THE NUTRITIONAL REQUIREMENTS OF

MICROCOCCUS LYSODEIKTICUS

By

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CHAPTER I

INTRODUCTION

The nutritional requirements of Micrococcus lysodeikticus have been studied for many years. At least four synthetic media have been proposed. Feiner, Meyer, and Steinberg (7) reported in 1946 that they were unable to cultivate any of the three immunologically distinct strains tested unless crude folvite liquors (impure folic acid) were used. When pure folic acid was added to synthetic media, growth did not occur. Some media formulations have been rather simple containing only minerals, glucose, an amino acid and biotin (25); others become more complex employing up to 16 amino acids, 8 B-vitamins, purines, pyrimidines, minerals and glucose (24). Regardless of the complexity of the media, the workers who obtained growth in synthetic media used biotin and glucose plus an amino acid or an amino acid mixture and mineral salts.

There appear to be at least 3 "strains" of M. lysodeikticus (Iowa State, Purdue and Western Reserve University strains), all of which appear to have different nutritional requirements. Whether they are all different strains of M. lysodeikticus is not known, since information is lacking relating to identification of this organism. It is merely described in Bergey's Manual (6th Edition) as a yellow micrococcus easily lysed by lysozyme. It is not listed in the 7th Edition of Bergey's Manual.

It had been demonstrated that the Purdue University strain of M. lysodeikticus required, for its growth, a substance present in peptone (5). This peptone "factor" could not be replaced by any combination of vitamins and amino acids, nor could it be replaced by purines, pyrimidines or carbohydrates (3). These preliminary studies indicated that the substance in peptone which was responsible for growth was either completely new or a compound not usually added to bacteriological media. The main purpose of this research was to devise a chemically defined medium for the Purdue University strain of M. lysodeikticus, which would allow good growth and, if possible, to identify the unknown substance present in peptone.

CHAPTER II

HISTORICAL

Micrococcus lysodeikticus was isolated from the air in a laboratory and first described by Fleming (8). The organism, a Gram-positive micrococcus, grows well on ordinary media within a range of pH 7.0 to 7.6 and has an optimum temperature of 37 C. The colonies are characteristically yellow, convex, opaque, smooth, and glistening. Reactions in the usual biochemical media are generally negative. Gelatin is not liquefied. Nitrates are not reduced. Litmus milk becomes slightly alkaline with no other changes. Glucose, lactose, maltose, saccharose, mannitol, and salicin are not fermented. Indole and hydrogen sulphide are not produced and starch is not hydrolyzed (7).

This organism is important because it is easily lysed by lysozyme, an enzyme present in tears, phagocytic cells and other body secretions. In the body, lysozyme functions in a non-specific manner to protect mammals from bacterial invasion by causing lysis and/or death of many bacteria. Because lysozyme is an important body defense mechanism, any organism susceptible to its action becomes important as a "tool" to study the lytic mechanism of the enzyme. Also, susceptible bacteria become important because they can be used for study of the synthesis of the lysozyme-substrate in the cell wall of bacteria. In those circumstances where the substrate comprises the bulk of the cell wall, they can be used to study synthesis of the cell wall. Studies, thus far, show that the substrate of lysozyme is an aminopolysaccharide (14, 6)

located exclusively in the cell wall of bacteria (20, 22, 9, 10, 23). Depolymerization and subsequent degradation of this material by lysozyme leads to lysis of the cells (14). Once removal of the cell wall has occurred leaving the cytoplasmic membrane as the only external protective structure, plasmoptysis easily occurs unless hypertonic solutions are employed to protect and stabilize the remaining protoplasts (22).

Before studies can be initiated which relate to the synthesis of the cell wall of M. lysodeikticus or, more specifically, the lysozyme substrate in the wall, it would be desirable to grow the organism in a completely synthetic medium. Such a synthetic medium would simplify studies that could relate differences between nutrition and the susceptibility of cells to lysis by lysozyme (cell wall composition). Isotopic tagging for study of specific cell wall precursor substances could also be done more accurately using synthetic media. Several synthetic media formulations for M. lysodeikticus have been reported.

Feiner, Meyer and Steinberg (7) made the first attempt to devise a synthetic medium for M. lysodeikticus, using three immunologically distinct strains. Their attempt was unsuccessful; they were able to obtain good growth only when they used a Lactobacillus casei factor (a folvite liquor which has been shown to be impure folic acid). When they substituted pure folic acid, growth did not occur. They also observed that no stimulation of growth occurred when any of the B-vitamins including biotin were added.

Wessman, Allen and Werkman (24) reported that their strain of M. lysodeikticus (Iowa State University) grew well in a synthetic medium containing 16 amino acids, 8 B-vitamins, purines, pyrimidines, salts and

glucose. They observed that biotin was stimulatory to growth (see Appendix for composition of their medium).

In 1955, at the meeting of the Society of American Bacteriologists in New York City, Wolin and Naylor (25) reported a synthetic medium for M. lysodeikticus. The medium contained biotin, glucose, glutamic acid and minerals (see Appendix for composition of their medium).

In the same year, the formulation of a synthetic medium for M. lysodeikticus was obtained from Dr. Heden of Sweden (personal communication to Dr. E. A. Grula). The synthetic medium contained only minerals, glucose and casamino acids. Biotin was not used (see Appendix for composition of the medium).

Also in 1955, Gerhardt and Britt (4) reported using a "synthetic" medium during their studies on the lysine "pool" of M. lysodeikticus. The source of their strain is not known, however, they stated that their medium actually required the addition of yeast extract. Also, the organism grew abundantly in 1 or 2 per cent peptone water.

Litwack and Pramer (13) also reported that their strain (Rutgers University stock culture) grew abundantly in 1 per cent peptone. They attempted to grow the organism in 10 different peptones and observed that neopeptone or protone allowed no growth whatever. With regard to further work concerning the synthesis of the lysozyme-substrate, it is interesting to note that they observed different amounts of lysozyme-lysis after growing the organism in the presence of various peptones (13). Although Repaske (19) investigated only Gram-negative bacteria, he has also reported that variation in medium composition (all complex media) resulted in changed response of cells of lysozyme (19).

A recent publication, regarding the nutritional requirements of M. lysodeikticus, was reported by Wolin and Naylor (26). Their synthetic medium contained minerals, biotin, glucose and monosodium glutamate. They reported that this medium allowed better growth of the organism than complex media. When the Purdue University strain of M. lysodeikticus was inoculated into these media, growth did not occur (3).

Brock (5) using the Purdue University strain of M. lysodeikticus (a subculture of the original organism isolated by Fleming), was able to obtain growth in a medium containing only amino acids and mineral salts when a "factor" present in either peptone or trypticase was present. Brock found that the factor was dialysable, heat stable (withstood boiling for 1 hour in neutral solutions) and resistant to digestion by trypsin, chymotrypsin and pepsin (5).

Brickler (3) reported that he was able to obtain the factor present in peptone in a relatively concentrated state by selective elution from a Dowex-2 anion exchange resin column with 0.3N HCl. Some properties of the factor as reported by Brickler may be summarized as follows:

1. Very soluble in water.
2. Stable to acid and alkali and heat (cannot be destroyed when heated to boiling for 60 minutes at pH 1.0 to 11.9).
3. Soluble in 70 and 95 per cent alcohol.
4. Not soluble in benzene or ether. (The latter refluxed for 24 hours continuously.)
5. Not steam distillable.
6. The factor is organic, since ashing destroyed growth promoting activity.
7. It is not a naturally occurring amino acid.

8. It is a small dialyzable molecule.

9. It is not a sugar.

10. It is not a short chain fatty acid.

11. It is not a known vitamin.

12. It is not precipitated by 1, 2, 3, 4 or 5 volumes of 95 per cent alcohol.

Because the Purdue University strain of M. lysodeikticus does not grow in any of the reported synthetic media, we have assumed that there are differences in the nutritional requirements of the various strains. For that reason and because cell wall synthesis studies are planned, we have attempted to formulate a synthetic medium which would allow good growth of the Purdue University strain of M. lysodeikticus and also to identify, if possible, the "unknown" factor present in peptone.

CHAPTER III

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 12 years ago.

Inoculum: Young cultures, 18-24 hours old, grown on nutrient agar slants at 37 C, were washed twice in 10 ml of 0.85 per cent sterile sodium chloride solution (saline) and resuspended in 10 ml of sterile saline prior to inoculation. To inoculate the synthetic media, one drop of the cell suspension (65 per cent transmittance measured at 535 mμ) was inoculated into 5 ml of medium from a sterile 1 ml pipette. The inoculated media were then placed on a rotary shaker (240 r.p.m.) at 25 C for at least 24 hours. (Shaking conditions were always used because this organism is highly aerobic.)

Basal Medium: The basal medium consisted of 1 ml mineral solution, 1 ml potassium phosphate buffer solution (see Appendix) and 0.5 ml "Vitamin Free" casein hydrolyzate (enzymatic). The growth factor or test compounds and triple distilled water were added to the basal medium to make a final total volume of 5 ml. Autoclaving was at 12 lbs pressure for 10-15 minutes, unless otherwise indicated.

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 double distilled water prior to use.

Growth measurements: Growth was determined by measurement of the optical density or per cent transmittance of liquid cultures at 535 mμ in a Bausch and Lomb spectrophotometer, Type 33-29-40.

Fractionation of the growth factor from peptone using Dowex-2 anion exchange resin column: The growth factor used in all experiments was partially purified using a Dowex-2 anion exchange resin column. The column had been aged in distilled water at room temperature for approximately 8 months before using. A small amount of glass wool was placed at the bottom of a glass tube, 57 cm long and 26 mm in diameter tapered at one end to prevent the resin from washing out. Dowex-2 anion exchange resin suspended in triple distilled water was poured into the column to a point 25 cm above the glass wool. The column was washed with triple distilled water until the effluent was colorless and neutral. The resin column was then cleaned by allowing 100 ml of 1N HCl to run through at the rate of about 100 drops per minute. When the acid level reached the top of the resin, water was added and the resin was washed until the effluent was almost neutral. After the final wash, when the water level reached the top of the resin column, 50 ml of 2N NaOH heated to 75 C was added. When the NaOH level reached the top of the resin, the column was again washed with distilled water until neutral. After this treatment, the column was charged (negative charge) and ready for use.

