L. bulg.</u> This study 1952	<u>L. lactis</u> *
Gelatin	0.02	0.31	0.86		
Casein(vitamin- free)	1.00	1.00	1.00	1.00	1.00
Hemoglobin	1.2			2.26	4.25
Crystallin trypsin	2.6	1.3	1.2	2.1	
Crystallin insulin	8.0			0.55	0.41

* Converted the original data by the use of the activity of casein (vitamin free) as unity.

However, further work must be done by testing these factors in the L. casei assay before assuming identity or non-identity with strepogenin.

3. Properties. The properties of strepogenin were reported by Sprince and Woolley (1944). It is amphoteric, insoluble in alcohol, partially dialyzable, partially precipitated by lead acetate, and exists in acid hydrolyzed protein. Whole protein does not have strepogenin activity. Woolley's further study (1945) recognized that strepogenin had the nature of a peptide. Peptides from different sources had different activities. Further hydrolysis resulted in the reduction of the strepogenin activity. Mistry and Kodicek reported that strepogenin may contain more than one peptide. As compared with the properties of the growth factor for L. bulgaricus 09 and L. lactis 8000 the only difference was that Morite removed more of the growth factor for L. bulgaricus 09 and L. lactis 8000 than strepogenin.

4. So far as the composition is concerned strepogenin was reported to be rich in glutamic acid (Woolley 1946). Peptides containing glutamic acids also have strepogenin activity. In contract microbiological assay of the amino acid composition of fraction A of trypsinized globin showed that leucine and valine are present in higher concentration than glutamic acid in fraction A of trypsinized globin.

From the above four points it can be concluded that:

(1). If strepogenin is an unspecific term and includes several growth substances having peptide properties, the peptide growth factor (plate growth factor) for L. bulgaricus 09

and L. lactis 3000, therefore, might be classed as strepogenin.

(2). However, if the term strepogenin is specifically restricted to the substances that actively stimulated the growth of L. casei; if it is not absorbed by Norite; if it is highly concentrated in insulin and consists of an high percentage of glutamic acid, then the growth factor for L. bulgaricus 09 and L. lactis 3000 is an independent growth factor not related to strepogenin.

II. Growth factors for L. bulgaricus 09 and L. lactis 3000.

1. All source materials of the growth factor for L. bulgaricus 09 and L. lactis 3000 give positive biuret test, which indicates that the growth factor is peptide in nature. Paper chromatography showed that, the active peptide has a R_f value of 0.0, when water-saturated n-butanol is used as solvent and a R_f value between 0.7-0.8, when 80% cellosolve is used as solvent. This suggests that a single peptide or peptides having the same solubility in both solvents is responsible for the growth stimulation.

2. Asparagine and L.B.F. for L. lactis 3000. Table 7 reveals that asparagine and L.B.F. improved the tube assay of L. lactis 3000, and the specificity for the growth factor was retained. Asparagine and L. B. F. can be listed as

additional growth stimulating substances for L. lactis 8000. For L. casei Kodicek reported (1952) that asparagine had weak stimulation.

III. Inhibitors.

In the plate assay cyanide was found to have an inhibitory effect on the growth of L. lactis 8000, but no effect on the growth of L. bulgaricus 09. The cyanide inhibition could be reversed by the products from liver or by heme and heme has growth stimulating action to lactis in the presence of cyanide. This phenomenon is probably due to the poisoning bacterial enzymes. Heme and liver preparations may have similar structures as the enzyme and compete with the enzyme for KCN, thereby reversing the cyanide inhibition.

Heme had growth-inhibiting action on L. bulgaricus in plate assay, but failed to demonstrate a similar effect in plate assay of L. lactis. A straight line relationship was found when the amount of heme was plotted against the diameter in mm of the inhibiting zone (Figure 5).

The growth stimulating action of heme on various organisms in nutrient media was first reported by Kammer (1914). Later Lwoff (1948) reported that heme was a growth factor for certain bacteria, trypanosomes and even for certain blood-sucking insects. The specificity of the heme varied with the species, and their activity was found to be conditioned by the presence of vinyl groups in the molecule. The porphins other than protoporphine had an inhibitory effect on growth. This effect is competitive and required the presence of propanyl groups in the molecule (Example 1,3,5,

7-tetramethyl-2,4,6,8-tetrapropanyl porphin). Protoheme strongly inhibited certain bacteria and could even cause bacteriolysis. The sensitivity of organism varied considerably with species. The experiment was carried out on Strigomonas fasciculata, Haemophilus influenzae and Proteus vulgaricus. Van Heyninger (1948, 1951) reported that the growth of certain aerobic spore-forming bacilli was inhibited by heme in vitro. Recently Mick (1951) reported that sangunin, a less toxic substance prepared from blood cells, had a bacteriostatic effect on Streptococcus zooepidemicus.

