DEFINED MEDIUM FOR GROWTH OF *MICROCOCCUS LYSODEIKTICUS*; PURIFICATION AND IDENTIFICATION OF AN UNKNOWN GROWTH FACTOR

By

YUNG-CHIEH CHU

Bachelor of Science

Taiwan Provincial College of Agriculture

Taichung, Taiwan, China

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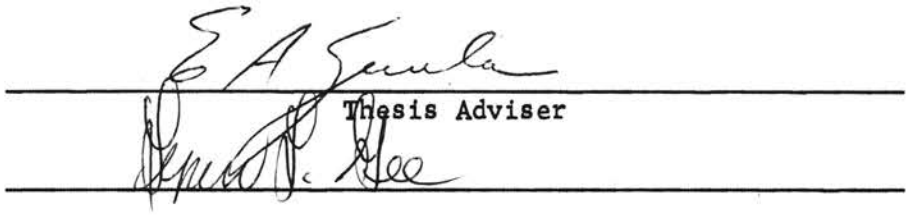

Thesis Adviser

Dean of the Graduate School

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PART I

CHAPTER I

INTRODUCTION

This portion of the thesis will report extended studies which relate to the nutrition of Micrococcus lysodeikticus, (13). Apparently several "nutritional types" of this organism exist since at least 3 different synthetic medium formulations (13, 26, 28, 29) and one semi-synthetic formulation (13) are known. Also, Feiner, Meyer, and Steinberg (8) reported they could not grow any of three immunologically distinct strains in any synthetic media which they compounded.

This thesis will not consider whether all of these "nutritional types" are different strains of M. lysodeikticus and if, therefore, some of them could be more appropriately classified. Bergey's Manual (6th Edition) lists the organism as a yellow micrococcus easily lysed by lysozyme; no other information is included. In the 7th Edition of Bergey's Manual the organism is not listed.

Luk (13) was able to grow this organism in a medium consisting of adenosine, biotin, 10 amino acids (L-arginine, L-cysteine, L-glutamic acid, L-isoleucine, L-leucine, DL-lysine, L-methionine, DL-phenylalanine, L-proline, and L-tyrosine), magnesium sulfate, potassium phosphate (dibasic) and ammonium chloride.

The synthetic medium formulated by Luk differs from other media as regards: amino acid composition, presence or absence of glucose, a

purine base and pH requirements. These differences are summarized in Table I.

Considering the media formulated either by Luk, Heden or by Wessman, et al., it would appear that M. lysodeikticus possesses specific amino acid requirements. This does not appear to be true with regard either to the Western Reserve medium or the formulation of Wolin and Naylor.

All of the media formulations contain biotin; however, only Luk reported that biotin was stimulatory rather than being absolutely required. His studies also demonstrated that biotin could be replaced by either oxybiotin or biocytin.

Luk could not demonstrate either a need for, or a stimulation of growth by glucose. In his formulation, amino acids apparently serve in a triple capacity (source for carbon, organic nitrogen and energy). Although all of the other reported media contain glucose, no statement regarding the essentiality of this substance for growth of the organism has appeared.

The pH requirements, when given, are obviously different; only Luk reported studies regarding pH manipulations.

The greatest and most striking difference relates to the finding, by Luk, that M. lysodeikticus possesses an absolute requirement for a purine base. This requirement can be satisfied by adenine or hypoxanthine, however increased growth stimulation occurs in the presence of the nucleoside form of these compounds (adenosine or inosine). Prior to the work by Luk, and since, no statement relating to a purine requirement by this organism has appeared.

Because 10 amino acids constitute a large number of amino acids; because amino acids are usually interchangeable by cells, and because

TABLE I

COMPARISON OF LUK'S FORMULATION WITH OTHER PROPOSED SYNTHETIC MEDIA FOR GROWTH OF M. LYSODEIKTICUS

No. of Amino Acids	Amount of Biotin per 100 ml	Amount of Glucose	pH	Purine Present	Pyrimidine Present
Wessman, Allen, and Werkman Medium (26)					
16*	1.0 µg	0.375%	7.0	adenine, guanine	uracil
Western Reserve Medium (13)					
1**	1.0 µg	0.5%	not given	hypoxanthine	-
Wolin and Naylor's Medium (28,29)					
1***	1.0 µg	0.7%	not given	-	-
Heden's Medium (13)					
Casamino acids	-	0.98%	7.5	-	-
Luk's Medium (13)					
10****	1.0 µg	-	7.6 - 7.8	adenosine or inosine	-

*DL-α-alanine, L-arginine.HCl, L-cysteine.HCl, glycine, L-glutamic acid, L-histidine.HCl, DL-isoleucine, L-leucine, L-lysine.HCl, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-tryptophan, L-tyrosine, DL-valine.

**Sodium glutamate.

***Monosodium glutamate.

****L-arginine, L-cysteine, L-glutamic acid, L-isoleucine, L-leucine, DL-lysine, L-methionine, DL-phenylalanine, L-proline, L-tyrosine.

Luk did not show the essentiality of all of these amino acids in the nutrition of M. lysodeikticus, a re-investigation of this segment of his studies appeared appropriate. A re-study of the biotin stimulation and the effects of glucose and other substances such as urea and iron was also undertaken.

Little or no attention has been directed toward the iron requirements of this organism. Townsley and Neilands (25) reported that iron chelates such as ferrichrome enhance growth when the organism is grown in the medium of Wolin and Naylor. Because the organism is highly aerobic (cytochrome-containing) and because it is one of the best sources for the enzyme catalase (iron containing), it can be assumed that iron will be required as a micro-nutrient or possibly, in larger amounts.

CHAPTER II

MATERIALS AND METHODS

Organism: The Purdue University strain of M. lysodeikticus is a subculture from the original culture isolated by Fleming in 1922 and sent to this country to Dr. S. E. Hartsell of Purdue University at least 14 years ago.

Inoculum: The organism was grown for 22-24 hours on nutrient agar slants at 37°C, washed twice in 10 ml of 0.85 per cent sterile sodium chloride solution (saline) and resuspended in approximately 10 ml of sterile saline. One drop of the washed suspension (O.D. 0.28-0.32 measured at 535 mμ) was inoculated into a total volume of 5 ml of the various media formulations. Because aeration is necessary for maximal growth, tubes (17 mm I.D.) were always incubated at 32-35°C at least 24 hours on a reciprocating shaker (100 strokes/min.).

Basal medium: The basal medium contained per 5 ml: ammonium chloride 5.0 mg, potassium phosphate (di-basic) 10.0 mg, magnesium sulfate 0.1 mg, inosine 0.3 mg, (or an equivalent amount of adenosine) and biotin 2.5 μg. Test compounds such as amino acids and finally triple distilled water were added to the basal medium to make a final total volume of 5 ml. The pH was adjusted to 7.6-7.8 using solid potassium hydroxide prior to autoclaving of the medium. Toluene was routinely added to all solutions during storage to prevent microbial growth. When heat labile compounds were added to the medium, they were

sterilized by passage through a sintered glass filter and added aseptically; otherwise the medium was sterilized by autoclaving at 115°C for 12 minutes.

Cleaning of equipment: All pipettes, test tubes, bottles and other glassware were allowed to remain in chromic acid cleaning solution for at least 24 hours and then rinsed 15 times with tap water and 5 times with triple distilled water prior to use.

Growth measurements: Growth was determined by measurement of optical density of liquid cultures at 535 mμ in a Bausch and Lomb "spectronic 20" spectrophotometer.

Amino acids: When arginine, cysteine, and lysine were used, the chloride-salt forms were employed. All amino acids were solubilized in water prior to their addition in the various media formulations. At times it was necessary to solubilize some amino acids (L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-tyrosine) by acidification with hydrochloric acid.

Potassium hydroxide was used to solubilize L-glutamic acid.

CHAPTER III

RESULTS AND DISCUSSION

Quantitation of adenosine and biotin for optimal growth of M. lysodeikticus: To establish the optimal levels of adenosine and biotin, parallel series of tubes were inoculated and incubated at 25°C and 33°C. Growth response and contents of the tubes are shown in Table II. It was observed that maximal growth was obtained when the concentration of adenosine was increased ten times over that used by Luk, and when the biotin concentration was increased fifty times. Also, more growth occurred when the cultures were grown at 33°C.

Substitutions involving biotin: Because several forms of biotin exist (biocytin, desthiobiotin, d-or l-biotin sulfoxide, d-or l-oxybiotin), all were tested in equal molar concentration (2.04×10^{-6} M) to determine if they could stimulate growth of the organism in a manner similar to biotin. These data are incorporated into Table III. It can be observed that biotin, biotin-d-sulfoxide or biocytin allowed almost equal growth. Desthiobiotin, dl-oxybiotin or biotin-l-sulfoxide, although also capable of stimulating growth, were inferior to biotin in this respect.

Variation in response to biotin analogues by microorganisms is not unusual. Firestone and Koser (9) studied the activity of biotin analogues using Candida albicans, and observed that although oxybiotin also supported good growth of the organism, about 10 times as much was required for comparable growth when present in suboptimal levels. Assuming

TABLE II

INFLUENCE OF TEMPERATURE AND DIFFERENT CONCENTRATIONS OF ADENOSINE AND BIOTIN ON GROWTH OF M. LYSODEIKTICUS*

Amount of Adenosine (mg) per 5 ml Medium	Amount of Biotin (µg) per 5 ml Medium	Optical Density** 24 hours	
		25°C	33°C
0.1	1.25	0.05	0.17
0.5	1.25	0.07	0.23
1.0	1.25	0.12	0.25
0.3	0.25	0.08	0.15
0.3	1.25	0.12	0.27
0.3	2.5	0.06	0.29
1.0	2.5	0.12	0.39
0.5	2.5	0.07	0.25

* All tubes contained the following per 5 ml of medium: ammonium chloride 5.0 mg, potassium phosphate (di-basic) 10.0 mg, magnesium sulfate 0.1 mg. L-arginine 3.28 mg, L-cysteine 2.4 mg, L-glutamic acid 17.92 mg, L-isoleucine 2.56 mg, L-leucine 7.36 mg, DL-lysine 6.56 mg, L-methionine 1.36 mg, L-phenylalanine 2.0 mg, L-proline 9.04 mg and L-tyrosine 5.04 mg.