Twenty-five ml of a 5 per cent peptone solution was passed through the column at the rate of about 100 drops per minute and the effluent was discarded. (~~presumably only neutral compounds such as sugars were present~~). The column was then washed with distilled water until neutral. Fifty ml of 1N acetic acid was then added and the effluent was again discarded (this step removed many charged compounds such as amino acids). When the acid level reached the top of the resin, the column was washed with distilled water until neutral.

Elution of the growth factor from Dowex-2 anion exchange resin: Fifty ml of 0.3N HCl was then passed through the column and the rate of flow maintained at about 100 drops per minute. When the acid level reached the top of the resin, water was added very slowly and collection of the effluent was immediately begun using an automatic fraction collector. The rate of flow was adjusted to 18 ml per hour, and collected in 10 ml fractions for 12 hours. The factor was detected by microbiological assay employing the liquid basal medium consisting of buffer, minerals, casamino acids and 0.5 ml of each collected fraction (see Appendix). A light brown color appeared to be associated with the presence of the factor.

Further purification of the factor after elution from the Dowex-2 anion exchange resin: The factor, which had been eluted from the column and neutralized with NaOH for microbiological assay, was evaporated to dryness in a vacuum oven at 60 C, resuspended to approximately one-fourth of its original volume with distilled water, and placed in the refrigerator. After 24 hours, a precipitate began to form and it was

removed from the supernatant after 3 days by centrifugation. The precipitate was discarded since tests proved that it did not contain the growth factor.

Although dried growth factor material could be solubilized in either 100 per cent ethanol or methanol, attempts to crystalize the factor using these solvents failed. Several other solvents were tested to determine solubility of the factor (pyridine, hexane, absolute alcohol, butanone, diethyl amine, acetone, t-butanol, methyl alcohol, n-butanol). These studies showed that the factor was soluble only in ethanol (up to 100 per cent) or methanol.

Further purification of the factor from the column using paper chromatography: (a) n-butanol/acetic acid/water system: About 0.05 ml of the supernatant obtained after refrigeration was deposited on a strip of Whatman No. 1 filter paper 22 inches long and 1½ inches wide, under a continual 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 (24). The papers were then removed and hung in an oven at 60 C for 10 hours (this step removed the acetic acid and butanol from the paper). The strips were then cut into two halves. 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: The factor from the column was 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 oven at 60 C for 12 hours to remove the phenol. A narrow strip was cut from the chromatogram, treated with ninhydrin, and heated in an oven at 105 C for 5 minutes. The remainder of the chromatogram was placed on a seeded bioautographic plate which was incubated at 37 C for 12-24 hours. After incubation the region on the plate that gave growth was marked and the Rf value determined. The Rf value of the growth factor in this system was 0.73-0.77.

Elution of the growth factor from paper chromatograms: The location of the factor on the chromatograms was determined by comparison with growth areas on bioautographic plates. The area containing the growth factor was then cut out, 6-8 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 in a vacuum oven at 60 C and kept for further purification.

Final purification of the factors: The dried factor obtained using chromatography in either phenol/water or butanol/acetic acid/water was resuspended in a small quantity of water and re-chromatographed for final purification using the n-butanol/acetic acid/water (4/1/5) system. Using the bioautographic plate method, two growth areas were located in a position of about Rf 0.3 and 0.5 on the chromatogram. The regions in the chromatograms containing the growth factors were then cut out., eluted with water and taken to dryness in the vacuum oven at 60 C.

Because the areas on the chromatograms which allowed growth as determined by the bioautographic technique did not react with any color-producing reagents and did not absorb under ultraviolet light, it was necessary to calculate their position prior to elution on the basis of Rf. Such procedures do not allow accurate work, however, it would have been physically impossible to check the Rf values of the growth areas on each chromatogram using the bioautographic technique.

Bioautographic plate test: Three grams of agar (Bacto) was added to 150 ml of the basal medium. The contents were autoclaved at 12 lbs pressure for 15 minutes. Ten ml of a cell suspension of M. lysodeikticus which had been washed 3 times in sterile physiological saline (transmittance 20 per cent) was then poured into the basal medium after it had cooled to about 50 C. The seeded medium was gently shaken for mixing and poured into a Pyrex glass rectangular plate (14" x 9" x 2") and covered with a flat glass plate to insure aseptic conditions. After the medium had solidified, the chromatogram to be tested was placed carefully on the surface. The seeded medium was incubated at 37 C for 12-24 hours. After incubation the chromatogram was removed with forceps and the plate was examined for growth under a subdued or indirect light source. The region on the plate that showed growth (usually a hazy diffuse zone) was marked with a glass marking pencil and the Rf value was then determined and compared to paper strips that had been treated with various spray reagents used for detection of different compounds.

Acid hydrolysis for the detection of amino acids: About 0.2 ml of the concentrated 0.5 Rf material was placed in a 2 ml ampule and evaporated to dryness. Two-tenth ml of 6N HCl was added. The ampule was then

sealed with a flame and autoclaved at 15 lbs pressure for 20 hours. The hydrolysate was evaporated to dryness in a vacuum oven at 60 C, resuspended in water and dried three times in order to cause evaporation of the acid.

Alkali hydrolysis for the detection of amino acids: Approximately 0.2 ml of 3N NaOH was added to a dried sample and the mixture was treated as previously described above. After hydrolysing for 20 hours at 15 lbs pressure, the hydrolysate was dried in a vacuum oven at 60 C. Sufficient 1N HCl was then added to make the final pH about 1.0. This procedure transforms the amino acids contained in the hydrolysate into a chloride form which are soluble in acetone. The contents were evaporated to dryness and extracted with dried acetone (salts and acid would not dissolve in acetone).

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 or alkali hydrolysate (10-20 µgm) was applied over ammonia vapors to the left hand bottom corner of the paper, 2 cm from the edges. Diameter of the spot was never more than ¼ cm. A constant flow of dry air from a hair dryer was played over the paper to prevent the liquid from spreading. The paper was folded into a cylinder 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, then dried at room temperature for 15 minutes. The chromatogram was then unfolded and re-folded at right angles to the former

axis and placed into solvent No. 2 (t-butanol/methyl ethyl ketone/water/diethyl amine (40/40/20/4)). When the solvent advanced to the top of the chromatogram (about 5 hours), it was taken out and steamed in an autoclave for 8 minutes with both exhaust valves open in order to eliminate diethyl amine which is a ninhydrin-positive substance. After autoclaving, the chromatogram was dried and 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 (18).

Pinchot's technique for the hydrolysis of nucleotides: (a) Acid hydrolysis: Equal volumes of 1N HCl and concentrated factor were placed in a test tube and heated in a hot water bath at 100 C for 20 minutes. (b) Alkali hydrolysis: Equal volumes of 0.3N KOH and the factor were mixed in a test tube and incubated at 37 C for 15 hours (17).

Partial hydrolysis of the growth factors using 2N sulphuric acid for qualitative analysis: About 0.2 ml of each factor was mixed with 2 ml of 2N sulphuric acid and heated for 2 hours at 100 C in a test tube. After cooling, the hydrolysates were neutralized with 1N barium hydroxide and the precipitates were removed by centrifugation. The supernatants were evaporated to dryness in a vacuum oven at 60 C and resuspended in 0.5 ml of triple distilled water.

CHAPTER IV

RESULTS AND DISCUSSIONS

Since an "unknown" growth factor in peptone was found to be required for growth of M. lysodeikticus (3), other materials were tested to see if they contained the growth factor or if it were present in amounts greater than found in peptone. The compounds were tested by adding 1 ml of a 1 per cent solution to the basal medium. The factor was present in heart infusion, liver extract, tryptone and tryptose. Casitone also contained a small amount, however, none was present in jack bean meal, milk whey, neopeptone or litmus milk. Whether the factor contained in these various substances is identical in chemical composition with the factor present in peptone has not been determined. Since these studies showed that peptone was a good natural source for the growth factor, we attempted concentration and purification of the factor using peptone as a starting material.

Fractionation and purification of the growth factor in peptone: A procedure was devised for the fractionation of the factor from peptone using a Dowex-2 anion exchange resin column which allowed relatively high yields to be obtained. Dowex-2 resin was ground and suspended in distilled water. The portion that did not settle in 20 seconds was discarded. The resin was cleaned and charged as previously described.

When the rate of flow during elution of the factor with 0.3N HCl was reduced to 18 ml per hour, a high concentration of the factor was obtained in the 90th to 110th ml of effluent. The assays were performed using the basal medium containing casamino acids, minerals, and the buffer solution, plus quantitated amounts of column effluents which had been neutralized with NaOH (Fig. 1). No attempts were made to quantitate the amount of purification that had been obtained.

It was observed that a further purification could be accomplished by drying the effluent from the column in a vacuum oven at 60 C, resuspending it in a small amount of distilled water and refrigerating for 3 days. During refrigeration, a heavy white amorphous precipitate formed which could be removed by centrifugation. Assay showed that the growth factor remained in the supernatant.