Summary and Conclusion

1. For the materials tested, trypsinized globin is the best source of a growth stimulating factor for L. bulgaricus 09 and L. lactis 8000.
2. A peptide, fraction A of trypsinized globin, prepared by the removal of the inactive substance from the lead acetate filtrate of trypsinized globin, was the fraction with highest activity.
3. The R_f value of the peptide growth factor for L. bulgaricus 09 and L. lactis 8000 was 0.0 using water saturated n-butanol as solvent, and 0.7-0.8 using 60 percent cellosolve as solvent.
4. The percentage composition of amino acids of fraction A of trypsinized globin is : Tyrosine 1.03, tryptophan 1.05, methionine 2.07, arginine 2.04, histidine 5.44, glycine 8.20, threonine 9.56, serine 10.74, glutamic acid 11.0, valine 19.32 and leucine 23.54.
5. None is an inhibitor for L. bulgaricus 09, but a growth stimulating factor for L. lactis 8000.
6. Cyanide is an inhibitor for L. lactis 8000, but not for L. bulgaricus.
7. The required growth factor for L. bulgaricus 09 consisted of two portions: i.e. the peptide factor and the agar factor.

8. The growth stimulating factor for L. lactis 8000 consisted of the peptide factor, agar factor, asparagine and L.D.F.
9. The fractionation of growth activity in trypsinized hemo-globin resulted in the division of activity. The removal of heme from globin resulted in an increase of the growth activity.
10. The structure of the peptide determines the activity towards different organisms.

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Appendix

I. L. bulgaricus O9 plate assay.

Expt. no.	Sample	Amount (ng)	Diameter of growth zone (mm)	Equivalency to the amount of standard (ng)	Unit (ng of std./ng of sample)
1	Trypsinized casein (Merck 823051)	1.5	32.0, 32.5		
		0.3	25.5		
		0.15	23.0, 23.0		
		0.075	20.0, 20.0		
	Tryp. globin	0.635	34.5, 34.0	2.25	4.16
		0.127	28.0, 28.0	0.53	
		0.063	26.0, 24.5	0.27	
		0.031	21.0, 23.0	0.125	
	Phytone eluate	3.322	34.0, 33.5	2.15	0.64
		0.664	26.0, 28.0	0.42	
		0.332	24.0, 25.0	0.225	
		0.166	21.0, 21.0	0.090	
	Soytone	1.5	29.5, 30.0	0.80	0.79
		0.3	25.0, 23.5	0.235	
		0.15	21.5, 22.5	0.123	
		0.075	19.5, 20.5	0.076	
2	Tryp. casein (Merck SR 3051)	1.5	37.0, 35.5		
		0.3	30.0, 29.0		
		0.15	25.5, 26.0		
		0.075	23.5, 23.5		
	Wilson's liver L	12.5	35.0, 36.0	1.35	0.084
		6.25	32.4, 30.5	0.54	
		2.5	28.0, 29.0	0.265	
		1.25	27.0, 25.5	0.16	
3	Tryp. casein (Merck SR 3051)	1.5	32.0, 32.0		
		0.3	27.0, 26.5		
		0.15	24.0, 22.0		
		0.075	21.0, 20.0		

EXPT. No.	SAMPLE	AMOUNT (mg)	Diameter of growth zone (mm)	Equivalency to the amt. of standard (ng)	Unit (ng of std./mg of sample)
3	Tryp. Zn-insulinate	0.3	23.0,	0.155	0.55
		0.15	21.5	0.088	
		0.06	19.0,		
		0.03	17.5,		
Tryp. hemoglobin	0.175	26.5,	0.31	2.26	
	0.088	25.0,	0.22		
	0.035	21.0	0.006		
	0.018	20.0,			
Pancreatin digested hemoglobin	0.17	25.0,	0.2	1.18	
	0.034	19.0,			
	0.017	16.0,			
	0.0035	15.0,			
Tryp. cytochrom C	0.38	23.0,	0.125	0.38	
	0.19	22.5,	0.082		
	0.076	17.5,			
	0.039	15.5,			
4	Tryp. casein (Merck 3051)	1.5	32.0,		
		0.3	24.0,		
		0.15	22.0,		
		0.075	19.0,		
Phytone	10.0	36.0,		0.19	
	2.0	26.0,	0.38		
	1.0	23.0,	0.19		
	0.2	21.0,	0.12		
Slide's peptide	1.5	29.0,	0.7	0.39	
	0.3	19.0,	0.094		
	0.15	17.0,			
Fraction A of tryp. hemoglobin	1.5	29.0,	1.3	0.92	
	0.3	23.5,	0.235		
	0.15	21.5,	0.147		
	0.075	19.0,	0.078		