** Reading made at 535 mµ against a distilled water blank.

TABLE III

EFFECT OF INOSITOL, FATTY ACIDS, AND VARIOUS FORMS OF BIOTIN ON GROWTH OF M. LYSODEIKTICUS*

Compound Tested**	Optical Density*** 24 hours
Control (no addition)	0.21
Biocytin	0.49
Biotin-d-sulfoxide	0.47
Biotin	0.47
Desthiobiotin	0.39
dl-oxybiotin	0.34
Biotin-l-sulfoxide	0.27
Valeric acid	0.20
Sodium oleate	0.15
Inositol	0.23
Pimelic acid	0.20

* All tubes contained the following per 5 ml: ammonium chloride 5.0 mg, potassium phosphate (di-basic) 10.0 mg, magnesium sulfate 0.1 mg, inosine 0.3 mg, one drop of sterile iron solution, L-glutamic acid 17.92 mg, L-phenylalanine 2.0 mg, and L-tyrosine 5.04 mg.

** Concentration of biotin compounds 2.04×10^{-6} M. Valeric acid 1.042 µg, sodium oleate 2.88 µg, inositol 5.0 µg and pimelic acid 1.63 µg per 5 ml of medium.

*** Measured at 535 mµ against a distilled water blank after growth at 34°C for 25 hours on a reciprocal shaker having 100 strokes per minute.

that only the d-form of dl-oxybiotin was active, then the activity of oxybiotin for C. albicans is about one-fifth that of biotin. These results are within the same ranges as the 20 to 25 per cent activity reported for Saccharomyces cerevisiae by Rubin, Flower, Rosen, and Drekter (18) and Winnick, Hofmann, Pilgrim, and Axelrod (27). The activity of desthiobiotin compared favorably with that of biotin in amounts up to about 0.1 mμg per ml, however larger amounts of desthiobiotin were inhibitory. Reports by other investigators have shown that the effects of desthiobiotin on microorganisms differ greatly with different species. For S. cerevisiae this sulphur-free analogue of biotin was found to be quite active, but for Lactobacillus casei it is inactive as a growth agent and also inhibits response to biotin (9). The work of Perlman (16) with several species of clostridia well illustrates the differences in response to desthiobiotin and oxybiotin by different species, even within the same genus.

For some microorganisms, the requirement for biotin may be abolished by compounds such as oleic or pimelic acid. Oleic acid and related compounds such as lecithin may, in the presence of aspartic acid, effectively replace biotin for L. casei and certain other lactobacilli (21). The growth of Corynebacterium diphtheriae is stimulated by pimelic acid. In some strains of this organism pimelic acid appears to be used for the synthesis of biotin (21).

The biotin requirement for M. lysodeikticus could not be replaced by pimelic or valeric acid or the sodium salt of oleic acid at equimolar, 10 or 50 times the biotin concentration. At 50 times concentration, oleate was toxic. Also, inositol could not replace biotin. These data are included in Table III.

Iron requirement of M. lysodeikticus: At times, lack of growth or poor growth has been traced to an apparent iron requirement. If iron is added as ferrous ammonium sulfate prior to autoclaving of the entire medium, a precipitate forms which interferes with optical density measurements. Addition of ethylenediaminetetraacetic acid (EDTA) as an iron chelate does not sufficiently reduce the precipitate. Growth occurs using coprogen, hemin, cytochrome c or ferrichrome at a concentration of 1 μg/ml. Thus, although a requirement for iron is evident, the requirement is not specific for the inorganic forms. In most instances, there appears to be enough iron contamination of materials such as phosphate, sodium chloride, etc. to allow growth of the organism. To maintain more constant conditions in routine work, iron has been added to the medium after first autoclaving a solution of ferrous ammonium sulfate containing 2.5 mg/ml, allowing the iron to precipitate, and then aseptically adding one drop of the clear portion to 5 ml of the autoclaved medium (see Table IV).

Tests for growth stimulation by glucose, iron, and urea: Although all synthetic formulations employ glucose, the essentiality of glucose for growth of M. lysodeikticus has never been demonstrated. Luk had noted that glucose did not stimulate growth during the first 24 hours. Although glucose is not essential and does not increase growth response during the first 24 hours, optical density of the culture is greater after 36 hours incubation (Table IV).

Because M. lysodeikticus possesses the ability to produce large amounts of urease, urea was also tested to determine if this compound could either stimulate growth during the first 24 hours of incubation or increase final cell mass. The essentially negative results are also

TABLE IV

THE GROWTH RESPONSE OF M. LYSODEIKTICUS TO GLUCOSE, IRON AND UREA*

Ferrous Ammonium Sulfate	Glucose**	Urea***	Optical Density**** 24 Hours	36 Hours
-	-	-	0.85	1.14
+	-	-	0.92	1.7
+	+	-	0.80	2.0
+	-	+	0.59	1.38

* All tubes contained the following per 5 ml of medium: ammonium chloride 5.0 mg, potassium phosphate (di-basic) 10.0 mg, magnesium sulfate 0.1 mg, inosine 0.3 mg, biotin 2.5 µg, and the following amino acids: L-arginine 3.28 mg, L-cysteine 2.4 mg, L-glutamic acid 17.92 mg, L-isoleucine 2.56 mg, L-leucine 7.36 mg, DL-lysine 6.56 mg, L-methionine 1.36 mg, L-phenylalanine 2.0 mg, L-proline 9.04 mg and L-tyrosine 5.04 mg.

** Prepared separately (4.0 mg per 5 ml of medium), autoclaved at 10 pounds for 12 minutes and added aseptically.

*** Prepared separately (0.2 mg per 5 ml of medium) and sterilized by passage through a sintered glass filter.

**** Reading made at 535 mµ against a distilled water blank.

included in Table IV.

Amino acid requirements: The continued study of the 10 amino acids used by Luk has shown that all of the amino acids need not be present for good growth response by this organism. The results of a 10 amino acid study are tabulated in Table V.

Because good growth occurred in the presence of only 3 amino acids (arginine, phenylalanine and tyrosine), it was decided to re-study the amino acid requirements using these 3 compounds. Glutamic acid was also included in these studies since experiments had shown that it allowed increased cell yields. Representative data are tabulated in Table VI. From these studies, it is apparent that at least 2 and probably 3 amino acids should be present to obtain what may be considered good growth response in 36 hours.

These data show that no one amino acid will allow more than very minimal growth. Although 2 amino acids will allow fairly good growth. (e.g. phenylalanine and tyrosine; tyrosine and arginine, glutamic acid and phenylalanine), none is absolutely required since the amino acids can be interchanged. Apparently the bulk of amino acids synthesis in this organism occurs using glutamic acid as a precursor; however, glutamic acid alone will not satisfy the amino acid requirement. The presence of an aromatic acid (phenylalanine or tyrosine) appears to be essential for good growth in 36 hours. In further media manipulations, the combination of phenylalanine, tyrosine and glutamic acid has given satisfactory and reproducible growth responses.

Although glutamine can substitute for glutamic acid, chromatography of glutamine we used showed that at least 4 other ninhydrin-positive compounds were present as impurities.

TABLE V

RESPONSE OF *M. LYSODEIKTICUS* TO VARIOUS COMBINATIONS OF 10 AMINO ACIDS*

Amino acids**										Optical Density***	
Arginine	Cysteine	Glutamic Acid	Iso-leucine	Leucine	Lysine	Methionine	Phenyl-alanine	Proline	Tyrosine	24 Hours	36 Hours
+	-	+	+	+	+	+	+	+	+	1.30	1.40
+	-	-	+	-	-	-	+	-	+	0.045	0.17
+	-	-	+	+	-	-	+	-	+	0.19	0.525
+	+	+	+	+	+	+	+	+	+	1.30	1.42
+	-	+	+	+	-	-	+	-	+	0.51	1.07
+	-	+	+	-	-	-	+	-	+	0.37	0.95
+	-	-	+	+	-	-	+	-	-	0.085	0.275
+	-	-	+	-	-	-	+	-	-	0.075	0.155
+	-	-	+	-	-	-	-	-	+	0.11	0.165
+	-	-	+	+	-	-	-	-	+	0.17	0.455
+	-	-	-	+	-	-	+	-	+	0.165	0.375
+	-	-	-	-	-	-	+	-	+	0.395	1.14
-	-	-	+	-	-	-	+	-	+	0.125	0.205

TABLE V (CONTINUED)

Arginine	Cysteine	Glutamic Acid	Iso-leucine	Leucine	Lysine	Methionine	Phenyl-alanine	Proline	Tyrosine	Optical Density*** 24 Hours	Optical Density*** 36 Hours
-	-	-	+	+	-	-	+	-	+	0.01	0.075
-	-	+	+	+	-	-	+	-	+	0.28	0.425

* All tubes contained the following per 5 ml of medium: ammonium chloride 5.0 mg, potassium phosphate (di-basic) 10.0 mg, magnesium sulfate 0.1 mg, inosine 0.3 mg, biotin 2.5 µg and one drop of sterilized iron solution as explained in the text.

** Concentration of amino acids used: L-arginine 3.28 mg, L-cysteine 2.4 mg, L-glutamic acid 17.92 mg, L-isoleucine 2.56 mg, L-leucine 7.36 mg, DL-lysine 6.56 mg, L-methionine 1.36 mg, L-phenylalanine 2.0 mg, L-proline 9.04 mg, and L-tyrosine 5.04 mg per 5 ml of medium.