The partially purified factor was chromatographed using n-butanol/acetic acid/water (4/1/5) on Whatman No. 1 filter paper. After development with ninhydrin, five ninhydrin-positive bands of different Rf values appeared (0.05, 0.22, 0.35, 0.57 and 0.87). Also, at least 3 different bands which fluoresced under ultraviolet light were present (Rf values were not determined). In order to determine which of the separated materials stimulated growth, the chromatograms were cut in half. One half was treated with ninhydrin, and the remaining half was tested on a seeded bioautographic plate (see Materials and Methods section). The results showed that not one but two growth factors were present. One was nonreactive to either ninhydrin or ultraviolet light and had an Rf of approximately 0.3; the other appeared to be ninhydrin positive and had an Rf of approximately 0.5. Because the 0.3 Rf material was not well

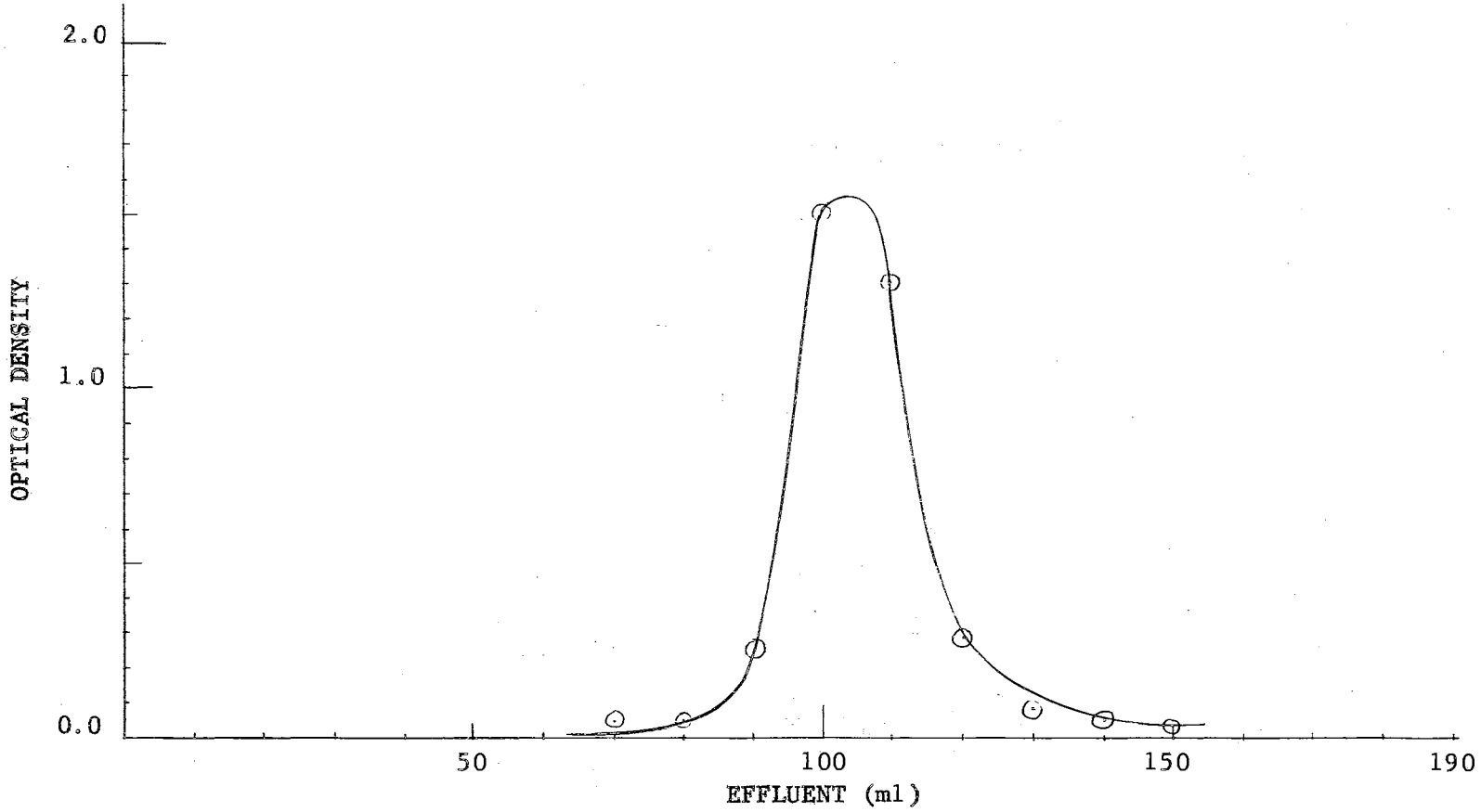

Fig. 1. Response of M. lysodeikticus to effluents collected from the Dowex-2 Anion Exchange Resin Column.

separated from some ninhydrin-positive materials, two other solvent systems were employed to aid in separation (phenol/water (7/3) and t-butanol/methyl ethyl ketone/water (4/4/1.5)). The phenol system ~~completely~~ failed to resolve the five ninhydrin-positive areas and the t-butanol/methyl ethyl ketone system allowed no better separation of the ninhydrin-positive materials than n-butanol. For these reasons, we returned to the butanol/acetic acid/water solvent and began purification of the 0.5 Rf ninhydrin-positive area since the butanol system allowed good separation of it.

When 1-2 mg of relatively pure 0.5 Rf material had been obtained, the material was re-chromatographed in the n-butanol/acetic acid/water system to check for purity. It was observed that a new ninhydrin-positive band always appeared having an Rf of approximately 0.18. This could be eliminated after 5 successive chromatographic runs. We did not determine if the 0.18 Rf material was a hydrolysis product of the 0.5 Rf material or if it was a contaminant that separated only after a certain level of purity was reached. At one time, it was thought that light was responsible for its appearance, however, elution of chromatographic strips in the dark did not stop it from appearing.

When enough of pure 0.5 Rf material was obtained, purity was re-checked using the 2-dimensional chromatographic method of Redfield (18). Using this system, only one ninhydrin-positive area was observed and it did not correspond to any known amino acid. Also, since the material was ninhydrin-positive, it was assumed to be a peptide. This material was hydrolyzed in 6N HCl for 20 hours in the autoclave at 15 lbs pressure and also in 3N NaOH under the same conditions to determine the amino acid

composition of the peptide (see Materials and Methods section). Alkali hydrolysis was used because some amino acids such as tryptophan are destroyed during acid hydrolysis. After elimination of the HCl and NaOH from the hydrolyzates, the acid hydrolyzates were again chromatographed using Redfield's method. Seven amino acids were detected: Alanine, glutamic acid, aspartic acid, methionine, tyrosine, cystine and glycine. Tryptophan was not detected in the alkaline hydrolyzates. Pure unhydrolyzed 0.5 Rf peptide was then dried, weighed, and added to the basal medium to quantitate the amount of peptide needed for growth. Now that the peptide was chromatographically pure, it did not stimulate growth. Either purification had altered its structure and therefore its growth promoting ability or the wrong material had been isolated.

On several occasions it had been noted that the factor detected by the bioautographic method did not exactly coincide with the 0.5 Rf ninhydrin-positive band since only the upper portion of the band stimulated growth. However, because growth on the plates was very diffuse, an accurate measurement was difficult. After the peptide was found not to be the factor, another solvent system was employed to determine if a growth substance could be separated from the 0.5 Rf peptide. The factor from the column was chromatographed in phenol/water (7/3) and again re-checked using the bioautographic technique and ninhydrin. It was observed that growth now occurred in a large area (Rf 0.73-0.77) located behind all of the unseparated and fast flowing ninhydrin-positive materials. After elution of the ninhydrin-negative area, the material was re-chromatographed in the n-butanol system. Bioautographic analysis showed that the material separated into two distinct bands of Rf 0.3 and 0.47. It was

again apparent that two growth substances were present in the column effluent, neither was ninhydrin-positive and the 0.47 Rf material had not been detected in previous studies because it migrated almost equally with the 0.5 Rf peptide in the butanol system. The two factors were then purified using chromatography in phenol/water (7/3) followed by methanol extraction of dried samples and re-chromatography in phenol/water followed by chromatography in the n-butanol/acetic acid/water (4/1/5) system.

Substitution of known compounds in place of the growth factors in the basal medium: While studies relating to purification of the unknown factors progressed, we continued to add known compounds to the basal medium to obtain growth. Although vitamins, purines, pyrimidines, carbohydrates, and fatty acids had been checked repeatedly, it was possible that a combination of these materials was required and that the correct combination was being missed. The full impact of this type of thinking came to our attention during an extended experiment employing purine and pyrimidine compounds. We had added 0.5 ml (1.0 mg per ml solution) of thymine, uracil, cytosine, guanine or adenine to the basal medium. After inoculation, the tubes were incubated at 25 C on the rotary shaker, however, this time the incubation was extended beyond the normal 48 hour limit that had been routinely employed.

It was observed (at 96 hours) that the medium containing adenine allowed some stimulation of growth (78 per cent transmittance). The observation that some growth occurred in the presence of adenine was immediately followed up since growth had not occurred previously unless peptone or the partially purified factor from peptone was present in the medium.

When adenosine (0.2 mg per ml of medium) was substituted for adenine in the basal medium, the growth was even better (58 per cent transmittance in 24 hours). Equal molar concentrations of adenosine (0.01875M), diphosphopyridine nucleotide, adenine, d(-)ribose and adenine plus d(-)ribose were then incorporated separately into the basal medium. Adenosine allowed growth in 24 hours, whereas adenine or diphosphopyridine nucleotide or adenine plus d(-)ribose did not permit growth until after 48 hours, and d(-)ribose alone could not stimulate growth.

Further tests were done using guanine, guanosine, guanylic acid, adenylic acid, flavin mononucleotide, adenosine triphosphate, diphosphopyridine nucleotide, and inosine. Representative results are shown in Figure 2.

These studies proved that the organism possessed a purine requirement that could be satisfied by adenine, however, it is to be emphasized that feeding of the nucleoside (purine plus ribose) or the complete nucleotide (purine plus ribose plus phosphate) allowed for increased growth. The purine requirement is not specific in that adenosine (6-aminopurine plus ribose) or inosine (6-ketopurine plus ribose) can substitute equally well for each other. Although hypoxanthine (6-ketopurine) also stimulated growth, the stimulation was somewhat inferior to that obtained when the nucleoside form (inosine) was used. Some specificity was shown, however, since guanosine (2-amino-6-ketopurine plus ribose) did not stimulate growth. Pyrimidines would not substitute for purines. Orotic acid (a pyrimidine precursor) also failed to allow growth.