Expt. no.	Sample	Amount (mg)	Diameter of growth zone (mm)	Equivalency to the amt. of standard (mg)	Unit (mg of std./mg of sample)	
5	Tryp. casein (Merck BR 3051)	1.5	26.0, 27.0			
		0.3	22.5, 21.0			
		0.15	19.0, 19.0			
		0.075	17.5, -			
	Edaminc	1.0	21.5	0.27	0.335	
		0.2	17.5	0.066		
		0.1	15.0			
	Papain	1.0	20.0	0.17	0.25	
		0.2	17.0	0.066		
	Pancreatin	1.0	21.5	0.27	0.27	
		0.2	15.5			
	Pepsin	1.0	23.5	0.53	0.43	
		0.2	17.0	0.066		
	Trypsin	1.0	30.5		2.10	
0.2		24.0	0.42			
N-Z-case	1.0	23.0	0.43	0.57		
	0.2	19.0	0.125			
	0.1	17.0	0.066			
6	Trypsinized casein (Merck BR3051)	0.5	36.0, 36.0			
		0.1	27.0, 27.0			
		0.05	24.0, 23.0			
		0.025	- , -			
	Nytone dialysate	3.43	40.0, 38.0		0.61	
		0.687	34.0, 34.0	0.35		
		0.343	32.0, 30.0	0.20		
		0.172	29.0, 28.0	0.125		
	7	Tryp. casein (Merck BR 3051)	1.5	34.0, 33.0		
			0.3	26.0, 25.0		
0.15			23.0, 22.0			
0.075			20.0, 20.0			

Expt. no.	Sample	Amount (mg)	Diameter of growth zone (mm)	Equivalency to the amt. of standard (mg)	Unit (mg of std./mg of sample)
7	Tryp. hemoglobin lead acetate filtrate	6.08	46.0, 43.0	2.1	1.72
		1.216	35.0, 35.0		
		0.61	32.0, 32.0		
		0.24	27.0, 27.0		
8	Tryp. casein (Merck 3R 3051)	1.5	35.0	0.45 0.185 0.084	1.28
		0.3	27.0		
		0.15	23.5		
		0.075	21.0		
	Fraction A of tryp. globin	1.5	38.0		
		0.3*	29.0		
		0.15	25.0		
		0.075	21.5		
9	Tryp. casein (Merck 3R 3051)	1.5	29.0	0.145 0.145 0.145 0.21 0.235 0.27 0.35 0.40 0.52 0.59	0.29 0.29 0.29 0.42 0.47 0.54 0.70 0.80 1.04 1.18
		0.3	23.5		
		0.15	19.0		
		0.075	17.0		
	Takadiastase	0.5	15.0*		
	Zn-insulinate	0.094	16.5*		
	Pancreatin	0.5	17.0*		
	Polidase-S	0.5	17.0*		
	Mylase-P	0.5	18.0*		
	Gelatin	0.5	20.0		
	Casein NBC	0.5	20.0		
	Alpha-protein	0.5	20.0		
	Pepsin	0.5	21.5		
	Yeast	0.5	22.0		
	Lactamin	0.5	22.5		
	Trypsin	0.5	23.5		
	Zein	0.5	24.0		
	Blood albumin	0.5	25.0		
	Hemoglobin	0.5	25.5		

* light growth zone (smaller colonies).