*** Reading made at 535 mµ against a distilled water blank.

TABLE VI

RESPONSE OF M. LYSODEIKTICUS TO VARIOUS COMBINATIONS OF 4 AMINO ACIDS*

Amino Acids**				Optical Density***	
Arginine	Glutamic Acid	Phenylalanine	Tyrosine	24 Hours	36 Hours
+	-	-	-	0.01	0.01
-	-	+	-	0.04	0.05
-	-	-	+	0.06	0.08
-	+	-	-	0.05	0.08
+	-	+	-	0.05	0.05
+	-	-	+	0.10	0.34
+	+	-	-	0.06	0.09
-	-	+	+	0.08	0.23
-	+	-	+	0.08	0.18
-	+	+	-	0.09	0.67
-	+	+	+	0.10	0.74
+	+	+	-	0.10	0.60
+	+	-	+	0.12	0.65
+	-	+	+	0.09	0.70
+	+	+	+	0.51	1.40
-	-	-	-	0.02	0.02

* All tubes contained the following per 5 ml: ammonium chloride 5.0 mg, potassium phosphate (di-basic) 10.0 mg, magnesium sulfate 0.1 mg, inosine 0.3 mg, biotin 2.5 µg, and iron solution as explained in the text.

** Concentration of amino acids per 5 ml medium: L-arginine 3.28 mg, L-glutamic acid 17.92 mg, L-phenylalanine 2.0 mg, and L-tyrosine 5.04 mg.

*** Measured at 535 mµ against a distilled water blank. Cultures incubated on a reciprocating shaker (100 strokes/minute) at 34°C.

The results of these nutritional studies can perhaps be explained by the results of Rudman and Meister (19). These investigators fractionated extracts of a strain of Escherichia coli and obtained evidence for the existence of two major transaminase systems. "Transaminase A" which exhibits the principal α-keto-glutarate-transaminase activity toward aspartate and the aromatic amino acids; and "Transaminase B" which catalyzes transaminations between α-keto-glutarate and isoleucine leucine, valine, norvaline or norleucine. Reactions involving phenylalanine, tyrosine, tryptophan and aspartate were catalyzed almost exclusively by transaminase A. Similarly the aliphatic amino acids were much better substrates for transaminase B. The function of glutamate is unique since it reacts equally well with both transaminases.

Because M. lysodeikticus grows better in the presence of an aromatic amino acid, this would appear to indicate that the organism possesses a low level or improperly functional transaminase "A" enzyme system. Apparently some transaminase "A" is present and functional since the organism does not require tryptophan and does appear to be capable of synthesizing some of its own phenylalanine and tyrosine from glutamic acid albeit at a low or inefficient rate. On the other hand, the aliphatic amino acids can be dispensed with particularly when glutamic acid is present. Therefore, the transaminase "B" system would appear to be present in large amounts and properly functional.

PART II

CHAPTER I

INTRODUCTION

This portion of the thesis will report continued studies which relate to the unidentified growth factor requirements of Micrococcus lysodeikticus (13, 14).

Britt and Gerhardt (6) reported that their strain of M. lysodeikticus grew abundantly in 1 or 2 per cent peptone water.

Litwack and Pramer (12) also reported that their strain (Rutgers University stock culture) grew abundantly in 1 per cent peptone water. They attempted to grow the organism in 10 different peptones and observed that neopeptone or protone allowed no growth whatever. Although the largest amount of growth occurred using tryptose, growth was preceded by a lag period of approximately 25 hours. Shortest lag period occurred using peptone. Thiotone allowed fair growth, while lactalysate, trypticase, N-Z amine and gelsate gave poor growth. Luk and Grula (14) reported that a factor present in peptone is also present in heart infusion, tryptose, liver extract and tryptone; a small amount is also present in casitone.

Brock (7) using the Purdue University strain of M. lysodeikticus was able to obtain growth in a medium containing only amino acids and mineral salts when a small amount of either peptone or trypticase was present. Brock found that the material present in peptone (unidentified

factor) was dialyzable, heat-stable (withstood boiling for 1 hour in neutral solutions) and resistant to digestion by trypsin, chymotrypsin or pepsin.

Brickler (5) reported that the factor present in peptone could be partially purified by selective elution from a Dowex-2 anion exchange resin column using 0.3 N HCl. Some properties of the factor as reported by Brickler may be summarized as follows:

1. Very soluble in water.
2. Relatively stable to acid and alkali (growth activity remains when heated to boiling for 60 minutes at pH 1.0 or 11.9).
3. Soluble in 70 and 95 per cent ethyl alcohol.
4. Not soluble in benzene or ether. (The latter refluxed for 24 hours continually).
5. Not steam distillable.
6. The factor is organic, since ashing destroys growth promoting activity.
7. It is a small dialyzable molecule.
8. It is not precipitated by 1, 2, 3, 4, or 5 volumes of 95 per cent alcohol.

Luk (13) was the first to observe that our strain of M. lysodeikticus requires for growth either of two factors present in peptone. The factors were fractionated and partially purified by selective elution from a Dowex-2 anion exchange resin column, and purified further by paper chromatography using phenol water (7/3) followed by n-butanol/acetic acid/water(4/1/5). The Rf of both factors is 0.73-0.77 in phenol/water, (does not separate into 2 bands); whereas in n-butanol/acetic acid/water, two distinct bands are present. One band has an Rf

of 0.3 and the other Rf of 0.47. Preliminary qualitative tests showed that neither of the factors was a sugar. Oxidized or reduced sulphur was not present. After partial hydrolysis (2N H_2SO_4 at 100°C for 2 hours) the factors gave a positive test for sugar phosphate. No pronounced absorption peaks could be detected using the Beckman D.U. spectrophotometer in the range of 200-400 mμ.

Either of the factors may be replaced by adenosine, inosine or hypoxanthine; however, using the techniques of light absorption in the region of 260 mμ, chromatography and hydrolysis tests, Luk concluded that the factors are not purines or purine-containing compounds.

The study of the factor has been difficult because of the lack of purified material. Most of the studies have employed material eluted from chromatography papers. Such material can be obtained only in small amounts and is usually contaminated with some ninhydrin positive materials that chromatography will not remove.

Therefore, the purpose of this portion of the research effort was to obtain larger amounts of purified material and to attempt identification.

CHAPTER II

MATERIALS AND METHODS

Basal medium: The basal medium contained per 5 ml: ammonium chloride 5.0 mg, potassium phosphate (K_2HPO_4) 10.0 mg, magnesium sulfate 0.1 mg, and 0.5 ml of a 5.0 per cent "Vitamin Free" casein hydrolyzate solution adjusted to pH 7.6-7.8 using solid KOH. Since all of the above materials were pipetted into tubes in a total volume of 1.5 ml, the growth factor or test compounds and triple distilled water were added to the basal medium to make a final total volume of 5.0 ml. Toluene was routinely added to all solutions during storage to prevent growth of contaminating organisms. Autoclaving was at 115°C for 10 minutes unless otherwise indicated.

Growth measurements: Growth was determined by measurement of optical density of optical cultures at 535 mμ in a "spectronic 20" spectrophotometer.

Paper chromatography:

(a) n-butanol/acetic acid/water system: The materials for testing were deposited on a strip of Whatman No. 1 filter paper 22 inches long and 1 1/2 inches wide, under a continuous stream of hot air from a hair-dryer. The papers were then equilibrated for 1 hour by letting them hang suspended in the chromatography jar in the presence of the developing solvent which was placed in the bottom of the jar in a petri-dish. Developer, n-butanol/acetic acid/water (4/1/5), was then added to the troughs and allowed to flow over the papers for 22-24 hours (13).

The papers were removed, dried and then cut into two halves parallel to the long axis of the paper. One half was dipped into ninhydrin dissolved in acetone (125 mg per 100 ml), then heated in an oven at 105°C for 5 minutes to permit color development. The other half was reserved for the bioautographic plate test.

(b) Phenol/water system: All materials to be tested were placed on the paper as previously described, and then chromatographed in phenol/water (7/3) for about 12 hours at 25°C. The chromatograms were then heated in an exhaust hood at 60°C for 12 hours to remove the phenol. A narrow strip was cut from the chromatogram and tested with ninhydrin as above. The remainder of the chromatogram was placed on a seeded bioautographic plate which was incubated at 37°C for 12 to 24 hours. The technique regarding the bioautographic plate test was the same as described by Luk (13).

Elution of the growth factor from paper chromatograms: The location of the factor on the chromatograms was determined by observation of growth areas on bioautographic plates (growth occurred only in those areas where factor material was present). The area of the paper chromatogram containing the growth factor was then cut out, 6 to 8 such pieces were stapled together and eluted with flowing water for at least 8 to 12 hours in a dark and closed system. The eluate was evaporated to dryness either by placing it in a vacuum oven adjusted to 60°C or by placing it in an exhaust hood under a continuous stream of hot air from a hair dryer.

Hydrolysis of the growth factor using sulphuric acid or hydrochloric acid for qualitative analysis: About 50 µg of dry factor material was mixed with 3 ml of 2N sulphuric acid and heated for 2 hours at 100°C in a

sealed tube. After cooling, the hydrolyzate was neutralized with 1N barium hydroxide and the precipitate was removed by centrifugation. The supernatant was evaporated to dryness in an exhaust hood, under a continuous flow of hot air. Hydrolysis using HCl (1N for 1 hour at 121° C or 6N for 8 or 22 hours at 121°C) was carried out in the same manner except neutralization with barium hydroxide was not necessary. Instead, the hydrolyzates were evaporated to dryness, resuspended in water and taken to dryness three times in order to ensure evaporation of the acid.