Test for growth stimulation by B-vitamins in the presence of adenosine: Because we could now obtain growth in a semi-synthetic medium (minerals,

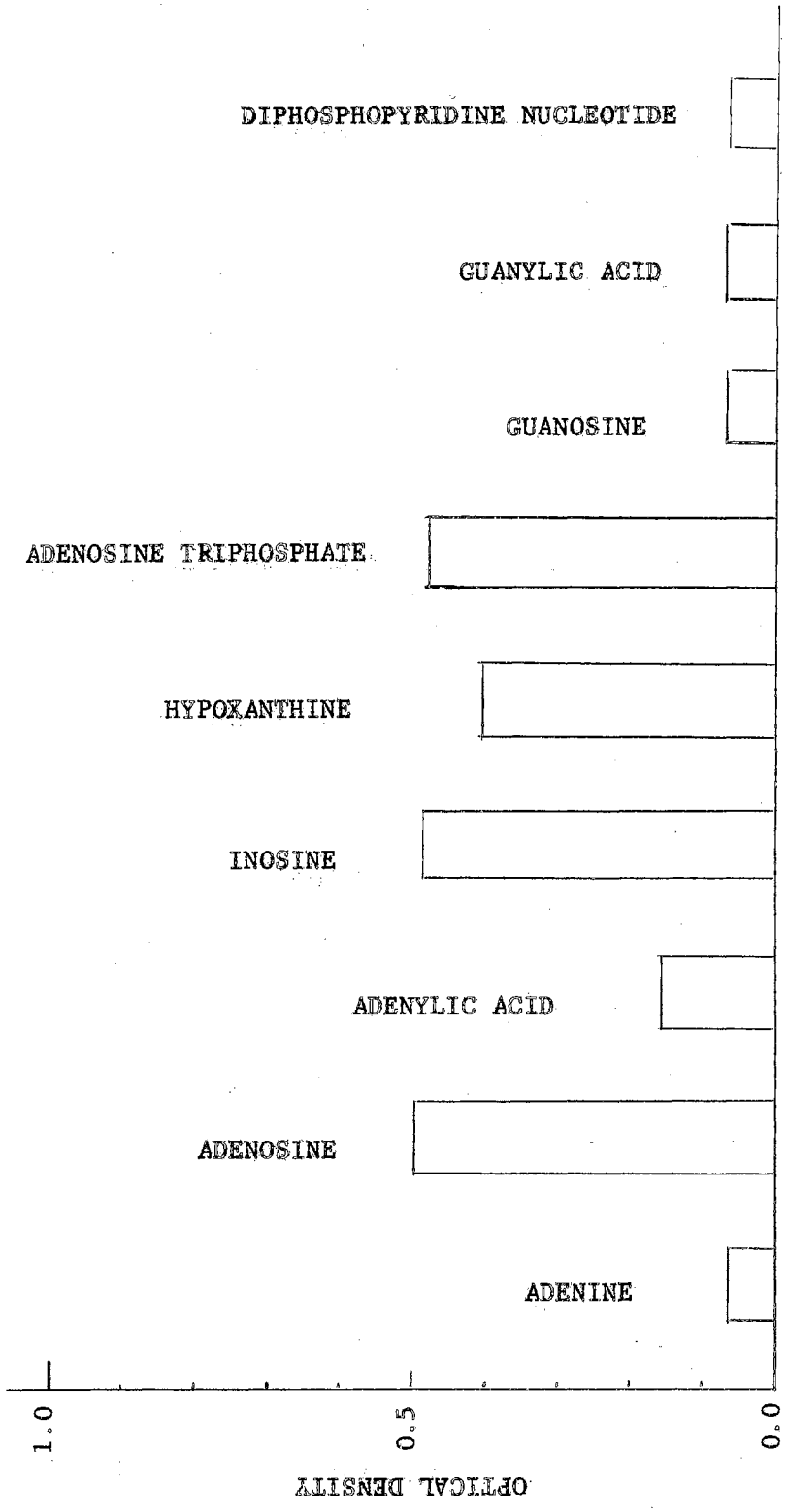

Fig. 2. Influence of purines and purine nucleosides and nucleotides on the growth of M. lysodeikticus. (All tubes contained the basal medium plus biotin.)

buffer, casamino acids plus adenosine) without incorporation of the factor from peptone, the B-vitamins were re-assayed in the presence of adenosine in order to determine if a further stimulation of growth was possible. Although the B-vitamins had been studied previously, they had not been studied in the presence of adenosine. One ml of biotin, B_{12}, choline, folic acid, inositol, p-aminobenzoic acid, calcium pantothenate, pyridoxal hydrochloride, riboflavin and thiamine chloride solutions (1 µgm per ml) were added separately to 4.0 ml of the basal medium containing 0.1 mg of adenosine. The media were autoclaved, inoculated, and incubated as previously described. These studies showed that only biotin allowed a further stimulation of growth during the first 24 hours; inositol also allowed some stimulation of growth, but it was not discernable until 48 hours. (Results incorporated into Fig. 4.)

Quantitation of adenosine for optimal growth of M. lysodeikticus: Differing amounts of adenosine (varying from 0.1 µgm to 500 µgm) were incorporated into separate tubes of basal medium (5 ml) containing biotin (0.05 µgm). The tubes were autoclaved, inoculated and incubated at 25 C on a rotary shaker for 24 hours. Growth response is shown in Fig. 3. Similar studies were also done in the absence of biotin. All studies showed that maximum growth of the organism occurred when adenosine was present at the level of 20 µgm/ml of medium.

Substitutions involving biotin and inositol: Except for the use of casamino acids, it was now possible to obtain excellent growth of the organism in defined media. Because several forms of biotin exist (oxybiotin, d or l biotin sulfoxide, desthiobiotin and biocytin), all, except the l and d sulfoxides of biotin were tested in equal molar

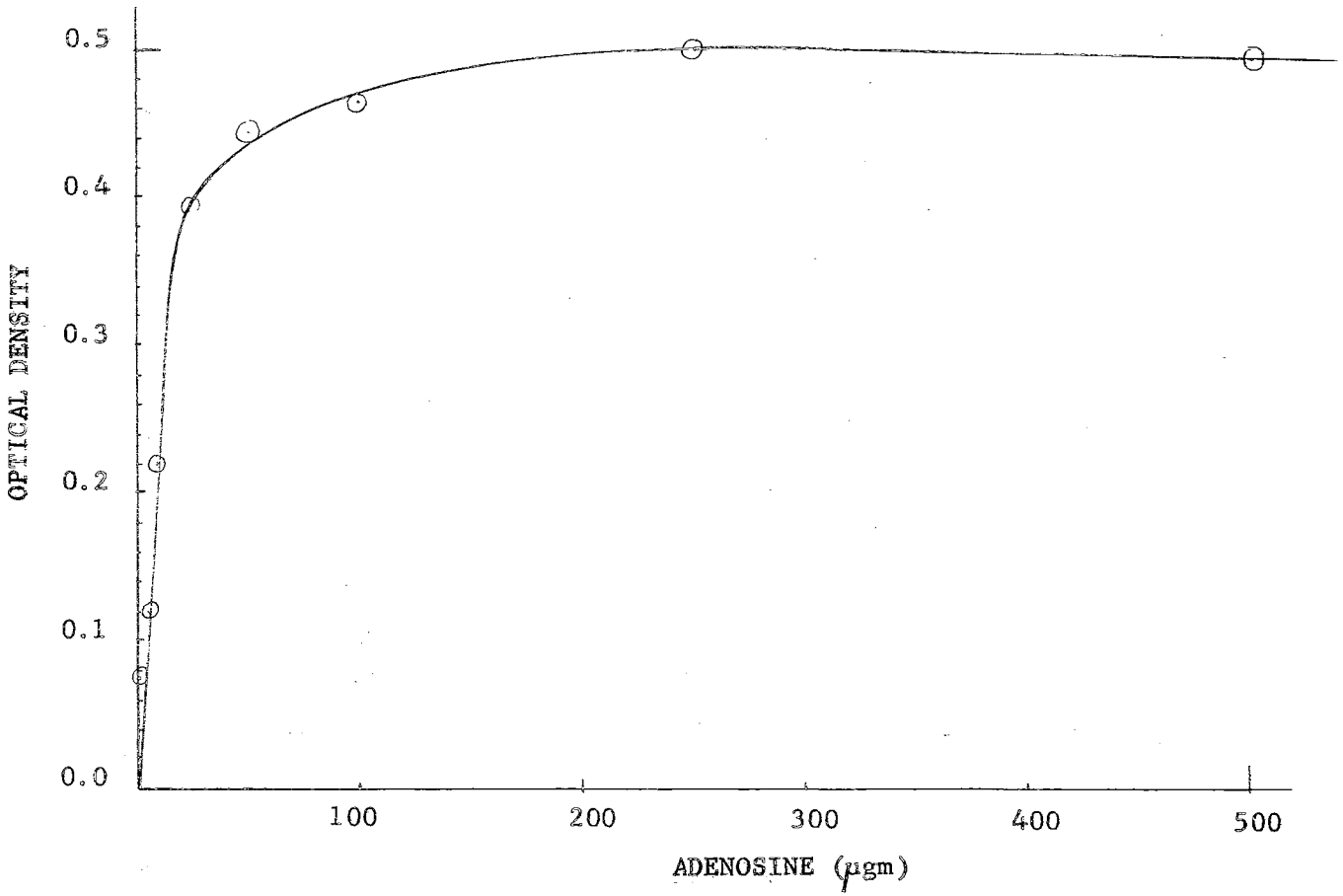

Fig. 3. The growth of M. lysodeikticus in the presence of varying amounts of adenosine.