II. L. bulgaricus 09 tube assay.

Expt. no.	Sample	Amount (mg)	Galvanometer reading	Equivalency to the amt. of standard (mg)	Unit (mg of std./mg of sample)
10	Tryp. casein (Merck 8R 3051)	15.0	42		
		5.0	62, 60		
		1.0	81, 33		
		0.5	87, 88		
		0.1	90, 91		
		0.05	90, 92		
		0.0	93, 94		
	Phytone eluate	19.9	50, 53	7.5	0.21
		10.0	81, -	1.25	
		5.0	88, 85	0.66	
		3.0	87, 90	0.62	
		1.0	91, 90		
		0.5	91, -		
		0.0	93, 94		
	Slade's pep- tide	10.0	57, 60	5.1	0.56
		5.0	65, 66	2.9	
		1.0	86, 88	0.59	
		0.5	92, 93		
		0.2	93, 94		
		0.0	93, 94		
11	Tryp. casein (Merck 8R 3051)	25.0	62		
		15.0	78		
		5.0	91		
		1.0	93		
		0.5	96		
		0.1	97		
		0.0	100		
	Phytone dia- lysate	20.0	75	15.0	0.875
		10.0	82	10.0	
		5.0	93		
		1.0	97		
		0.0	100		

Expt. no.	Sample	Amount (mg)	Galvanometer reading	Equivalency to the amt. of standard (mg)	Unit (mg of std./mg of sample)
11	Fraction A of trypt. hemo-globin	20.0	86	6.9	0.144
		10.0	90	5.3	
		5.0	96		
		1.0	97		
		0.0	100		
	Fraction A of trypt. globin	10.0	90	5.3	0.53
		1.0	96		
		0.1	97		
		0.01	99		
		0.0	100		

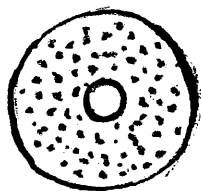
III. L. lactis 8000 plate assay.

Expt. no.	Sample	Amount (mg)	Diameter of growth zone (mm)	Equivalency to the amt. of standard (mg)	Unit (mg of std./mg of sample)
12	Phytone eluate	3.322	29.0, 29.0		
		0.664	25.0, 24.0		
		0.332	23.5, 22.0		
		0.166	20.0, 20.0		
Wilson's liver L	Wilson's liver L	12.5	30.0, 31.0	2.5	0.12
		6.25	26.0, 27.0	0.5	
		2.5	25.0, 23.5	0.2	
		1.25	22.0, 23.0		
Trypt. chicken globin	Trypt. chicken globin	0.84	30.0	4.6	6.33
		0.17	26.0	1.22	
		0.084	24.0	0.62	
		0.042	21.0	0.23	
Trypt. Zn-insu- linate	Trypt. Zn-insu- linate	0.3	22.0	0.34	1.1
		0.06	?		
		0.03	-		

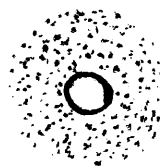
EXPT. no.	Sample	Amount (mg)	Diameter of growth zone (mm)	Equivalency to the amt. of standard (mg)	Unit (mg of std./mg of sample)	
12	Tryp. cytochrom C	0.38	24.0	0.62	1.63	
		0.07	18.0			
		0.04	17.0			
		0.02	16.0			
	Tryp. hemoglobin	0.23	25.0, 26.0	1.03	11.4	
		0.046	24.0, 23.0	0.54		
		0.023	21.0, 21.0	0.23		
		0.011	19.0, 20.0	0.14		
	13	Phytone eluate	3.322	30.0, 31.0		
			0.664	28.0, 27.5		
0.332			25.0, 25.0			
0.166			23.5, 24.0			
Tryp. casein (Merck 8R 3051)		1.5	33.5, 31.0	4.3	2.68	
		0.3	28.0, 28.0	0.8		
		0.15	25.0, 26.5	0.38		
		0.075	24.0, 25.0	0.24		
14		Phytone elu- ate	3.322	36.0, 36.0		
			0.664	32.0, 31.0		
	0.332		28.0, 29.0			
	0.166		26.0, 27.0			
	Tischkoff's peptide #1	1.0	- , -			
		0.2	- , -			
		0.1	- , -			
	Tischkoff's peptide #2	1.0	- , -			
		0.2	- , -			
		0.1	- , -			
		0.05	- , -			
	Slade's pep- tide	1.5	31.0, 31.0	0.66	0.43	
		0.3	25.0, 25.0			
		0.15	23.0, 21.0			