Redfield's technique for the identification of amino acids (2-dimensional chromatography): Whatman No. 1 filter paper was cut into an 8-inch square. Acid hydrolyzates were applied to the left hand bottom corner of the paper 2 cm from the edges; the diameter of the spot was never more than 1/4 cm. A constant flow of hot air was directed on the area where the material was deposited. Also, all depositions were made directly over NH_4OH. The paper was then folded into a cylindrical shape and fixed in position with staples at both ends. Solvent No. 1 (methanol/water/pyridine (80/20/4)) was poured into a jar to a depth of 1 cm and the chromatogram was placed in the solvent. When the solvent ascended to the top of the chromatogram (approximately 2 hours) the chromatogram was removed and dried at room temperature for 15 minutes. The chromatogram was then unfolded, refolded at right angles to the former axis and placed into Solvent No. 2 (t-butanol/methyl-ethyl ketone/water/diethylamine (40/40/20/4)). When the solvent advanced to the top of the chromatogram (about 4 hours), it was taken out, dried over night, then steamed in an autoclave for 8-10 minutes with both exhaust valves open in order to remove diethylamine which is a ninhydrin-positive substance. After steaming in the autoclave, the chromatogram was dried,

then dipped into ninhydrin in acetone (125 mg/100 ml) and heated in an oven at 105°C for 5 minutes. The colored spots were compared with a standard chart of known amino acids (17).

Phosphorylated intermediates: When the barium and calcium salts of phosphorylated intermediates were studied, the metallic cations were removed by reacting the compounds with a weak solution of H_2SO_4. After sedimenting the insoluble calcium and barium salts by centrifugation, the remaining solutions were neutralized using potassium hydroxide.

CHAPTER III

RESULTS AND DISCUSSION

Fractionation and purification of the growth factor: At the beginning of this series of studies wherein attempts were made to utilize organic solvent extraction, it was observed that the unidentified factor could be extracted from dried peptone by methanol. The degree of purity was not as good as the material eluted from a Dowex-2 anion exchange resin column however; greater quantities could be obtained in less time with minimal effort. Data showing the solubility of the factor in various solvents are shown in Table VII.

For the methanol extraction 200 gms of peptone were dried in an oven at 40-60°C for 24 hours and then kept in a desiccator. The dried peptone was then extracted using 500 ml methanol at 4°C for 24 hours, with occasional stirring. Three 24 hour extractions were employed; however, during the last two extractions a total of 100 ml of methanol was used. The methanol extracts were concentrated to dryness using a rotary evaporator at 60°C. The dried material was stored at approximately -18°C. Final yield was about 42 gms.

Because the dried methanol extract contained many impurities (determined by ninhydrin treatment of paper chromatograms), studies were conducted using several solvents to determine if other solvents would allow further purification of the factor. These tests were done using aqueous methanol extracts (dried methanol extract resuspended in

TABLE VII

EXTRACTION OF THE UNIDENTIFIED FACTOR FROM DRIED PEPTONE USING VARIOUS SOLVENTS*

Solvent	Factor Solubility
Ether	Not soluble
Acetone	Not soluble
Toluene	Not soluble
Chloroform	Not soluble
2-Butanone	Not soluble
Hexane	Not soluble
n-Amyl alcohol	Not soluble
n-Butanol	Not soluble
Ethanol (70 to 98 per cent)	Soluble
Methanol	Soluble

* In each case, 100 mg of dry peptone was extracted for 1 hour at room temperature with 10 ml of each organic solvent. Solid material was separated by centrifugation and after evaporation of the solvents; the dry residues were redissolved in a total volume of 10 ml of water. One ml of the water solubilized material was added to the basal medium.

water to a concentration of 1 gm/ml). Results of these studies are presented in Table VIII.

These data show that the factor can be further purified by extraction from water using acetone. Addition of acetone to water solubilized material resulted in the formation of 2 immiscible layers and a heavy amorphous precipitate. The acetone, containing some water and factor material, goes to the top of the tube; below that, another layer forms that is viscous and brown in color. Finally, at the bottom of the tube, a heavy amorphous precipitate settles out. The reaction is very striking and goes to completion in approximately 30 minutes at room temperature. Analysis showed that the bulk of factor material migrates into the acetone-water layer along with some ninhydrin-positive impurities.

Attempts to decrease the migration of impurities into the acetone-water layer by adding acid or alkali to the solubilized methanol extract were not successful. Almost complete migration of the factor could be obtained when the extract was adjusted to pH 1.0, however, a much larger amount of impurities also migrated into the layer. Therefore, although incomplete migration of the factor occurred at pH 5.0 (pH of the solubilized extract), there were less impurities migrating at this pH. Adjustment of the pH to an alkaline pH (about 10.0), resulted in a sharp decrease in the amount of factor which migrated into the acetone-water layer. For these reasons, this step was performed at pH 5.0 and the acetone treatment was adopted in the over-all treatment sequence since good purification was achieved using this solvent.

At this stage of the experimentation, it appeared that the factor was some form of acid material. Two reasons for this conclusion can be

TABLE VIII

SOLUBILITY OF THE UNIDENTIFIED FACTOR (METHANOL EXTRACT) IN VARIOUS SOLVENTS AS DETERMINED BY LIQUID ASSAY USING M. LYSODEIKTICUS*

Solvent Used	Fraction Tested	Optical Density 24 Hours
Control	Methanol extract	0.925
Acetone	Brown layer	0.18
	Acetone-water layer	0.85
	Brown precipitate	0.12
Benzene	Immiscible benzene layer	0.07
	Immiscible water layer	0.90
2-Butanone	Immiscible butanone layer	0.04
	Immiscible water layer	0.92
Chloroform	Immiscible water layer	0.64
	Immiscible chloroform layer	0.185
Toluene	Immiscible toluene layer	0.115
	Immiscible water layer	0.90

* Five ml of the aqueous methanol extract (1 gm/ml) was treated with an equal volume of solvent. All fractions were separated by centrifugation, the fractions were dried and resolubilized in 10 ml of water. Five-tenths ml of the water solubilized material was added to the basal medium for testing.

given.

(1) Chromatography of a neutral, an acid and a basic amino acid (alanine, glutamic acid and arginine) in the butanol/acetic acid/water system showed that the Rf of the unidentified factor could be most closely compared to the Rf of glutamic acid. (2) Because acidification of the material resulted in an increased migration into acetone, it was concluded that a lower pH allowed for formation of the free acid which is more soluble in a relatively non-polar solvent such as acetone. The salt form (present at higher pH ranges) should be far less soluble in a solvent such as acetone.

Because the factor appeared to be acidic, a basic solvent, pyridine, was added to dried acetone-water extracts. As expected, the factor material migrated into pyridine (presumably a factor-pyridine complex was formed); however, a heavy precipitate formed. The formation of the precipitate was fortuitous since additional impurities were removed by this treatment.

Further purification of the factor: Factor material, which had been treated with methanol, acetone and pyridine was again subjected to solvent extraction techniques in order to obtain greater purification. The solvents used included: ligroine, ether, acetone, toluene, chloroform, n-amyl alcohol, isopropyl alcohol, methanol and diethylamine. Results obtained using either isopropyl alcohol or diethylamine showed that the factor was almost completely soluble in these solvents and again, some ninhydrin positive materials precipitated. After analyzing both supernatant fractions via chromatography, it was decided to use diethylamine in this step rather than isopropyl alcohol since less ninhydrin-positive compounds migrated into the diethylamine.

After much effort, it was observed that further purification could be brought about if the diethylamine extract was dried and subsequently re-extracted with diethylamine. Such treatment again resulted in the precipitation of ninhydrin-positive amorphous materials which did not contain factor material.

When the diethylamine extract was separated from the precipitate, dried and resuspended in water, the material did not give a clear solution, but, instead, a milky turbid mixture resulted. It was hoped that treatment with an ether-chloroform mixture (1:4) might clarify the solution and dissolve what were assumed to be fat globules. When the ether-chloroform mixture was added, three layers formed. The top layer was a clear aqueous solution which contained factor material; the second layer contained "fluffy" material, and the layer at the bottom (ether-chloroform layer), contained several ninhydrin-positive impurities. The identity of the materials in the middle or fluffy layer was never determined. Although the factor material was now rather pure, three areas of ninhydrin-positive material could still be observed when the material was chromatographed in the phenol/water system. Because the factor was located in between two of the ninhydrin bands, it was decided to cut these areas out and elute the factor from the papers since all attempts to obtain further purification by solvent treatment or crystallization were unsuccessful.

Although the eluates from the chromatograms developed in phenol/water contained what appeared to be pure factor material (Rf 0.73-0.77), the factor did not separate into 2 bands in this system; for that reason, the eluates were rechromatographed in the butanol/acetic acid/water system. Instead of the usual 2 bands (Rf 0.3 and 0.47); which appeared in the

butanol system in approximately equal amounts, it was now observed that very little Rf 0.47 material was present. Nearly all factor material migrated with an Rf of 0.3. Subsequent work, to be reported, using pure factor material was therefore accomplished using this Rf 0.3 material.

The entire sequence for fractionation and purification of the unidentified factor is given in Table IX.

Some unsuccessful techniques for isolating the unidentified factor from peptone: Besides using different solvent extraction procedures to purify the unidentified growth factor from peptone, other batch chromatographic techniques were attempted (use of adsorbents).

Thirteen adsorbents were used. Factor material (15 ml of the dried methanol extract resuspended in water to a concentration of 24 mg/ml) was added to 4 gms of each of the adsorbents. The slurries were then placed on a rotary shaker at 25°C and shaken for 30 minutes. After separation of the particulate material by centrifugation, 0.7 ml of the supernatant was tested by the liquid assay procedure. Ninhydrin-positive impurities were also determined by employing paper chromatography of the liquid portions. The behavior of the various adsorbents is given in Table X.