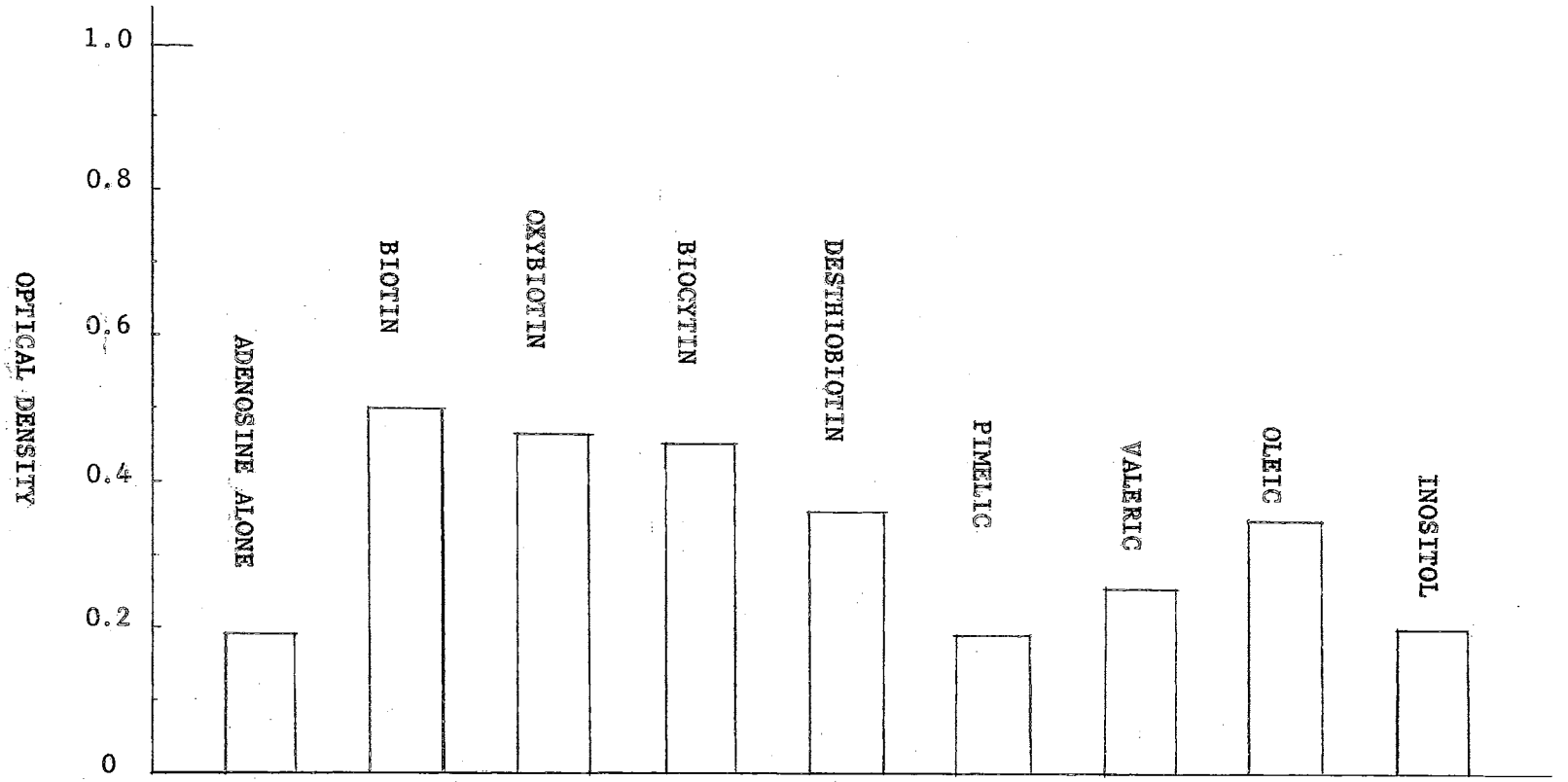

Fig. 4. Effect of various forms of biotin and fatty acids on the growth of M. lysodeikticus at 24 hours. (All tubes contained the basal medium plus adenosine.)

concentrations (0.041 μM/ml of medium) to determine if they could stimulate growth of the organism in a manner similar to biotin. Results are shown in Fig. 4. From these studies, it was apparent that oxybiotin or biocytin could completely replace biotin. Although the l and d sulfoxides of biotin were available, we possessed such small amounts that a quantitative study was not possible in liquid media. These forms of the vitamin were, however, employed in later studies involving chromatography of the unknown factors from peptone for comparison to the biotins.

Because some fatty acids, notably pimelic, oleic and valeric acids can replace biotin in some assay systems, these compounds were also added to the basal medium in the presence of adenosine to determine if they could substitute for biotin. Concentrations added ranged from equal molar amounts to 100 times the concentration of biotin. Data including these compounds is also included in Fig. 4. It can be seen that replacement of biotin by these fatty acids was not possible.

Inositol substitution by phytic acid was also studied since this vitamin can exist in the hydroxy (inositol) or phosphate (phytic acid) form in nature. Phytic acid was not able to substitute for inositol.

Therefore these studies had shown that an absolute purine requirement existed for growth of this organism, since stimulation by pyrimidines (uracil or cytosine) or a pyrimidine precursor (orotic acid) did not occur. Although adenine allowed some growth, the adenine requirement was better satisfied by either hypoxanthine or the nucleosides inosine or adenosine. Except for adenosine triphosphate, adenine-containing nucleotides (adenylic acid or diphosphopyridine nucleotide) did not stimulate growth to any great extent. Although nucleosides or nucleotides allowed growth,

this response appears to be specific for the hypoxanthine or adenine molecule since flavin mononucleotide (flavin-ribitol-phosphate) or guanine or guanylic acid did not permit growth to occur.

Although a stimulation by biotin did occur, the stimulation was only relatively specific since either biotin, oxybiotin or biocytin allowed good stimulation.

Determination of the amino acid requirements for M. lysodeikticus: Because the medium was now completely defined except for the amino acid requirements (a commercial preparation of enzymatically hydrolyzed casein had always been used), we next attempted to replace casamino acids with known amino acids. As mentioned previously, synthetic media for M. lysodeikticus have been reported that use just one amino acid (25) or as many as sixteen amino acids (24).

Studies were first attempted to determine if one or possibly two or more amino acids would allow good growth. One drop each of 20 sterile amino acid solutions (pH 7.0) was deposited on the surface of a seeded agar plate containing adenosine, biotin, minerals and the buffer solution, as a basal medium. The plate was divided into areas by marking the bottom of the plate with a marking pencil. Combinations of several amino acids were also deposited on separate areas. The plate was incubated at 37 C for 12-24 hours and the regions wherein growth occurred were marked. The amino acid combination that allowed a small amount of growth in this preliminary test included arginine, tyrosine, isoleucine, leucine, cysteine, proline and alanine.

Stock solutions of individual amino acids equivalent to 10 times the amount contained in a 4 per cent acid casein hydrolysate (12) were

made (see Appendix). Equal amounts (0.2 ml) of each solution were incorporated into the basal medium in place of casamino acids (enzymatic). The first tube of medium contained all amino acids, while each of the rest of the tubes were depleted three amino acids at a time (Table I). The results of this test and other similar elimination experiments showed that the combination of L arginine, L cysteine, L methionine, L proline, L alanine and L aspartic acid possessed some growth stimulating ability.

Although these 6 amino acids allowed some growth, we attempted to increase the growth response by adding other amino acids, singly and in various combinations. A relatively good growth response occurred when the following amino acid combination was present: DL lysine, L methionine, L arginine, L tyrosine, DL valine, L isoleucine, L leucine, DL phenylalanine, L cysteine, L proline, L alanine and L aspartic acid.

By eliminating these amino acids from the medium one at a time (Table II), and varying the quantity of each amino acid in the combination, we arrived at the final formulation of amino acid requirements given below (amounts per 100 ml of medium):

L Isoleucine	51.2 mg
L Leucine	147.2 mg
L Proline	180.8 mg
L Glutamic acid	358.4 mg
DL Phenylalanine	80.0 mg
L Tyrosine	100.8 mg
L Arginine	65.6 mg
L Cysteine	48.0 mg
L Methionine	27.2 mg

TABLE I

TYPICAL RESPONSE OF M. LYSODEIKTICUS IN ELIMINATION EXPERIMENTS USING KNOWN AMINO ACID MIXTURES

*Tube No.	Content	Growth (24 hrs.) Per cent Transmittance
1	18 amino acids	66
2	18 amino acids less histine, lysine and threonine	56
3	18 amino acids less arginine, cysteine, methionine	90
4	18 amino acids less phenylalanine, tyrosine, tryptophan	71
5	18 amino acids less glutamic acid, glycine, serine	65
6	18 amino acids less proline, alanine, aspartic acid	86
7	18 amino acids less isoleucine, leucine, valine	60

*All tubes contained minerals, adenosine, biotin and the buffer solution.

TABLE II

AMINO ACID ELIMINATION FOR GROWTH OF M. LYSODEIKTICUS

*Tube No.	Content	Growth (24 hrs.) Per cent Transmittance
1	12 amino acids	65
2	No. 1 less DL lysine	70
3	No. 2 less L methionine	72
4	No. 3 less DL valine	65
5	No. 4 less L isoleucine	69
6	No. 4 less L leucine	73
7	No. 6 less L isoleucine	76
8	No. 7 less DL phenylalanine	68
9	No. 8 less L proline	81
10	Control (casamino acids)	37

*All tubes contained adenosine 0.1 mg, biotin 0.05 µgm, phosphate buffer 1.0 ml, totalling 5 ml.

Equal volume of amino acid solutions (see Appendix) of the following: DL Lysine, L Methionine, L Arginine, L Tyrosine, DL Valine, L Isoleucine, L Leucine, DL Phenylalanine, L Cysteine, L Proline, L Alanine and L Aspartic acid.

DL Lysine 131.2 mg

It is to be noted that the final number of amino acids is ten in comparison to the sixteen used by Wessman et al. (24), however, total concentration (weight basis) is approximately 4 times greater.