Expt. no.	Sample	Amount (mg)	Diameter of growth zone (mm)	Equivalency to the ant. of standard (mg)	Unit (mg of std./mg of sample)	
14	Tryp. hemoglobin lead acetate filtrate	2.43	40.0, 41.0		2.305	
		1.216	40.0, 40.0			
		0.608	33.0, 35.0	1.65		
		0.243	29.0, 32.0	0.46		
	Fraction A of tryp. hemoglobin	1.5	38.0, 38.0		6.82	
		0.3	36.0, 35.0	2.65		
		0.15	33.0, 32.0	1.03		
		0.075	29.0, 29.0	0.36		
	15	Phytone dialysate	3.4	32.0, 32.0		
			1.7	30.0, 30.0		
0.85			27.0, 28.0			
0.425			25.0, 25.0			
0.213			22.0, -			
Asparagine			1.0	25.0, 25.0 D		
16	Wilson's liver L	12.5	33.0, 31.0			
		6.25	30.0, 29.0			
		2.5	26.0, 26.0			
		1.25	25.0, 25.0			
		0.625	22.0, 22.0			

D Diffused edge of growth zone.



Growth zone stimulated by phytone eluate



Growth zone stimulated by asparagine

Expt. no.	Sample	Amount (mg)	Diameter of growth zone (mm)	Equivalency to the amt. of standard (mg)	Unit (ng of std./mg of sample)	
16	Peptone	1.0	23.0, 24.0	0.94	0.112	
		0.1	- , -			
	H-Z-amine	1.0	31.0, 29.0	7.2	1.04	
0.1		24.0, 24.0	1.02			
	Edanine	1.0	28.0, 30.0	5.4	0.68	
		0.1	21.0, 23.0	0.6		
17	Phytone eluate	3.32	29.0, 30.0	0.94	1.47	
		0.664	25.0, 26.0			
		0.332	24.0, 24.0			
		0.166	22.0, 22.0			
	Phytone dialysate	3.433	30.0, 31.0	0.94	1.47	
		0.686	27.0, 26.0			
		0.343	25.0, 25.0			
		0.172	23.0, 23.0			
	18	Phytone dialysate	4.4	37.0, 37.0	0.78	2.35
			2.2	35.0, 35.0		
1.0			31.0, 32.0			
0.55			30.0, 30.0			
0.275			27.0, 26.0			
0.18			24.0, 24.0			
	Tryp. blood albumin	0.625	31.0, 32.0	0.24	6.28	
		0.121	27.0, 27.0			
		0.02	- , -			
	Tryp. blood fibrin	0.625	35.0, 35.0	2.45	6.28	
		0.121	30.0, 30.0			
		0.02	25.0, 26.0			
		0.01	- , -			
19	Phytone eluate	3.322	32.0, 31.5	0.57		
		0.664	28.0, 28.0			
		0.332	25.0, 26.4			
		0.166	23.5, 24.0			

Expt. no.	Sample	Amount (mg)	Diameter of growth zone (mm)		Equivalency to the amt. of standard (mg)	Unit (mg of std./mg of sample)
19	Tryp. globin	0.395	35.0,	31.0	5.4	14.7
		0.08	30.0,	29.0	1.45	
		0.039	26.0,	26.5	0.42	
		0.02	23.5,	22.5	0.13	
	Fraction A of trypt. globin	1.5	37.0,	37.0		8.39
		0.3	31.0,	31.0	2.6	
		0.15	30.0,	30.0	1.7	
		0.075	26.0,	26.0	0.39	
20	Phytone eluate	3.32	35.0,	33.5		
		0.664	29.0,	30.0		
		0.332	26.0,	26.5		
		0.166	23.5,	22.5		
Acid hydrolyzed proteins:						
	Gelatin	0.5	-	-		
	Takadiastase	0.5	-	-		
	Zn-insulinate	0.094	19.5,	20.0		
	Nylase-P	0.5	20.0,	20.0		
	Polidase-S	0.5	17.5,	18.5		
	Pepsin	0.5	19.0,	19.0		
	Pancreatin	0.5	20.0,	18.0		
	Yeast	0.5	25.5,	22.5	0.12	0.24
	Casein NBC	0.5	24.0,	25.0	0.16	0.28
	Zein	0.5	24.0,	26.0	0.165	0.33
	Alpha-protein	0.5	25.0,	25.0	0.165	0.33
	Blood albumin	0.5	25.5,	27.5	0.26	0.52
	Trypsin	0.5	29.0,	30.0	0.68	1.36
	Hemoglobin	0.5	29.0,	30.0	0.68	1.36
	Lactamine	0.5	29.0,	31.0	0.70	1.56