These data show that most of the adsorbents were not very useful. Some of the adsorbents, such as calcium phosphate, magnesium carbonate, lime (CaO), activated silicic acid and activated magnesium silicates will pick up part of the ninhydrin-positive materials, however, they also adsorb most of the factor material. Adsorbents such as starch, activated alumina and Fuller's earth pick up both ninhydrin-positive materials and the growth factor. Only sodium carbonate and potassium carbonate showed a good reaction, i.e., they picked up nearly all the

TABLE IX

FRACTIONATION AND PURIFICATION OF THE UNIDENTIFIED GROWTH FACTOR FROM PEPTONE

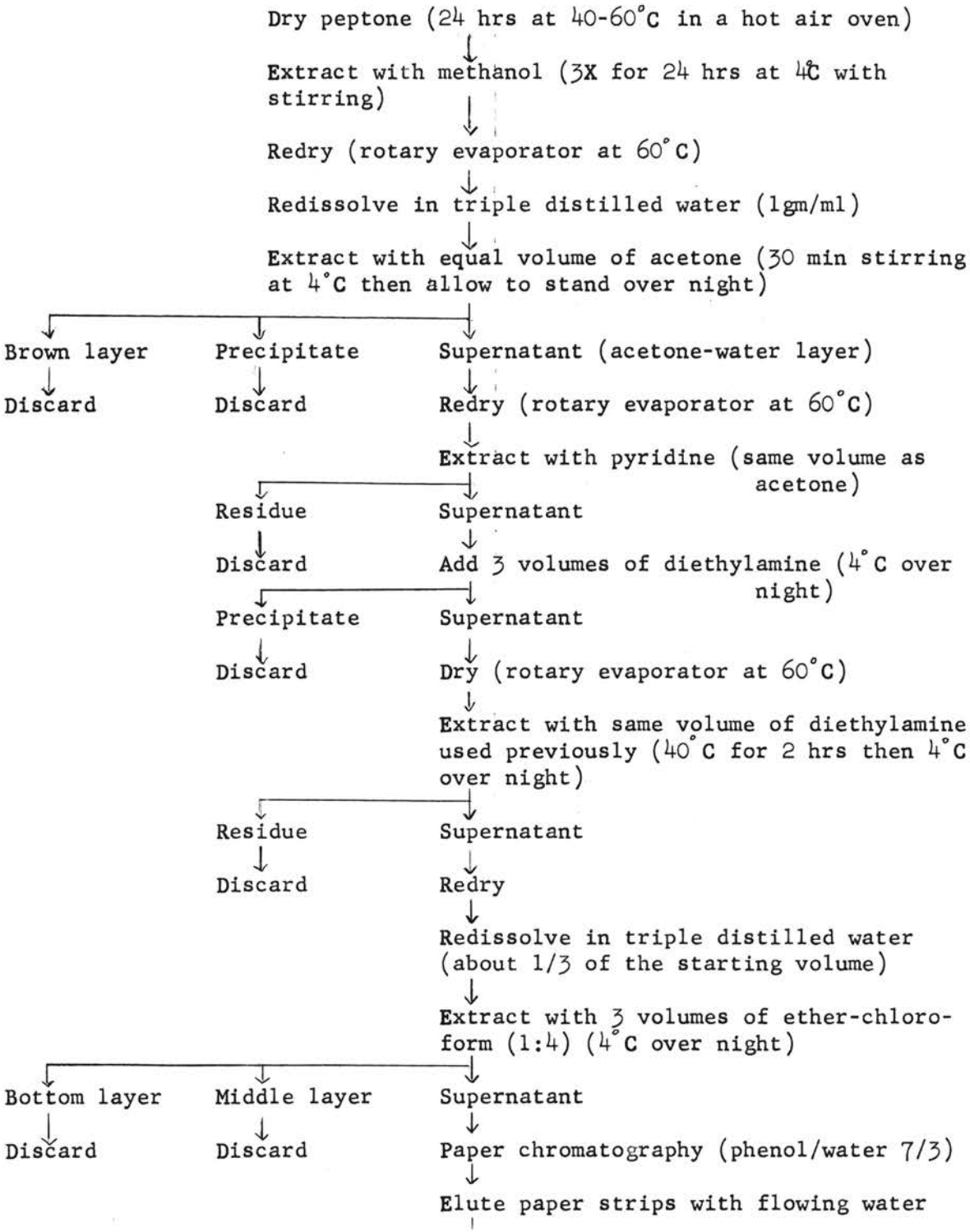

TABLE IX (CONTINUED)

↓ Concentrate eluate ↓ Re-chromatograph in butanol/acetic acid/ water (4/1/5) ↓ Elute with flowing water and dry

TABLE X

REMOVAL OF THE UNIDENTIFIED PEPTONE FACTOR OR NINHYDRIN-POSITIVE COMPOUNDS BY DIFFERENT ADSORBENTS*

Adsorbent Used	Ninhydrin Reaction**	Optical Density*** 48 Hours
None (Control)	++++	0.36
Starch	-	0.035
Mg-citrate	++++	0.06
Talc	+++	0.235
Na_2CO_3	+	0.25
K_2CO_3	+	0.30
$CaCO_3$	++++	0.215
$Ca_3(PO_4)_2$	++	0.125
$MgCO_3$	+++	0.12
CaO	++	0.185
Activated silicic acid	+++	0.125
Activated Mg-silicates	+++	0.115
Activated alumina	+	0.015
Fuller's earth	+	0.065

* All tubes contained the basal medium as given in materials and methods.

** (-) indicates no ninhydrin-positive materials on chromatogram; (++++) indicates many ninhydrin-positive impurities.

*** Reading made at 535 mμ against a distilled water blank.

ninhydrin-positive materials leaving the factor in solution. The results using magnesium citrate were also interesting since the factor was almost completely adsorbed leaving behind most of the ninhydrin-positive impurities. A procedure was therefore attempted wherein the methanol extract was treated with sodium carbonate to remove ninhydrin-positive materials. The eluate was then treated with magnesium citrate since this adsorbent takes the factor out leaving ninhydrin-positive materials in solution. However, once the factor had adhered to the magnesium citrate there was no way to elute the material even though several solvents such as methanol were used. Therefore, this type of procedure was regretfully discontinued.

Several attempts were made to aid purification by employing charcoal. It was observed that charcoal will adsorb both the growth factor and ninhydrin-positive materials. When eluted with different solvents at different temperatures (acetone, HCl, NH_4OH or pyridine at room temperature or 60°C), it was observed that only pyridine was capable of eluting the factor. Because ninhydrin-positive compounds were also eluted using pyridine, no appreciable purification resulted and use of charcoal was also discontinued.

Qualitative analysis of the growth factor: Since it was possible to replace the growth factor present in peptone with a purine (hypoxanthine) or the purine nucleosides (adenosine or inosine), and because stimulation of growth occurred using biotin, oxybiotin, or biocytin, it appeared appropriate to determine if the peptone factor was any of these known compounds. Two different procedures for identification of biotin were employed.

(1) Microbiological assay. Saccharomyces cerevisiae was used as an

assay organism. The organism was carried on nutrient agar slants containing 2 per cent sucrose. The composition of the test medium is given in the Appendix (24). The basal medium was made into separate stock solutions and pipetted together prior to autoclaving. Sucrose was autoclaved separately (10 lbs for 15 minutes), then aseptically added to the autoclaved medium. Early in these studies, it was observed that the sucrose needed to be of the best C.P. grade possible since most of the sucrose used contained enough biotin to allow growth of the test organism.

The organism was grown for 18 to 24 hours on 2 per cent sucrose nutrient agar slants at 37°C, washed twice in 10 ml of 0.85 per cent sterile sodium chloride solution (saline) and resuspended in approximately 10 ml of sterile saline. One drop of the washed suspension (O.D. 0.18 to 0.20) was inoculated into a total volume of 10 ml of the assay medium. Incubation was for 24 to 36 hours at 37°C. Vitamin B_6 must be present in the medium before any significant effect of biotin can be demonstrated (24).

Because some doubt has been expressed concerning the validity of the Saccharomyces cerevisiae assay procedure (2), Lactobacillus arabinosus was also used as an assay organism. The culture was carried in 1 per cent yeast extract agar stabs. Fresh transfers were incubated at 37 °C for 3 days and then held under refrigeration. Prior to the procedure leading to the determination of biotin, an inoculum is prepared by transferring the organism from the stock culture to a medium containing 1 per cent glucose and 1 per cent yeast extract and incubating at 37 °C for 18 to 36 hours. The cells are then centrifuged, washed twice with sterile saline and a faintly turbid saline suspension

prepared for seeding the assay medium. Composition of the basal medium is given in the Appendix (32).

All the solutions may be preserved under benzene without previous heat sterilization. Glucose was sterilized separately then aseptically added to the autoclaved assay medium. The final volume of the assay medium was 5 ml. The tubes were incubated without shaking at 37°C for 36 to 48 hours. Results are presented in Table XI.

From these data, it is apparent that some biotin activity was associated with the factor preparation. Biotin activity was more obvious using S. cerevisiae as the assay organism.

(2) Bioautographic plate method. Because there was no way to determine whether this growth response was due to the factor itself or to biotin present as an impurity, the bioautographic plate test, employing a p-aminobenzoic acid-less strain of Neurospora crassa, was also used. The composition of the basal medium for this assay is also given in the Appendix (31).

Stock cultures of the mutant were maintained on agar slants composed of the basal medium plus the following: agar 1.5 per cent, yeast extract 0.2 per cent, malt extract 0.2 per cent and choline 1 µg per ml.

