Unknown Growth Factors Required in the Nutrition of Micrococcus lysodeikticus[1]

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The nutritional requirements for *Micrococcus lysodeikticus* have been studied for 12 years. At least 6 different synthetic media have been proposed. A few authors, notably Feiner *et al.* (1946), reported they were unable to cultivate any of 3 immunologically distinct strains tested unless crude folvite liquors were used. Some media have been rather "simple" requiring only minerals, glucose, an amino acid and biotin (Wolin and Naylor, 1957); others become more complex employing up to 16 amino acids, 8 B vitamins, purines, pyrimidines, minerals and glucose (Wessman, *et al.* (1954). Regardless of the complexity of the media, all investigators who obtained growth in synthetic media used biotin and glucose plus an amino acid or acids and mineral salts. This report will consider the nutritional requirements for the Purdue University strain of *M. lysodeikticus*. This strain comes from the original isolation of the organism made by Fleming in 1922, and it will not grow on any of the synthetic media devised for other strains of *M. lysodeikticus*.

When the initial work with the organism was in progress, known substances were incorporated into a glucose-mineral-salts medium to see if they would support growth. No growth was found to occur with any of the B vitamins, which were tested individually, in groups and all together. Thirteen carbon-energy sources were tried. These included mono-, di-, tri- and polysaccharides, glucosamine and a few sugar alcohols such as mannitol and dulcitol; in no instance was growth obtained. Casamino acids (acid or enzymatically hydrolyzed) did not support growth either alone, in combination with B vitamins, or in combination with glucose and vitamins. Addition of purines and pyrimidines to media containing casamino acids, glucose and mineral salts also yielded negative results. Good growth occurred in mineral salts, casamino acids, glucose media only when peptone was added. The peptone "factor" could not be replaced by any combination of vitamins nor could it be replaced by any purine, pyrimidine or carbohydrate. It, therefore, appeared that the factor in peptone was either new or a compound not usually employed to obtain bacterial growth. The peptone factor is also present in varying amounts in tryptose, heart infusion, liver extract, tryptone and casitone. It is not found in milk whey, neopeptone, litmus milk, peptonized milk or jack bean meal.

To study the problem, either of 2 approaches could have been followed: (1) We could continue substituting known organic acids, alcohols,

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peptides etc. in a synthetic basal medium, or (2) we could attempt some purification and identification of the peptone factor. Because the number of compounds that could be tested were too numerous and because there was a possibility that the factor might be an unknown, the latter method was chosen.

The factor was fractionated from peptone using a Dowexl-2 anion exchange resin (OH-form) with 0.3N HCl as the eluting solvent. Approximately 200 ml. of effluent were collected in 10 hours at a flow rate of about 1 ml. in 3 minutes. The threshold volume of the fraction occurred between the 90th to 110th ml. of the effluent.

On biological assay in liquid media, the material appeared to be relatively pure. Purity was determined by chromatographing the purest fractions obtained on Whatman #1 paper using *n*-butanol - acetic acid - water (4-1-5) as the developing solvent for 24 hours at 25 C. Analysis revealed that 5 well separated ninhydrin-positive bands and approximately 5 U. V. fluorescing bands were present. No carbohydrates were detected.

The chromatographic strips were then tested, employing autobiographic assay in a basal medium containing only mineral salts and casamino acids. Such autobiographic data revealed that not one but two growth factors were present. One was non-reactive to either ninhydrin or U. V. 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 separated from ninhydrin-positive materials, two other solvent systems were employed to aid in separation (phenol-water; 7-3 and *tertiary*-butanol - methyl ethyl ketone - water; 4-4-1.5). The *tert*-butanol-methyl ethyl ketone system allowed no better separation than butanol-acetic acid-water; phenol completely failed to resolve any of the five ninhydrin-positive areas. For these reasons, we returned to the butanol-acetic acid-water system and began purification of the 0.5 Rf ninhydrin-positive area.

Prior to chromatography, preliminary purification of the effluent from the column was accomplished by drying the material 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 that could easily be spun out. Assay showed that the growth factors remained in the supernatant. We then chromatographed the supernatant in the butanol-acetic acid system and followed migration of the 0.5 Rf ninhydrin positive factor using ninhydrin dipping on a thin cut out edge from each paper strip. The remaining portion of the paper containing the factor was then cut out, several pieces stapeled together, and the paper strips eluted with flowing water for 6 hours at room temperature in a dark and closed system. During chromatographic purification, a new ninhydrin-positive band always appeared, having an Rf of approximately 0.18. This material could be eliminated after 5 successive chromatographic runs.