IV. L. lactis 8000 tube assay.

Expt. no.	Sample	Amount (mg)	Galvanometer reading	Equivalency to the amt. of standard (mg)	Unit (mg of std./mg of sample)
21	Phytone eluate	10.0	43, 41		
		5.0	49, 47		
		1.0	61, 59		
		0.5	71, 63		
		0.1	77, 70		
		0.0	91		
	Tryp. Casein (Merck 8R 3051)	5.0	44, 43	8.0	2.11
		1.0	56, 57	1.77	
		0.5	60, 56	1.48	
		0.1	64	0.74	
		0.0	91		
	Wilson's liver L	5.0	40, 38	13.5	1.02
		3.0	55, 48	3.2	
		1.0	65, 61	0.84	
		0.5	68, 64	0.58	
0.1		80, 78			
22	Phytone eluate	10.0	31, 29		
		5.0	38, 40		
		3.0	41, 46		
		1.0	56, 54		
		0.5	65, 62		
		0.2	71, 69		
		0.0	91, 87		
	N-E-amine	10.0	32, 35	5.2	0.46
		1.0	58, 54	0.4	
		0.5	62, 59		
		0.1	66, 66		
		0.05	68, 70		
	0.0	91, 87			

Expt. no.	Sample	Amount (mg)	Galvanometer reading	Equivalency to the amt. of standard (ng)	Unit (ng of std./mg of sample)
23	Phytone eluate	10.0	41		
		5.0	49, 52		
		3.0	51, 53		
		1.0	66, 66		
		0.5	71, 73		
		0.2	77, 73		
		0.0	94, 91, 95		
		Tryp. hemoglobin	3.56	49, 52	4.4
		0.365	81, 79	0.2	
		0.0	94, 91, 95		

V. Growth curves of fraction A of trypsinized globin and trypsinized hemoglobin in the plate assay of L. bulgaricus 09 and L. lactis 8000.

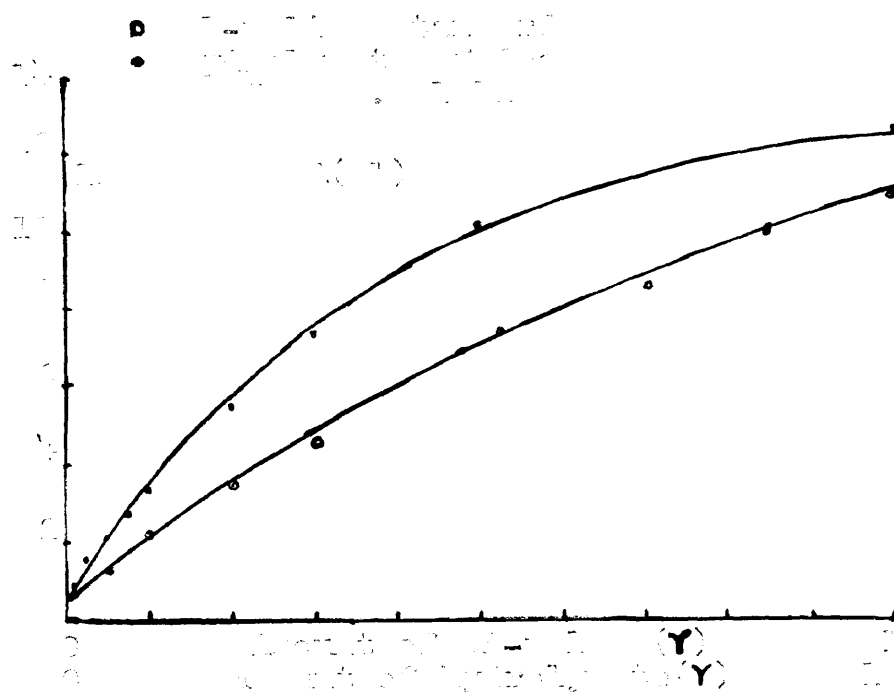
Sample	Amount (mg)	Diameter of growth zone (mm)	
		<u>L. lactis</u>	<u>L. bulgaricus</u>
Fraction A of trypsinized hemoglobin	1.5	38.0, 34.0	29.5
	0.3	29.0, 28.0	23.0
	0.15	28.0, 26.0	22.0
	0.075	24.5, 23.5	19.0
Fraction A of trypsinized globin	1.5	37.0, 37.0	34.5, 33.0
	0.3	31.0, 30.0	27.0, 26.0
	0.15	28.0, 28.0	23.0, 23.5
	0.075	26.0, 24.0	21.0, 21.0