The spores used in the test procedure were obtained by growing the organism on agar slants at 25°C for at least 4 days. The spore suspension of N. crassa was prepared by washing the spores 3 times in sterile saline. Such a suspension (10 ml) was then poured into 150 ml of cooled basal medium which contained 2.0 per cent washed agar. After the medium had solidified, dried paper chromatographic strips containing known biotin compounds and strips containing the unidentified factor were placed on the seeded agar and the entire assembly incubated at 25°C

TABLE XI

ASSAY FOR BIOTIN USING *SACCHAROMYCES CEREVISIAE* AND *LACTOBACILLUS ARABINOSUS*

Assay Organism	Biotin Present*	Factor Present**	Optical Density*** 48 Hours
S. cerevisiae	-	-	0.53
	+	-	1.43
	-	+	0.95
L. arabinosus	-	-	0.235
	+	-	0.39
	-	+	0.265

* Biotin concentration 2.5 µg/10 ml.

** 0.25 ml of factor was used (eluted from paper chromatograms which had been developed two times using the butanol/acetic acid/water system).

*** Reading made at 535 mµ for *S. cerevisiae* against a distilled water blank; for *L. arabinosus* a tube of uninoculated broth medium was used as a blank.

for 12 to 18 hours. Seeded bioautographic plates containing M. lysodeikticus were always run concurrently. Results are given in Tables XII and XIII.

Data in Table XIII show that our factor is not one of the biotins but rather, that our preparation was contaminated with biocytin and biotin. Because of the small quantity, S. cerevisiae was able to respond better than L. arabinosus in the liquid assay procedure since the former is supposed to be more sensitive (2).

It should be pointed out that all of these assay determinations were done early in the experimentation using factor material that was not as pure as we eventually obtained. No free biotin compounds are present in the purified Rf 0.3 factor material or in any hydrolyzates made using this material (see Table XV). Therefore these studies are presented to emphasize the point that the factor material itself is not one of the biotins.

Excellent ancillary evidence to confirm this point is presented in Table XIV. These data show that when the bioautographic plate test is performed using the completely defined medium, biotin cannot support growth in the absence of a purine conjugate or the factor. These data also demonstrate that when factor material is used to obtain growth, subsequent growth is equal to that obtained when inosine alone is used and no additive effects are apparent using either inosine or biotin.

Attempted substitution of the Rf 0.3 factor using known compounds: Although much data had been compiled in an attempt to relate the factor to known compounds, it was very difficult to catagorize the factor. The best information available appeared to be solubility of the factor in different solvents. The material is very soluble in water and insoluble in ether. It can be suspected that polar groups are present.

TABLE XII

COMPARISON OF THE Rf VALUES FOR VARIOUS FORMS OF BIOTIN USING N. CRASSA

Compound Tested	Wright's Data*	Rf Values We Obtained
Biocytin	0.37 & 0.20	0.35
Biotin	0.83	0.84
Biotin-d-sulfoxide	0.53	0.49
Biotin-l-sulfoxide	0.43	0.38
Desthiobiotin	0.90	0.88
Oxybiotin	0.78	0.77

* Rf values taken from reference No. 30.

TABLE XIII

COMPARISON OF Rf VALUES WHEREIN GROWTH OCCURRED USING *M. LYSODEIKTICUS* AND *N. CRASSA*

Test Organism	Rf Values of Growth Areas*
M. lysodeikticus	0.24; 0.41
N. crassa	0.35; 0.85

* Chromatograms used in this test were developed in n-butanol/ acetic acid/water (4/1/5).

TABLE XIV

COMPARATIVE GROWTH OF M. LYSODEIKTICUS IN THE PRESENCE OF BIOTIN, INOSINE AND THE Rf 0.3 FACTOR USING A DEFINED MEDIUM

Compound Tested	Growth Response
Factor	3+
Factor + inosine	3+
Factor + biotin	3+
Inosine + biotin	3+
Biotin	-
Inosine	3+

(-) indicates no growth; (3+) indicates maximal growth response

The types of compounds could therefore be polyhydroxy alcohols, diamines, carbohydrates, amino salts, metal salts, polybasic acids, hydroxyaldehydes, ketones or amino acids (22). Therefore, several tests were run using some of these groups of compounds as reference chemicals.

Because keto acids might have been involved, substitution tests for the unidentified factor were run using the bioautographic plate test. The keto acids tested included: α-keto-glutaric, pyruvic and oxalacetic acids. None of these compounds allow growth.

Other compounds such as malic acid, sodium succinate, fructose-1, 6-diphosphate (Ca-salt), DL-glyceraldehyde-3-phosphate (Ba-salt), triose-phosphate ester (Na-salt), 3-phospho-glyceric acid (Ca-salt), 2-phospho-glyceric acid (Ba-salt), DL-glyceraldehyde, and ribose-5-phosphate were also tested. The bioautographic plate test showed that none of the above was the growth factor.

Imidazole tests. Because the factor did not appear to be a free purine or purine conjugate, a purine precursor (4-amino-5-imidazole carboxamide.HCl) was tested to determine if it would allow growth and thereby substitute for the unidentified factor. Results were negative.

Qualitative analysis of the growth factor after acid hydrolysis: Acid hydrolysis was used to fragment the factor material since unhydrolyzed Rf 0.3 factor material reacted poorly with color-producing reagents. Enzyme digestions had been attempted using trypsin, chymotrysin, pepsin or alkaline phosphatase (intestinal), however, no fragmentation or diminution of growth promoting ability had occurred. The factor material could be partially cleaved employing acid hydrolysis. Growth response of *M. lysodeikticus* showed that the factor was only partially destroyed after hydrolysis in N HCl (1 hour at 121°C) but was completely

destroyed after hydrolysis in 6N HCl for 8 hours at 121°C (Table XV).

The products of hydrolysis were studied employing several colorimetric tests in order to determine the nature of the fragments.

(1) Identification of ninhydrin material. It was observed that the factor material contains a peptide which can be partially cleaved after hydrolysis in 1N HCl (1 hour at 121°C) or completely broken down after hydrolysis in 6N HCl (8 or 22 hours at 121°C). Chromatography of the 8 or 22 hour hydrolyzates in the Redfield two-dimensional system revealed 5 ninhydrin-positive areas. By comparing these Rf values to the Rf values obtained using known amino acids, we thought the compounds could be the amino acids alanine, aspartic acid, glutamic acid, glycine and serine. Co-chromatography of these five known amino acids with a sample of the 22 hour hydrolyzates showed that this conclusion was justified. All Rf values are given in Table XVI.

(2) Sugar tests. Only unhydrolyzed pure factor material and the hydrolyzate using 1N HCl (1 hour at 121°C) or 2N H_2SO_4 (2 hours at 100° C) gave a positive reaction when tested with the p-anisidine spray reagent. The reaction was, however, very weak and occurred only when the factor was spotted directly on filter paper. Sugar could not be detected after chromatography even though relatively large amounts were spotted. Using the spot test (spray with p-anisidine; see Appendix (15)) a light brown color was developed. For this reason, aldohexoses or deoxy sugars were suspected, since aldopentoses give a dark brown color reaction, ketohexoses are yellow, uronic acids are red-pink and methyl pentoses are green colored using this method. The sugar moiety could also be detected (in spot tests only) using the polyhydric alcohol reagents. This was fortuitous since a deoxy sugar which we

TABLE XV

GROWTH RESPONSE AND COLORIMETRIC REACTIONS OF UNHYDROLYZED AND HYDROLYZED Rf 0.3 FACTOR MATERIAL

Test Employed	Unhydrolyzed Factor	Factor Hydrolyzed in 1N HCl for 1 Hour at 121°C	Factor Hydrolyzed in 6N HCl for 8 Hours at 121°C	Factor Hydrolyzed in 6N HCl for 22 Hours at 121°C
Bioautographic growth response	4+	1.5+	None	None
Rf*	0.27	0.41	None detectable	
Rf***	0.42	0.53	None detectable	
Ninhydrin reaction**	None	4 positive areas	5 positive areas	5 positive areas
Sugar test	Trace	Trace	None	None
Rf*	None detectable			
Polyhydric alcohol	None	0.5+	4+	None
Rf*	None	0.14	0.16	None
Iodine test	Quenching	Quenching	Quenching	Quenching
Rf*	0.24	0.083; 0.13	0.13; 0.19	Not tested
Diazo color	Pink	Pink	Pink	Pink
Rf***	0.47	0.18	0.19	0.17
Keto acid	None	None	Trace	Trace
Rf*	None detectable			
Biotin assay using N. crassa	None	None	None	None
Rf*	None detectable			
Sugar-phosphate	None	Trace	None	None
Rf*	None detectable			

TABLE XV (CONTINUED)

* Chromatograms developed in butanol/acetic acid/water (4/1/5 v/v/v)

** Chromatograms developed in Redfield two dimensional system.
Solvent 1. Methanol/water/pyridine (80/20/4 v/v/v).
Solvent 2. t-butanol/methyl-ethyl ketone/water/diethylamine (40/40/20/4 v/v/v/v). (see Table XVI for Rf values).