Although growth using this amino acid combination is not as good in 24 hours as it is in a medium containing casamino acids (Table III), it is to be noted that growth, using our amino acid mixture, is equivalent (after 48 hours) to that obtained using casamino acids.

The problem of growth stimulation during the first 24 hours of incubation had always been difficult to overcome. Preliminary studies had shown that acid hydrolyzed casein (6N HCl for 20 hours at 15 lbs pressure) could support growth in a manner similar to enzymatically hydrolyzed casein. It was known, however, that charcoal treatment of acid hydrolyzed casein removed growth promoting ability of the amino acid solution[1]. This approach was then followed in an attempt to partially resolve the situation.

Acid hydrolyzed casein was passed through a small charcoal column (5 ml per 2 gm charcoal) which had been washed with 10 ml of triple distilled water. The effluent was collected in a 50 ml beaker and evaporated at 60 C in a vacuum oven to the original volume (5 ml), and re-tested for growth promoting ability as previously described. Growth did not occur. Since charcoal treatment can remove aromatic amino acids

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[1]We wish to thank Dr. Charles Crane of the Department of Biochemistry, Oklahoma State University for samples of charcoal treated and non-charcoal treated acid hydrolyzed casein.

TABLE III

COMPARISON OF GROWTH STIMULATION IN MEDIA CONTAINING CASAMINO ACIDS OR THE 10 AMINO ACID MIXTURE

Tube No.	Content	Growth Per cent Transmittance	
		24 hrs.	48 hrs.
1	Casamino acids	16	5
2	10 amino acid mixture	38	6

Tube No. 1: Adenosine 0.1 mg, biotin 0.05 µgm, phosphate buffer 1.0 ml, casamino acid 0.5 ml, distilled water to 5 ml.

Tube No. 2: Adenosine 0.1 mg, biotin 0.05 µgm, 10 amino acid mixture 2.0 ml, distilled water to 5.0 ml.

such as tyrosine, tryptophan and phenylalanine, it appeared that these amino acids might be essential for the growth of the organism. These three amino acids were then added back to the charcoal-treated amino acid mixture, singly and in combinations of two and three and re-tested for growth. The results showed that some stimulation occurred in the presence of tyrosine and phenylalanine (Table IV), however, growth obtained from this re-constituted casein hydrolysate was not equal to that obtained from untreated material. It seemed therefore that the charcoal had taken something out of casamino acids besides the aromatic amino acids. Elution of the charcoal column (after hydrolyzed casein had been passed through) using either 95 per cent ethanol, 1N HCl or ammonium hydroxide (1-10 dilution), failed to remove the growth stimulating material from charcoal.

These studies have not been continued. It can be pointed out, however, that although the material(s) present in non-charcoal treated casamino acids is not essential for growth; it is capable of stimulating growth during the first 24 hours of incubation.

Analysis of requirements for magnesium, inorganic nitrogen and phosphate: Although the buffer solution had always been present in the basal medium, it had never been determined whether any or all of the constituents therein were required. Therefore, media were made up containing casamino acids, biotin and adenosine. The constituents of the buffer solution ($MgSO_4 \cdot 7H_2O$, K_2HPO_4 and NH_4Cl) were made up individually and one of each was left out of the medium while the other two were added. These studies showed that each of these compounds was needed since growth did not occur when they were absent.

TABLE IV

GROWTH OF M. LYSODEIKTICUS IN CHARCOAL TREATED CASEIN HYDROLYZATE WITH AND WITHOUT ADDED AROMATIC AMINO ACIDS

*Tube No.	Content	Growth (24 hrs.) Per cent Transmittance
1	Charcoal treated acid hydrolyzed casein	93
2	Same as No. 1 plus tyrosine (12.6 mg)	77
3	Same as No. 2 plus phenylalanine (20.0 mg)	71
4	Same as No. 3 plus tryptophan (2.4 mg)	79

*All materials tested were added to the basal medium consisting of adenosine 0.1 mg, biotin 0.05 µgm and 1.0 ml of the buffer solution.

Study of optimum pH conditions: Although we had always used a pH of about 7.4-7.6 in early studies, a determination of optimum pH had not been made. Therefore, basal media were adjusted to various pH readings (7.0, 7.2, 7.4, 7.6, 7.8 and 8.0) prior to autoclaving of the media. Response of the organisms is shown in Table V. From these data, it is apparent that the pH optimum for this organism is 7.6-7.8.

Study of carbon-energy requirements: Although all synthetic medium formulations for this organism employ glucose, a statement has never appeared regarding the essentiality of glucose for the growth of M. lysodeikticus. During our early studies, it had been noted that cell growth was not affected in the presence or absence of glucose. Because no stimulation by glucose was evident, it had been omitted from all medium formulations and casamino acids served not only as a source of organic nitrogen, but also as a carbon-energy source.

Because it is well established that an organism can, in many instances, utilize fatty acids resulting from the breakdown of glucose as primary sources for carbon and energy, it was decided to test several fatty acids to determine if they could function as carbon-energy sources and perhaps stimulate growth of this organism. The following compounds were added to the basal medium containing casamino acids, biotin, adenosine and the buffer solution (each was added to a concentration of 100 µgm/ml of medium).

Formic acid

Potassium fumarate

Malic acid

Sodium butyrate

TABLE V

INFLUENCE OF pH ON THE GROWTH OF M. LYSODEIKTICUS IN A SEMI-SYNTHETIC MEDIUM

Tube No.	pH	Growth (24 hrs.) Per cent Transmittance
1	7.0	63
2	7.2	50
3	7.4	47
4	7.6	41
5	7.8	41
6	8.0	44

All tubes contained casamino acids, biotin, adenosine and the buffer solution.

Succinic acid

Sodium acetate

Sodium pyruvate

Data from this experiment are shown in Table VI. From these data, it is apparent that none of the compounds were capable of increasing the growth response of this organism. These compounds were also added to the amino acid synthetic medium. No additional stimulation was observed.

Therefore, the summation of these studies has led to the following synthetic formulation for the growth of M. lysodeikticus.

Adenosine	2.0 mg
Biotin	1.0 µgm
L Isoleucine	51.2 mg
L Leucine	147.2 mg
L Proline	180.8 mg
L Glutamic acid	358.4 mg
DL Phenylalanine	80.0 mg
L Tyrosine	100.8 mg
L Arginine	65.6 mg
L Cysteine	48.0 mg
L Methionine	27.2 mg
DL Lysine	131.2 mg
NH_4Cl	100.0 mg
K_2HPO_4	200.0 mg
$MgSO_4 \cdot 7H_2O$	2.0 mg

Distilled water to 100 ml and pH adjust to 7.6-7.8.

TABLE VI

*EFFECT OF FATTY ACIDS ON GROWTH OF M. LYSODEIKTICUS IN A SEMI-SYNTHETIC MEDIUM

Tube No.	Content	Growth (24 hrs.) Per cent Transmittance
1	Control	17
2	Formic acid	22
3	Potassium fumarate	20
4	Malic	31
5	Sodium butyrate	24
6	Sodium pyruvate	20
7	Succinic acid	23
8	Sodium acetate	19

All tubes contained adenosine 0.1 mg, biotin 0.05 µgm, casamino acids and the buffer solution.

*All of the compounds were sterilized by filtration through sintered glass and adjusted to pH 7.4 ± 0.2.

Adenosine can be replaced by:

Inosine

Hypoxanthine

Biotin can be replaced by:

Oxybiotin

Biocytin

Qualitative analysis of the growth factors: Since it was possible to replace the factors present in peptone with a purine (hypoxanthine) or the purine nucleosides (adenosine or inosine), and either biotin, oxybiotin or biocytin, it appeared appropriate to determine if the peptone factors were any of these known compounds. Although the factors have not been identified, the following data have been compiled, in an attempt to relate them to compounds known to function in the growth medium. It is to be emphasized that study of the factors has been made difficult because of the lack of purified material. Most of the studies that have been done have employed chromatographically-pure materials (see Materials and Methods section).

Pinchot (17) published a technique for hydrolysis of nucleosides and nucleotides as outlined in the Materials and Methods section. Using these techniques and comparing the factors to adenosine, it was concluded that our factor was not a nucleoside nor a nucleotide since destruction of growth promoting ability did not occur after either acid or alkali hydrolysis.

Because biotin, biocytin, l and d sulfoxides of biotin, oxybiotin, desthiobiotin and inositol could all stimulate growth to some extent, each of these compounds was chromatographed using the n-butanol/acetic acid/water

system. This technique was employed since Wright (27) reported the Rf values of all the different forms of biotin in bioautographic systems using Neurospora crassa as the test organism. We employed M. lysodeikticus in our system leaving adenosine and biotin out of the basal medium since the factors from peptone apparently could replace both of them. Growth did not occur in any areas on the plates when adenosine was left out of the medium. Addition of adenosine to the basal medium allowed growth to be too heavy over the entire plate and stimulation by the biotins was not discernable. For these reasons and also because the known Rf values of the different biotins do not correspond to the Rf values of our 2 factors, it was concluded that neither of the factors is a free biotin. Whether or not, biotin exists in a bound form in the molecule has not been determined.

Although the results using the hydrolysis techniques are meaningful, a more elegant rule out of purine compounds was accomplished employing different chromatographic systems in conjunction with the bioautographic technique. Data are presented in Table VII. Although some similarities were noted in some systems with adenosine and hypoxanthine, it can be concluded that our factors and these compounds are different.

As an additional study, the purified 0.3 and 0.47 Rf materials were analyzed in a Beckman D.U. spectrophotometer at wave lengths from 200-440 mμ (Fig. 5). Because no pronounced absorption peaks were present the data do not provide any meaningful clues with regard to the identity of our compounds.