VI. Inhibition curves of heme and calcium chloride in the L. bulgaricus 09 plate assay.

SAMPLE	Amount (ng)	Diameter of inhibition zone(mm)	
		Plate containing tryp. casein	Plate containing fraction A of tryp. hemoglobin
Calcium chloride	20.0	40.0	-
	4.0	30.0	27.0
	2.0	25.0	17.0
	1.0	-	-
Heme	0.67	20.0	18.0
	0.134	16.5	14.0
	0.067	14.0	-
	0.034	12.0	-

VII. Microbiological assay of amino acids in reaction A of tryptophan-synthesised globin.

Example of the microbiological assay of DL-valine.



Amount of hydrolysate (Y)	Amount of DL-valine in the standard (Y)	Percentage of DL-valine in the hydrolysate (%)	Average (%)
500	25.0	17.0	10.32
300	15.5	17.2	
200	37.5	19.3	
100	20.0	21.0	
75	14.5	21.6	
50	10.5	20.1	
25	5.0	20.0	

A. Titrimetric method.

Amino acid	Amount (γ)	1N NaOH used (ml)	Amt. of hydro- lysate of fra- ction A used (γ)	1N NaOH used (ml)	Percentage of amino acid in fraction A
DL-valine	uninocu- lated	0.25	10	0.95	19.32
	0	0.50	25	1.65	
	10	2.35	50	2.25	
	20	3.50	75	2.75	
	30	4.64	100	3.40	
	40	5.25	200	5.50	
	55	7.55	300	7.50	
	70	8.70	500	10.31	
	85	10.15	1000	12.70	
	100	11.00			
Glutamic acid	uninocu- lated	0.50	500	1.25	11.00
	0	0.70	1000	1.75	
	50	1.20	3000	3.50	
	100	1.95	4000	3.40	
	200	2.85			
	400	3.20			
	800	3.60			
DL-methio- nine	uninocu- lated	2.00	10	3.65	2.07
	0	3.75	25	3.95	
	10	6.60	50	4.50	
	20	8.40	75	4.20	
	30	9.90	100	4.70	
	40	11.10	200	5.15	
	55	11.30	300	5.75	
	70	11.05	500	6.95	
	85	11.65	1000	9.00	
	100	12.10			
L(-)tyrosine	uninocu- lated	0.90	10	1.80	1.03
	0	1.45	25	1.60	
	10	3.80	50	1.75	
	20	7.10	75	1.75	
	30	9.20	100	1.85	

Amino acid	Amount (γ)	1N NaOH used (ml)	Amt. of hydro- lysate of fra- ction A used (γ)	1N NaOH used (ml)	Percentage of amino acid in fraction A
L(-)tyrosine	40	7.40	200	1.40	
	55	9.00	300	1.90	
	70	9.80	500	2.55	
	85	10.20	1000	2.75	
	100	10.60			
L(-)arginine	unino- culated	0.10	10	0.60	2.04
	0	1.00	25	0.64	
	10	2.40	50	1.00	
	20	3.10	75	1.00	
	30	5.55	100	1.05	
	40	7.40	200	1.40	
	55	9.00	300	1.90	
	70	9.80	500	2.55	
	85	10.20	1000	2.75	
	100	10.60			
L(-)histidine	unino- culated	0.05	10	1.50	5.44
	0	0.76	25	2.10	
	5	4.80	50	3.30	
	10	7.15	75	4.20	
	15	8.60	100	4.70	
	20	9.35	200	7.10	
	25	10.20	300	8.20	
	30	11.65	500	11.40	
	40	11.70	1000	12.00	
	50	11.70			
L(-)trypto- phan	unino- culated	0.05	10	1.60	1.05
	0	1.60	25	2.40	
	1	2.80	50	2.30	
	2	3.90	75	2.75	
	3	4.80	100	2.85	
	5	6.50	200	3.35	
	7	8.15	300	4.25	
	10	9.70	500	6.55	
	15	10.85	1000	8.10	
	25	9.65			