*** Chromatograms developed in n-propyl alcohol/N acetic acid (3/1 v/v).

TABLE XVI

Rf VALUES OF KNOWN AMINO ACIDS AND HYDROLYZATES OBTAINED FROM THE Rf 0.3 FACTOR*

Known Amino Acids			Hydrolyzed in 1N HCl for 1 hour at 121°C		Hydrolyzed in 6N HCl for 8 hours at 121°C		Hydrolyzed in 6N HCl for 22 hours at 121°C	
	#1	#2	#1	#2	#1	#2	#1	#2
Glutamic Acid	0.45	0.06	0.41	0.34	0.41	0.34	0.44	0.06
Aspartic Acid	0.36	0.07	0.33	0.34	0.33	0.33	0.33	0.06
Glycine	0.41	0.20	0.37	0.44	0.37	0.44	0.45	0.19
Serine**	0.45	0.33	0.46	0.53	0.41	0.54	0.51	0.30
Alanine**	0.58	0.25			0.55	0.48	0.58	0.25

* Solvent #1: Methanol/water/pyridine (80/20/4 v/v/v)
Solvent #2: t-butanol/methyl-ethyl ketone/water/diethylamine (40/40/20/4 v/v/v/v)

** The Rf value for both serine and alanine in the 1N HCl hydrolyzate is listed as one Rf value since they did not appear to separate using these conditions

tested (deoxyribose) did not react with this reagent. According to Gortner and Gortner (10) three aldohexose compounds are found in nature (glucose, galactose and mannose). Of the three possibilities, glucose was ruled out because no reaction occurred using the glucose oxidase enzyme reaction as modified by Salton (20). Further work to determine if the sugar is mannose or galactose has not been done. Whether or not further effort in this direction is justified is debatable since the sugar reaction is extremely weak. This observation makes it appear reasonable to conclude that the sugar exists as an impurity in the preparation and is, therefore, not a part of the growth factor molecule.

(3) Identification of the polyhydric alcohol moiety. Hydrolysis in 6N HCl (8 hours at 121°C) will maximally release a compound from the Rf 0.3 material that gives a positive test for polyhydric alcohol. The indicator spray for detection of polyhydric alcohols is given in the Appendix (4). It appears that, at the time this polyhydric alcohol group is maximally released, the factor is completely destroyed as indicated by loss of growth supporting ability.

Several known compounds were studied to determine if they gave positive reactions using this test procedure. Compounds included N-acetylglucosamine, allose, deoxyribose, dulcitol, galactose, glucose, inositol, lactic acid, mannitol, mannose, pyruvic acid, raffinose, rhamnose, ribose, sorbitol, sucrose, and xylose. All gave positive reactions except N-acetylglucosamine, deoxyribose, raffinose, rhamnose, and sucrose. Because the Rf value of the fragment released by hydrolysis in 6N HCl (8 hours at 121°C) is 0.16, compounds known to give a positive reaction with the polyhydric alcohol spray reagent were chromatographed in the same system (butanol/acetic acid/water) and a

comparison of Rf values was made. These data are given in Tables XV and XVII. The fragment released by hydrolysis in 6N HCl migrated most closely to dulcitol, galactose and glucose. It is highly improbable that the compound is either galactose or glucose since the sugar test (p-anisidine reagent) using the 6N HCl hydrolyzate (8 hours at 121°C) is completely negative. Therefore, this compound should be dulcitol. This was confirmed by co-chromatography of the hydrolyzate with dulcitol in the butanol system.

(4) Imidazole compounds. Unhydrolyzed and hydrolyzed factor material was also spotted to detect the presence or absence of imidazole compounds. The first technique used was to spot the material onto a strip of Whatman #1 filter paper and first treat it with the vapors from ammonium hydroxide. The paper was then neutralized, dipped into 1 per cent iodine in carbon tetrachloride, dried and observed using ultra-violet light.

Imidazole compounds show a strong quenching action after this treatment (23). It appears that the unidentified factor could have an imidazole group since quenching was observed. The quenching was more obvious after hydrolysis of the factor in 6N HCl for 8 hours. Results of these studies are incorporated into Table XV.

Chromatography of the hydrolyzates showed that the quenching material had two Rf values (0.13 and 0.19 in the butanol/acetic acid/water system), however, most of it was present in the area of Rf 0.13. Because there are no published Rf values of imidazole compounds available for comparison using the butanol system, another system was adopted which employs ascending chromatography in n-propyl alcohol/N acetic acid (3/1 v/v). Because a reagent is known which gives different

TABLE XVII

COMPARISON OF Rf VALUES OF KNOWN COMPOUNDS WITH THE FRAGMENT RELEASED FROM THE Rf 0.3 MATERIAL AFTER ACID HYDROLYSIS (6N HCl FOR 8 HOURS AT 121°C)*

Compound Tested**	Rf Value
Hydrolyzate fragment	0.16
Allose	0.22
Dulcitol	0.17
Galactose	0.18
Glucose	0.16
Inositol	0.09
Lactic acid	0.77
Mannitol	0.22
Mannose	0.21
Pyruvic acid	0.74
Ribose	0.31
Sorbitol	0.21
Xylose	0.26

* Chromatographed in butanol/acetic acid/water (4/1/5 v/v/v).

** Detected using the polyhydric alcohol indicator spray reagent.

color reactions with different imidazole-containing compounds (diazonium spray reagent; (1)) this spray reagent was substituted for the iodine test. Using the diazo-spray reagent, a pink color and an Rf value for the fragment were obtained. Rf values are incorporated into Table XV.

Again, other known imidazole compounds were compared with the factor fragment both for color and Rf values. We have not been able to identify this imidazole-containing fragment for two reasons. (1) The color developed with our fragment is pink rather than red. No pink colored compounds are listed in the reference material we employed (1). The Rf value of the imidazole reacting compound is 0.47 using the unhydrolyzed factor molecule, but it has an Rf value of 0.18 after hydrolysis for 22 hours in 6N HCl. Because of the change in Rf value, it can be concluded that the imidazole-reacting compound has been split off from a larger molecule. (2) The Rf value of 0.18 does not correspond either to histidine (Rf 0.22 and red color) or guanine (Rf 0.37 and orange color), both of which were tested. The compound cannot be adenine (yellow color) or hypoxanthine (no color). Further, the fragment is neither free imidazole (Rf value of free imidazole is 0.58) nor can it be 4-amino-5-carboxamide imidazole (blue color and Rf 0.51). Because the diazo color reaction is extremely weak (dulcitol and amino acid reactions are very pronounced), we favor the view that this compound is not a part of the factor molecule. Its presence could be explained as: (1) an impurity in the preparation or, (2) an unexplained color artifact.

Whatever the identity of the imidazole compound, good evidence is presented in Table XV showing that it is probably an impurity. These data show that the Rf value of the imidazole compound differs from the

Rf value of the growth factor in the n-propyl/acetic acid system. Also, of importance is the observation that after hydrolysis in N HCl (1 hour at 121°C), the Rf value of the imidazole compound is far removed from the area in which growth occurs and no imidazole reaction is associated with this area of growth.

(5) Determinations for keto acids. A positive test for keto-acid was obtained, using the spot test, however the positive result occurred only after hydrolysis of the factor in 6N HCl for 8 hours at 121°C. Although too small an amount was present to be detected by chromatography, several known keto acids (α-keto glutaric, α-keto isovaleric, pyruvic and oxalactic acids) were chromatographed and tested for growth promoting ability using the bioautographic plate technique. None of these compounds could support growth of M. lysodeikticus.

Because a small amount of sugar (galactose or mannose) is present in the factor preparation, and since sugars can, under conditions of acid hydrolysis give rise to keto compounds, it is concluded that the keto acid is an artifact arising from the sugar impurity present in the factor preparation.

An additional impurity appears to be present in that after hydrolysis in N HCl for 1 hour at 121°C, a weak positive test for sugar phosphate is apparent. Again the material exists in amounts too small to be detected by chromatography and disappears completely when stronger conditions for acid hydrolysis are employed.

It is entirely conceivable that an imidazole sugar phosphate compound is present with our factor compound in very small amounts. This would explain all the reaction obtained and limit the impurities in

the factor preparation to one molecular species. Further studies involving crystallization and/or additional chromatographic separations of our factor material are required to resolve this particular point.

On the basis of data obtained via acid hydrolysis of the factor material it appears appropriate to conclude that the compound is a complex of dulcitol and a peptide made up of five amino acids. Judging from the intensity of color reactions developed on paper, the molar ratio for the amino acids aspartic acid, glutamic acid, alanine, serine, and glycine would be 1-1-1-1-3.

No information relating either to isomeric configuration or sequence of amino acids is yet available, therefore no statement can be made except to point out that under relatively weak hydrolytic conditions (N HCl for 1 hour at 121°C) serine and alanine do not separate well. It can be assumed therefore, that these two amino acids are bonded together in the peptide chain.

A question to be considered relates to the ninhydrin negative character of the peptide. Assuming that the factor is a peptide-dulcitol complex only, then its ninhydrin-negative character could be explained by two structural types of molecules. (1) The complex is cyclic; therefore no free amino and carboxyl groups are exposed to react with ninhydrin. (2) The peptide is an aliphatic chain with the terminal group on one end being the carboxyl group of an amino acid and the other end amino acid being attached to dulcitol, through its amino group. This type of structure would also have no free amino groups to react with ninhydrin.

The presence of free amino groups appears to be necessary for the ninhydrin reaction. As an example: we tested glucosamine and N-acetyl-

glucosamine using ninhydrin during the portion of the work dealing with amino acid identification. Although glucosamine reacts very well with ninhydrin, N-acetylglucosamine gives no color. The only difference in the molecules relates to the N-acetyl group present in N-acetylglucosamine.

On the basis of the data collected thus far, it is not possible to characterize the molecule as to whether it is a cyclic or aliphatic structure.

Although it would be desirable to quantitate the amount of factor required for growth, no statement can be made at this time. The amounts of factor material obtained have been too small to allow accurate measurement.

A remarkable feature of the factor is its ability to completely replace purine (free purine such as hypoxanthine or the nucleosides inosine or adenosine) in the nutrition of M. lysodeikticus. Data thus far obtained justify the conclusion that no purine moiety exists in the factor. Therefore, the organism is not hydrolyzing the factor to release a purine and thereby satisfy the purine requirement.