When enough of the 0.5 Rf material had been obtained, purity was rechecked using the 2 dimensional chromatographic method of Redfield (1953). In this system, only 1 ninhydrin-positive spot was observed and it did not correspond to any known amino acid. We, therefore, hydrolyzed this material in 6N HCl for 20 hours in the autocalve at 15 pounds pressure and also in 3N NaOH for 20 hours in the autoclave at 15 pounds pressure to determine the amino acid composition of the peptide. After getting rid of the HCl or NaOH, the hydrolyzates were chromatographed using the Redfield method. Seven amino acids were present in the peptide hydrolyzate: alanine, glutamic acid, aspartic acid, methionine, tyrosine, cystine, and glycine. Peptide was then weighed and added to the basal medium to quantitate the amount needed for growth. The chromatog-

raphically-pure peptide did not stimulate growth. Either purification had altered its structure, and therefore its growth-promoting ability, or the wrong material had been isolated.

Because it was known that the phenol-water system did not separate the ninhydrin-positive materials in the column effluent, it had been discarded as a technique. Also, we were not sure that enough of the excess phenol could be removed to allow autobiographic studies. We now returned to the system and rechecked the effluent from the column, after chromatography in phenol, using the autobiographic technique. It was observed that growth occurred in a large area that was located behind all the ninhydrin-positive materials. After elution of the ninhydrin-negative area, the material was re-chromatographed in the butanol system. Autobiographic analysis showed that the material again separated into two distinct bands of Rf 0.3 and 0.47, but neither was ninhydrin-positive. The 0.47 Rf material was missed in previous studies because the factor had been followed, using ninhydrin. It might be thought that autobiographic techniques would have avoided missing the 0.47 Rf material, however, it should be pointed out that growth in this system is diffuse and, because of the closeness of the true factor and the peptide, their individual Rf values could not be distinguished by autobiography of papers chromatographed in the butanol system. The 2 factors have now been purified using chromatography in phenol followed by methanol extraction and re-chromatography in phenol followed by chromatography in the butanol-acetic acid-water system. Preliminary studies have shown that neither factor is a sugar nor a fatty acid. There is still doubt regarding their classification as nucleotides. Phosphorus and sulfur determinations on either hydrolyzed or unhydrolyzed samples have not been done.

During purification of the effluent from the column, we had continued to study the purine-pyrimidine requirements of this organism. It had been noted that adenine was required for growth. Although adenine allowed some growth in 72 hours, adenosine was superior and allowed growth in 24 hours. The adenosine could be replaced with either adenylic acid, hypoxanthine or inosine, but not with orotic acid or pyrimidines. Because some growth could be obtained in a semi-synthetic medium at this point (it had never been possible unless the peptone factors were present), we re-assayed the need for B vitamins in the presence of adenosine. Two of the B vitamins (biotin and inositol) stimulated growth. Therefore it was possible to substitute for either one or both peptone factors using adenosine and biotin. Biotin could be replaced by desthiobiotin, oxybiotin and biocytin, but not by oleic or pimelic acid. The problem to be answered was whether or not either of the petone factors was adenosine or any of its replacements or biotin or its replacements. Chromatography of adenosine, inositol, biotin, biocytin, inosine, hypoxanthine or desthiobiotin in conjunction with the autobiographic technique has shown that our factors are not any of these compounds. We have further attempted to rule-out biotin by employing microbiological assay, using *Lactobacillus arabinosus* and *Saccharomyces cerevisiae*. Thus far, a mixture of the 0.3 and 0.47 Rf materials will replace biotin for *S. cerevisiae* but not for *L. arabinosus*. Further studies with purified factors are continuing in this direction employing autobiographic assay using *Neurospora crassa*.

Although biotin and adenosine or our purified factors will allow excellent growth in a basal medium, the medium is not chemically defined because casamino acids must be present. Thus far, it has not been possible to completely substitute for casamino acids using known solutions of amino acids. Either acid hydrolyzed or enzymatically hydrolyzed casein will allow excellent growth in 24 hours; however, charcoal treatment will remove growth promoting activity of the amino acid hydrolyzates. Because charcoal removes aromatic amino acids, known amounts of tyrosine, trypto-

phane and phenylalanine were added to our synthetic amino acid mixtures. All attempts have been unsuccessful in the sense that we cannot obtain growth equivalent to casamino acids in 24 hours. However, using a mixture of 10 amino acids, (glutamic acid, cysteine, methionine, arginine, tyrosine, isoleucine, leucine, lysine, proline, phenylalanine), good growth occurs in 48 hours.

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