Amino acid	Amount (γ)	LN NaOH used (ml)	Amt. of hydro- lysate of fra- ction A used (γ)	LN NaOH used (ml)	Percentage of amino acid in fraction A
DL-serine	uninocu- lated	0.60	25	-	
	0	7.00	50	15.10	10.74
	10	16.70	100	16.40	
	20	17.90	150	17.50	
	40	18.50	200	17.50	
	70	20.40	250	17.90	
	85	20.60	300	18.30	
			400	19.30	
			450	19.10	
			500	19.60	
DL-leucine	uninocu- lated	0.30	25	10.15	
	0	10.75	50	12.20	
	10	11.32	100	13.73	
	20	13.59	150	15.52	
	30	14.70	200	16.70	
	40	15.50	250	17.30	
	50	17.25	300	18.10	
	70	18.50	400	19.60	
	85	18.95	450	20.10	
	100	19.85	500	20.45	
DL-threo- nine	uninocu- lated	0.40	50	1.25	2.56
	0	0.65	100	3.05	
	10	2.95	150	5.35	
	20	7.10	200	6.10	
	30	9.10	250	7.90	
	40	10.65	300	8.80	
	55	13.10	400	10.60	
	70	15.15	500	11.25	
	85	16.85			
	100	18.70			

B. Turbidimetric method.

Amino acid	Amount (γ)	Galvano- meter reading	Amount of hy- drolysate of Fraction A used (γ)	Galvano- meter reading	Percentage of amino acid in Fraction A
Glycine	0	74, 72, 71	10	69, 72	3.20
	10	65, 63	25	68, 69	
	20	60, 60	50	67, 68	
	30	58, 58	75	60, 66	
	40	58, 58	100	65, 65	
	55	57, 60	200	62, 61	
	70	58, 57	300	60, 60	
	85	58, 58	500	57, 56	
	100	56, 56	1000	55, 56	
	L(+)-lysine	0	-	20	
1000		---	100	-	
			1000	-	
DL-aspartic acid	0	-	20	-	0.0
	1000	---	100	-	
			1000	-	
L(-)-cystine	0	-	20	-	0.0
	1000	---	100	-	
			1000	-	
DL-alanine	0	-	20	-	0.0
	1000	---	100	-	
			1000	-	

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Publication:

1. Studies on the Effect of α -Aminoisobutane Sulphonic Acid in the Rat. M.S. thesis, 1950, Purdue University, West Lafayette, Indiana.
2. Factors Influencing the Microbiological Assay of Vitamin B₁₂. Mary S. Shorb, Kung Y.T. Kao, and W.H. Scott, Scientific Paper No. A364, Contribution No. 2352 of Md. Agric. Exptl. Station, Dept. of Poultry, College Park, Md.

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ABSTRACT

Kung Ying Tang Kao, Doctor of Philosophy, 1953.
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Title of thesis: Fractionation and Concentration of Growth
Factors for L. bulgaricus 09 and L. lactis 8000.

Thesis directed by Dr. Fletcher P. Veitch and Dr. Mary S. Shorb.

Major: Biochemistry.

Minor: Physical Chemistry and Inorganic Chemistry.

Page of thesis: 99. Words in abstract, 267.

An improved assay for streptogenin based on the essential nature of trypsinized protein for L. bulgaricus 09 was suggested by Wright and the complexity of the growth factor for L. lactis 8000 was reported by Shorb and Silver. The present investigation was undertaken to study the nature of the growth factors for these two organisms.

The growth factors for L. bulgaricus 09 and L. lactis 8000 are similar. The growth required factors for L. bulgaricus 09 consists of two portions; i.e. a peptide or peptides growth factor from trypsinized protein and an agar factor. The growth stimulating factor for L. lactis 8000 consists of a peptide growth factor, an agar factor, asparagine and L. F. The peptide growth factor is different from other known growth factors. It can be differentiated from streptogenin by their amino acid composition, being rich in leucine

and valine. The R_f value of the peptide growth factor is 0.0 using water-saturated n-butanol as solvent and 0.7-0.8 using 60 percent cellosolve as solvent. Trypsinized globin is the best source of this peptide growth factor. The removal of the heme from hemoglobin results in an increase of the growth promoting activity.

A peptide, fraction A of trypsinized globin, prepared by the removal of the inactive substance from the lead acetate filtrate of trypsinized globin, is the fraction with highest activity. The amino acid composition of fraction A has been determined chromatographically and microbiologically.

Heme is an inhibitor for L. bulgaricus 09, but a growth factor for L. lactis 8000. Cyanide is an inhibitor for L. lactis 8000 in the plate assay, but not for L. bulgaricus 09.