The factor, although a new molecule, most nearly resembles a cell wall peptide; however important differences are apparent. Both aspartic and glutamic acids are present whereas either one or the other is present in cell wall peptides. Also, no basic amino acid such as lysine is present. The biggest difference exists in the dulcitol moiety. In cell wall peptides, the peptide fragment is attached to a uridine-phosphate-amino sugar residue; this type of residue has not been found in our factor. Whether or not the dulcitol-peptide is used in cell wall synthesis remains a problem for further study.

Another feature of the factor which will require close scrutiny relates to the growth promoting potential of the molecule after partial hydrolysis. If the entire molecule contains only dulcitol and the five amino acids, how many of the amino acids can be split off before growth stimulation fails to occur? Data in Table XV make it obvious that dulcitol alone will not support growth.

SUMMARY

1. A chemically defined medium for growth of M. lysodeikticus is presented. It includes ammonium chloride, potassium phosphate (di-basic) magnesium sulfate, L-glutamic acid, L-phenylalanine, L-tyrosine, inosine, biotin and iron. A requirement for iron is evident, the requirement is not specific for the inorganic forms. Growth also occurs using coprogen, hemin, cytochrome c or ferrichrome. The organism possesses a specific requirement for purines. Although hypoxanthine (free base of inosine) allow growth almost equivalent to inosine, far less growth occurs when the free base adenine is substituted for adenosine. Although biotin stimulates growth, equally good growth occurs using biocytin or biotin-d-sulfoxide; less stimulation is apparent using desthiobiotin, dl-oxybiotin, or biotin-l-sulfoxide.

2. The unidentified growth factor which is present in peptone and required for growth by M. lysodeikticus was fractionated and purified using organic solvent extraction techniques. Further purification was accomplished using paper chromatography in phenol/water (7/3) followed by n-butanol/acetic acid/water (4/1/5).

Hydrolysis in 6N HCl, (121°C for 8 or 22 hours) showed that the growth factor appears to be a mixed peptide containing at least 5 amino acids (aspartic and glutamic acids, alanine, glycine and serine) and dulcitol. Its growth promoting ability is completely destroyed after hydrolysis in 6N HCl for 8 hours at 121° C.

Possible structures relating to the ninhydrin-negative characteristic

of the dulcitol peptide are discussed.

A remarkable feature of the factor is its ability to completely replace a purine (free purine such as hypoxanthine or the nucleosides, adenosine or inosine) in the nutrition of M. lysodeikticus. Data obtained thus far justify the conclusion that no purine moiety exists in the factor molecule.

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APPENDIX

Snell, Eakin and Williams Medium (24):

C. P. Sucrose	20.0 gm
$(NH_4)_2SO_4$	3.0 gm
KH_2PO_4	2.0 gm
$MgSO_4 \cdot 7H_2O$	0.25 gm
$CaCl_2 \cdot 2H_2O$	0.25 gm
H_3BO_3	1.0 mg
$ZnSO_4$	1.0 mg
$MnCl_2$	1.0 mg
$TlCl_3$	1.0 mg
$FeCl_3$	0.5 mg
$CuSO_4 \cdot 5H_2O$	0.1 mg
KI	0.1 mg
L-aspartic acid	0.1 gm
Inositol	5.0 mg
β-alanine	0.5 mg
Thiamine	20.0 µg
Vitamin B_6	20.0 µg
Distilled Water	1,000.0 ml

Wright and Skeggs Medium (32):

Hydrochloric acid-hydrolyzed, Norite-treated, vitamin free casein	0.5 %
Tryptophan	0.01 %

Cystine*	0.01	%
Glucose	2.0	%
Sodium acetate (anhydrous)	0.6	%
K_2HPO_4	0.05	%
KH_2PO_4	0.05	%
NaCl	0.001	%
$MgSO_4 \cdot 7H_2O$	0.02	%
$FeSO_4 \cdot 7H_2O$	0.001	%
$MnSO_4 \cdot 4H_2O$	0.001	%
Adenine sulfate**	5.0	ppm
Guanine hydrochloride**	5.0	ppm
Xanthine	5.0	ppm
Uracil	5.0	ppm
Thiamine	1.0	ppm
Calcium pantothenate	1.0	ppm
Pyridoxine hydrochloride	2.0	ppm
Riboflavin	1.0	ppm
Nicotinic acid	1.0	ppm
Para-aminobenzoic acid	0.1	ppm

* Dissolved separately with a small amount of concentrated HCl.

** Dissolved separately by heating with hydrochloric acid.

Wright, Cresson and Driscoll Medium (31):

Sucrose	20.0	gm
Ammonium tartrate	5.0	gm
Ammonium nitrate	1.0	gm

Dihydrogen potassium phosphate (KH_2PO_4)	1.0	gm
Magnesium sulfate (heptahydrate)	0.5	gm
Sodium chloride	0.1	gm
Calcium chloride	0.086	gm
Trace elements*	1.0	ml
p-aminobenzoic acid	10.0	mg

* Horowitz, N. H., and Beadle, G. W. 1943. A microbiological method for the determination of choline by use of a mutant of neurospora. J. Biol. Chem., 150, 325-333.

Trace elements added as salts in mg per liter:

B	0.01
Mo	0.02
Fe	0.2
Cu	0.1
Mn	0.02
Zn	2.0

Reagent used for the detection of sugars:

p-Anisidine HCl (15):

0.5 gm of p-anisidine is dissolved in 2 ml of H_3PO_4 (sp. gr. 1.75), and the solution is diluted to 50 ml with ethanol. The precipitated anisidine phosphate is removed by filtration. The filtrate (A) is saved. The precipitate is dissolved in a minimum quantity of water and diluted with an equal volume of alcohol. Phosphoric acid is added to a final concentration of 2 per cent (B). A and B are mixed, and the chromatogram is sprayed and heated at 95-100°C for 3-5 minutes. Several sugars can be identified by the color of the spots, which remain stable for over a week.

Sugar	Color reaction
Aldohexoses	Light brown
Aldopentoses	Brown
Ketohexoses	Yellow
Uronic acids	Red-pink
Methyl pentoses	Green
Deoxy sugars	Light brown

Indicator spray used for the detection of polyhydric alcohols (4):

Bromocresol purple	40.0 mg
Ethanol (95%)	100.0 ml
Boric acid	100.0 mg
Aqueous borax solution (1%)	7.5 ml

Sorbitol, mannitol, dulcitol and other sugar alcohols appear as yellow spots on a blue background; owing to a pH change by the sugar-borate complex; the color fades with time.

Reagents used for the detection of imidazole compounds:

(a) Iodine spray (23).

Iodine, 1 per cent, in carbon tetrachloride. Papers, which should be neutral, are dipped in the reagent and suspended in a hood. As the solvent evaporates, transient brown spots are formed which are marked immediately in pencil. Papers may be dipped again and the spots will re-appear. It is useful to examine the chromatograms in ultra-violet light before and after dipping in the iodine solution. After dipping, the imidazoles appear as dark spots.

(b) Diazonium spray (1):

Solution A: 25 ml of freshly prepared 5 per cent $NaNO_2$ is slowly added at 0 C to 5.0 ml of a sulfanilic acid solution (0.9 gm of sulfanilic acid plus 9 ml of concentrated HCl, diluted to 100 ml, with distilled water).

Solution B: 5 per cent Na_2CO_3.

The dried chromatogram is lightly sprayed with solution A, and while the paper is still damp it is sprayed with solution B.

Diazo colors for imidazoles (1):

Compound	Diazo Color
Histidine	Red
4(5)-carboxyimidazole	Yellow
Carnosine	Red
2-Thiolhistidine	Red
[Imidazolyl-4 (5)]-lactic acid	Red
Ergothionine	Red
Guanine	Orange
[Imadzole-4(5)]-pyruvic acid	Red
4(5)-Amino-5(4)-carboxamide imidazole	Blue
Histamine	Red
4(5)-Hydroxymethyl-imidazole	Red
Imidazole	Red-orange

Spray reagent used for the detection of free α-keto acids (3):

o-phenylenediamine	0.05 gm
Aqueous CCl_3COOH (10%)	100.0 ml

The dry filter paper is sprayed with a solution of o-phenylene-diamine and heated for 2 minutes at 100 C. If the paper is over heated, the whole strip fluoresces. α-keto acids are identified as yellow-green spots which fluoresce under U. V. illumination.

Reagent for detection of sugar phosphate (11):

$HClO_4$ (60% w/w)	5.0 ml
1N HCl	10.0 ml
$(NH_4)_2MoO_4$ (25% solution)	25.0 ml
Water	60.0 ml

The chromatograms are sprayed at the rate of 1 ml of the reagent per 100 cm_2, and then autoclaved for 2 minutes at 8-10 pounds pressure. After autoclaving and while still damp, the chromatograms are exposed to ammonium vapors. The blue color from the reagent will be bleached on contact with ammonium vapors, however, in the area containing sugar phosphate, the blue color will remain unbleached.

VITA

Yung-Chieh Chu

Candidate for the Degree of

Master of Science

Thesis: DEFINED MEDIUM FOR GROWTH OF MICROCOCCUS LYSODEIKTICUS; PURIFICATION AND IDENTIFICATION OF AN UNKNOWN GROWTH FACTOR

Major Field: Bacteriology

Biographical:

Personal Data: Born at Chekiang, China, December 14, 1934.

Education: Graduated from Taichung Girl's High School, Taichung, Taiwan, China in 1952. Received the Bachelor of Science degree from Taiwan Provincial College of Agriculture, Taichung, Taiwan, China in 1956.

Experience: Full time graduate Assistant, Taiwan Provincial College of Agriculture, Taichung, Taiwan, China June 1956-July 1957. Graduate Teaching Assistant, Department of Bacteriology, Oklahoma State University, Stillwater, September 1957 - May, 1959. Graduate Research Assistant, Department of Bacteriology, Oklahoma State University, Stillwater, 1959-1960.

Organization: Phi Sigma Biological Society.