Because both the 0.3 and 0.47 Rf materials became adsorbed to an anion exchange resin, a negatively charged polar group is present at near

TABLE VII

Rf VALUES OF KNOWN PURINE COMPOUNDS AND THE GROWTH FACTORS FROM PEPTONE IN DIFFERENT SOLVENT SYSTEMS

Material	A	B	C	D
0.3 factor	0.3	0.73-0.77	0.62	0.29
0.47 factor	0.47	0.73-0.77	0.62	0.39
Adenosine	0.44	- - -	0.61	0.13
Inosine	0.26	- - -	0.71	- - -
Hypoxanthine	0.47	- - -	0.63	0.44
Adenosine triphosphate	0.03	- - -	- - -	- - -

A: n-Butanol/acetic acid/water (4/1/5).

B: Phenol/water (7/3).

C: Ethanol/1.0M ammonium acetate pH 7.5 (7/3).

D: 0.1M phosphate pH 6.8/ammonium sulphate/n-propanol (100/60/2).

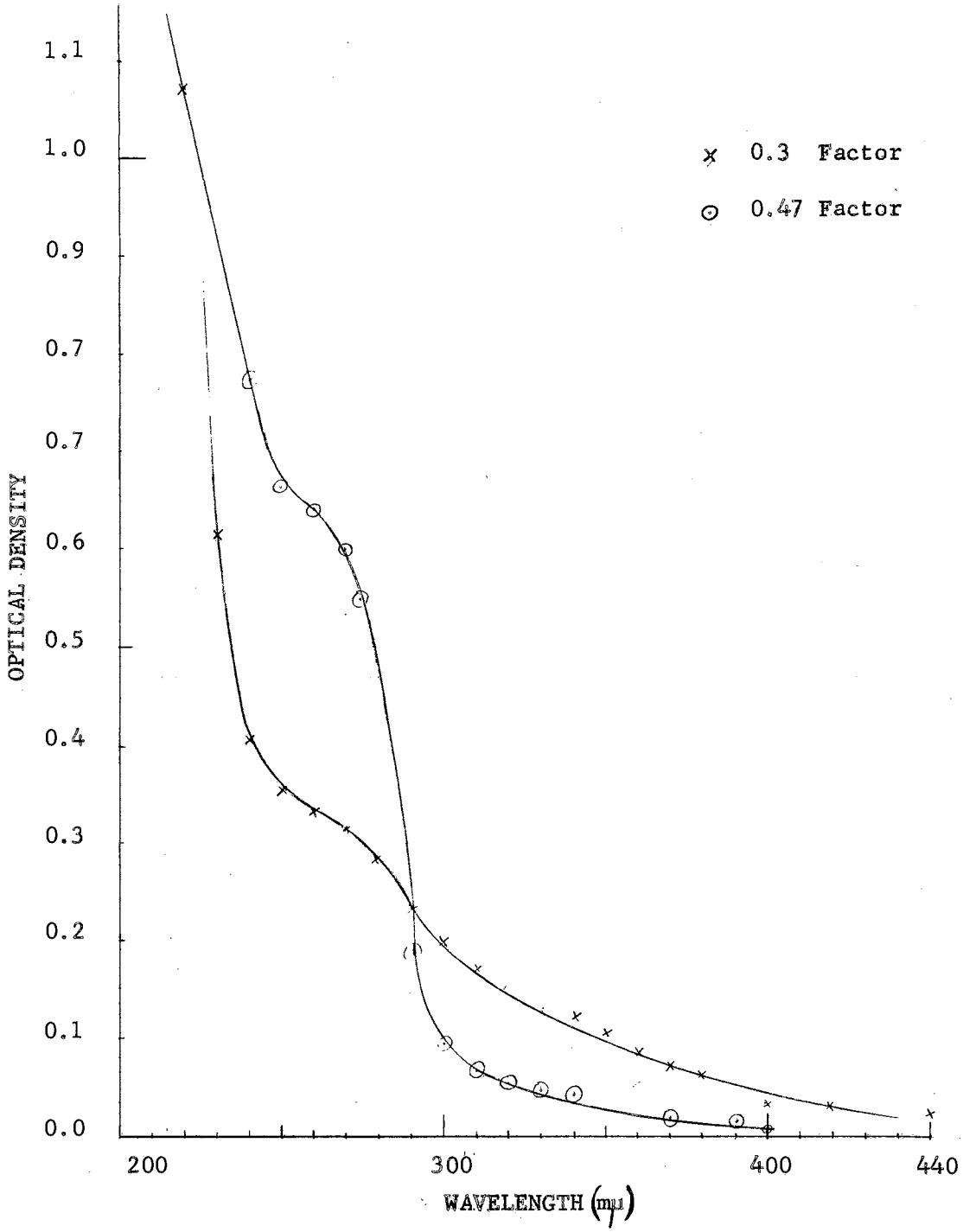

Fig. 5. Ultraviolet absorption spectra of 0.3 and 0.47 Rf materials measured in a Beckman D.U. Spectrophotometer.

neutral pH ranges. Apparently, a positively charged group also exists since the compounds were also adsorbed on Dowex-50 resin in the H^+ form. (These studies were done by adding the factors to a Dowex-50 column and checking the effluent for their presence. Because they were never actually eluted from the column it cannot be determined whether they were adsorbed to the column resin or destroyed.)

Throughout these studies, it had been determined that the 0.3 and 0.47 Rf materials were negative to ninhydrin, negative in determinations for sugars (anthrone and aniline phthalate), and did not show "quenching" (darkened areas) under ultraviolet light. Also, they were negative for sugar phosphate, negative to $FeCl_2$ reagents for detection of the aromatic structure with an OH group in the meta position and they were negative for the presence of oxidized or reduced sulfur. The techniques and results to substantiate these conclusions are given below.

(a) Test for cysteine or reduced sulphur. Chromatographically-pure Rf 0.3 or 0.47 material was concentrated on chromatography paper under a stream of continual flowing heat after which the paper was dipped into sodium nitroprusside reagent (see Appendix) using cysteine as a control. Formation of a crimson color did not occur except in the cysteine control (21).

(b) Test for cystine. Papers prepared as above were dipped into sodium nitroprusside reagent and dried slightly. While the paper was still damp, it was dipped into sodium cyanide reagent and observed for the formation of a red color. These tests were negative (21).

(c) Test for sugar phosphates. Chromatographic papers containing concentrated areas of the factors were sprayed at the rate of 1 ml per

100 cm^2 with chloric acid reagent (see Appendix) autoclaved for 2 minutes at 8-10 lbs pressure, after which they were exposed to ammonia vapors. The presence of a blue color indicates the presence of sugar phosphate (11). (The blue color on the paper caused by the reagent disappears on exposure to NH_3, however, if sugar phosphate is present the blue color remains unchanged.) These tests were also negative.

(d) Test for phosphates. Chromatographic papers containing concentrated areas of the factors were sprayed with ammonium molybdate reagent (see Appendix) and heated at 80 C for 5-10 minutes. When dried, they were sprayed with 0.05 per cent benzidine in 10 per cent acetic acid and then exposed to ammonia vapors. Meta-and ortho-phosphates give a blue color while pyrophosphate becomes purplish red (2). These tests were also negative.

(e) Anthrone method for analysis of sugars (pentoses and hexoses). Twelve ml of anthrone reagent (see Appendix) was pipetted into a test tube and cooled in cracked ice. Two ml of the concentrated factor was added with no mixing. The tube was cooled in an ice bath, mixed rapidly, and then heated to 80 C. The appearance of green color indicates the presence of sugars (1).

(f) Aniline hydrogen phthalate test for reducing sugars. Chromatograms or paper strips containing an area of concentrated factors were sprayed with the reagent (see Appendix) and heated in an oven at 105 C for 5 minutes (16). Reducing substances other than sugars will not give any positive reaction in this test.

(g) Growth promoting ability of either of the 0.3 or 0.47 Rf material was not destroyed after digestion with relatively purified

lipase (Nutritional Biochemicals Corp.) for 2 hours at 37 C.

Because the growth factors did not react with any of the tests employed for the detection of various compounds they were hydrolyzed with 2N sulphuric acid for 2 hours at 100 C and then tested again for the presence of sugar, sugar phosphate, phosphates, cysteine and cystine as previously described.

After hydrolysis, all tests were again negative except the test for sugar phosphate which was positive for both the 0.3 and 0.47 Rf factors. This result could explain the negative charge of the compounds (ionized phosphate groups). Obviously, however, there must be another part(s) in the compounds since the sugar phosphate must be unmasked by hydrolysis. Tests, thus far, have given no indication of the nature of the other part(s) of the molecules.

Aside from the lack of positive information concerning the chemical nature of the unknown compounds, it is interesting to note that both give the same chemical reactions and both allow growth of the organism. The only difference noted between them throughout these studies has been their migration in chromatographic systems.

SUMMARY

1. M. lysodeikticus requires for growth either of 2 factors present in peptone.

2. The factors were fractionated and partially purified by selective elution from a Dowex-2 anion exchange resin column, and further purified 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; in n-butanol/acetic acid/water, one factor has an Rf of 0.3, the other an Rf of 0.47.

3. Preliminary qualitative tests showed that the factors contained no sugars, phosphate or oxidized or reduced sulphur. After partial hydrolysis (2N H_2SO_4 at 100 C for 2 hours) the factors gave a positive test only for sugar phosphate. No pronounced absorption peaks could be detected using the Beckman D. U. spectrophotometer in the range of 200-440 mμ.

4. Substitution of the factors could be accomplished using either adenosine, inosine or hypoxanthine together with biotin, oxybiotin, or biocytin. The unknown factors are not any of these compounds.

5. The organism possesses a specific requirement for purines (adenine or hypoxanthine). Although the free base will allow growth, increased growth occurs when the nucleoside form of these purines is used. Biotin and biotin-like compounds (biocytin and oxybiotin) are not required for growth; They are however, greatly stimulatory.

6. A chemically-defined synthetic medium was successfully devised for the

growth of M. lysodeikticus. The medium contained adenosine, biotin, 10 amino acids (L glutamic acid, DL lysine, L methionine, L arginine, L tyrosine, L isoleucine, L leucine, DL phenylalanine, L cysteine, L proline), magnesium sulphate, potassium phosphate (di-basic) and ammonium chloride.

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APPENDIX

Basal Medium:

Solution I: Contents per 1 liter of distilled water and adjust to pH 7.6-7.8.

NH_4Cl	5.0 gm
K_2HPO_4	10.0 gm
$MgSO_4 \cdot 7H_2O$	0.1 gm

Solution II: Contents per 1 liter of triple distilled water. Adjust pH to 7.6-7.8 and dilute 1-50 before use.

H_3BO_3	12.5 mg
$CaCO_3$	25.0 mg
$CuSO_4 \cdot 5H_2O$	2.5 mg
$Fe(NH_4)_2SO_4 \cdot 6H_2O$	125.0 mg
KI	2.5 mg
$MnSO_4 \cdot H_2O$	5.0 mg
MoO_3	2.5 mg
$ZnSO_4 \cdot 7H_2O$	12.5 mg

"Vit. Free" Casein Hydrolyzate (Enzymatic). Nutritional Biochemicals Corporation. Adjust pH to 7.6.

Basal medium will total 5 ml as follows:

Solution I	1.0 ml
Solution II (1-50)	1.0 ml

Casein hydrolyzate	0.5 ml
Triple distilled water	2.5 ml

Proposed synthetic media for M. lysodeikticus:

Wessman, Allen and Werkman Medium (24):

DL Alanine	100.0 mg
L Arginine HCl	100.0 mg
L Cysteine HCl	25.0 mg
Glycine	50.0 mg
L Glutamic acid	100.0 mg
L Histidine HCl	50.0 mg
DL Isoleucine	100.0 mg
L Leucine	100.0 mg
L Lysine HCl	100.0 mg
DL Methionine	50.0 mg
DL Phenylalanine	50.0 mg
L Proline	100.0 mg
DL Serine	25.0 mg
DL Tryptophan	25.0 mg
L Tyrosine	100.0 mg
DL Valine	100.0 mg
Adenine sulphate	5.0 mg
Guanine HCl	5.0 mg
Uracil	5.0 mg
Glucose	1,500.0 mg
NH_4Cl	500.0 mg

$MgSO_4 \cdot 7H_2O$	100.0 mg
$FeSO_4 \cdot 7H_2O$	5.0 mg
$MnSO_4 \cdot 4H_2O$	10.0 mg
NaCl	5.0 mg
K_2HPO_4	300.0 mg
KH_2PO_4	300.0 mg
p-Aminobenzoic acid	50.0 µgm
Calcium Pantothenate	250.0 µgm
Nicotinic acid	250.0 µgm
Pyridoxine HCl	500.0 µgm
Riboflavin	250.0 µgm
Thiamin HCl	250.0 µgm
Folic acid	1.0 µgm
Biotin	4.0 µgm

distilled water to 400 ml and adjust pH to 7.0.

Heden's Medium: (Personal communication to Dr. E. A. Grula)

Solution I, II and III are prepared separately, adjusted pH to 7.5, autoclaved and then mixed together aseptically after being cooled.

Solution I:

Casamino acid	10.0 gm
Glucose	10.0 gm
Triple distilled water	900.0 ml

Solution II:

NH_4Cl	1,000.0 mg

K_2HPO_4	700.0 mg
KH_2PO_4	300.0 mg
Na_2SO_4	100.0 mg
Triple distilled water	100.0 ml

Solution III:

$MgSO_4$	20.0 mg
Triple distilled water	20.0 ml

Western Reserve Medium: (Personal communication to Dr. E. A. Grula)

Glucose	0.5 gm
Sodium Glutamate	1.2 gm
Hypoxanthine	1.0 µgm
Biotin	1.0 µgm
Salts of Snell A & B	1.0 ml
Triple distilled water	100.0 ml

Wolin & Naylor's Medium: (25, 26)

Glucose	0.7 %
Monosodium Glutamate	1.0 %
K_2HPO_4	0.2 %
NH_4Cl	0.1 %
$MgSO_4 \cdot 7H_2O$	0.01%
$FeSO_4 \cdot 7H_2O$	0.0004%
$MnCl_2 \cdot 4H_2O$	0.0002%
Biotin	10.0 µgm/liter

Amino acid stock solutions (contents per 10 ml)

L Arginine	164.0 mg
L Histidine	124.0 mg
DL Lysine	328.0 mg
L Cysteine	120.0 mg
L Methionine	68.0 mg
DL Threonine	40.0 mg
L Serine	88.0 mg
L Leucine	368.0 mg
L Isoleucine	128.0 mg
L Valine	288.0 mg
L Glutamic acid	896.0 mg
L Aspartic acid	140.0 mg
Glycine	108.0 mg
L Alanine	60.0 mg
L Proline	452.0 mg
DL Phenylalanine	200.0 mg
L Tyrosine	252.0 mg
L Tryptophan	24.0 mg

Adjust pH to 7.6-7.8 after mixing.

Solvent systems used for development of chromatograms:

1. n-Butanol/acetic acid/water (4/1/5)
2. Phenol/water (7/3)
3. Redfield system (18):

(a) Methanol/water/pyridine (80/20/4)

(b) t-Butanol/methyl ethyl ketone/water/diethyl amine (40/40/20/4).

4. Pabst solvent systems for 5'-ribonucleotides (15):

(a) System No. II: Ethanol/1.0 M ammonium acetate pH 7.5 (7/3).

Dissolve 77 grams of ammonium acetate in approximately 750 ml of water, adjust to pH 7.5 with ammonium hydroxide and dilute to 1 liter. Mix 300 ml of the latter solution with 700 ml of 95 per cent ethanol.

(b) System No. III: 0.1 M Phosphate pH 6.8/ammonium sulphate/ n-propanol (100/60/2).

Dissolve 600 grams of ammonium sulphate in 1 liter of 0.1 M sodium phosphate buffer, pH 6.8, add 20 ml of n-propanol and mix.

Reagents used for the detection of sugars:

Aniline reagent (16):

Aniline (re-distilled)	0.93 gm
Phthalic acid	1.66 gm
n-Butanol (water saturated)	100.0 ml

After spraying with this reagent, the chromatograms were heated in an oven at 105 C for five minutes. Aldopentoses will give a bright red color and aldo-hexoses, desoxy sugars and uronic acid will give various shades of green and brown.

Anthrone reagent (1):

Sulphuric acid (concentrated)	1,000 ml
Anthrone	1.0 gm

One liter of concentrated sulphuric acid is mixed with 290 gms of ice. Dissolve 1 gm anthrone in the mixture when cooled. Store in dark at 4 C. Stable for 2 months.

Twelve ml of the anthrone reagent was poured into a test tube and cooled in ice. A 2.0 ml test sample was then added to the reagent. When cooled, mixed rapidly, and heated to 80 C, a green color indicates the presence of pentoses and hexoses.

Reagents for the detection of reduced and oxidized sulphur (21):

Solution No. 1:

Sodium nitroprusside	1.5 gm
Sulphuric acid 2N	5.0 ml
Methanol (absolute)	95.0 ml
Ammonium hydroxide (28%)	10.0 ml

Sodium nitroprusside was dissolved in 5 ml of 2N sulphuric acid. Methanol (95 ml) and 10 ml ammonium hydroxide were then added. The mixture was filtered and stored in the refrigerator.

Solution No. 2:

Sodium cyanide	2.0 gm
Water	5.0 ml
Methanol (absolute)	95.0 ml

Test for cysteine: Air-dried chromatograms were dipped into solution No. 1. The appearance of bright red color indicates the presence of cysteine.

Test for cystine: The chromatograms were first dipped into solution No. 1 and then dried slightly. While still damp, they were dipped into solution No. 2. Color for cystine is red.

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

Reagent for detection of inorganic phosphate (2):

Solution I:

Ammonium molybdate	0.4 gm
Nitric acid (8%)	100.0 ml

Solution II:

Benzidine	0.05 gm
Acetic acid (10%)	100.0 ml

The chromatograms were first sprayed with solution I and heated in an oven at 80 C for 5-10 minutes. After drying, they were sprayed with solution II and exposed to ammonia vapors while still damp. Meta- and orthophosphate will result in blue spots, while the color for pyrophosphate is purplish red.

Distillation of n-Butanol: About 50 gm of anhydrous potassium carbonate was added to 1 liter of n-butanol contained in a glass distilling apparatus and distilled at 117 C using a heating jacket (electrical) to avoid explosion. Anhydrous potassium carbonate will remove water as well as other impurities such as aldehydes and carbonyl compounds from butanol.

Detection of compounds under ultraviolet light (15): The chromatograms were placed under a short wave ultraviolet light in the dark. Substances such as 5'-ribonucleotides that absorb U. V. light will appear as dark spots, whereas other substances which reflect U. V. light will appear as fluorescent materials.

Instruments used:

Short Wave Ultra Violet Lamp, Model SL 2537, Mineral Light Ultra Violet Products Inc., South Pasadena, Calif.

Beckman DU Spectrophotometer, Model 2400, Beckman Instruments Inc., Fullerton, Calif.

Beckman pH Meter, Model 96, Beckman Instruments, Inc., Fullerton, Calif.

Spectrophotometer, Type 33-29-40, Bausch & Lomb Optical Co., Rochester, New York.

Miscellaneous:

Whatman No. 1 Filter Paper, F3784 (1½"). Purchased from Schaar & Co., 7300 N. Montrose Ave., Chicago 34, Ill.

Dowex-2 Anion Exchange Resin, 20 x 50 mesh. The Dow Chemical Company, Midland, Michigan.

Dowex-50 Cation Exchange Resin, 20 x 50 mesh. The Dow Chemical Company, Midland, Michigan.

Charcoal G-60, Darco Dept., Atlas Powder Co., 60 E. 42nd Street, New York 17, N. Y.

Pyrex Rectangular Plate (14" x 9" x 2") .

"Vit. Free" casein hydrolyzate (enzymatic), Control No. 4498, Nutritional Biochemicals Corporation, Cleveland, Ohio.

VITA

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