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A CHEMICALLY DEFINED MEDIUM FOR GROWTH OF RHIZOBIUM

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ABSTRACT

A new synthetic medium was developed for growth of rhizobia. The medium consisted of mineral salts amended with 0.5% mannitol and one or two growth factors. The vitamins calcium pantothenate, thiamine mononitrate, inositol, and biotin stimulated rhizobial growth. In particular, calcium pantothenate was found to be almost as effective as a combination of all the other vitamins in promoting growth of the root nodule bacteria studied. Three strains of *Rhizobium* proliferated readily in this simple medium. For instance, a cowpea *Rhizobium* strain KO4SRPR grew in this medium with a generation time of 4.5 hours compared to a generation time of 4.1 in the conventional yeast extract mannitol broth. Therefore this yeast extract-free, synthetic, medium would be useful for the study of the metabolism and the physiology of the root nodule bacteria.

INTRODUCTION

Yeast extract mannitol (YEM) is the conventional medium used for growing root nodule bacteria in the laboratory (Vincent, 1970). This medium consists mainly of mineral salts and mannitol enriched with yeast extract. However, since the exact chemical composition of yeast extract is not known, YEM is unsatisfactory for growth of rhizobia whenever it is desired to study the physiological and biochemical characteristics of these bacteria, or whenever it is necessary to examine the metabolism of chemical compounds by these organisms. To surmount this problem some authors have used certain types of defined media. For example, Valera and Alexander (1965) used a medium consisting of mineral salts-mannitol plus about eight different vitamins, to study the nodulation factor for *Rhizobium*-legume symbiosis. A similar broth was also employed by Phillips and Torrey (1970) and Peters and Alexander (1966) to characterize some physiological properties of the root nodule bacteria.

The purpose of this study, therefore, was to determine the relative importance of each of the common growth factors to rhizobial proliferation, and consequently establish a new, simple, synthetic medium which would be employed readily to study the physiology, biochemistry, and ecology of this group of bacteria.

MATERIALS AND METHODS

In order to replace the complex, yeast extract-containing YEM medium with a simple mineral salts-with-growth factors medium a broth was prepared as follows. Vincent's (1970) mineral salts medium was modified to contain the following macro – and micro-nutrients per liter of distilled water: 10.0g mannitol, 1.0g K^2HPO_4, 1.0g KH_2PO_4, 1.0g KNO_3, 0.20g NaH_2PO_4 $2H_20$, 0.18g $MgSO_4$, 0.13g $CaSO_4.2H_20$, 0.10g $Fe(NO_3)_2.9H_20$, 0.2mg $NaMo0_4.2H_20$, 0.2mg $ZnSO_4.7H_20$, 0.2mg H_3BO_3, 0.2mg $MnSO_4H_20$, 0.015mg $CuSO_4$, and 0.001mg $Co(NO_3)_2.6H_20$.

Portions (15.0ml) of this mineral salts-mannitol medium were placed in 25ml test tubes, and 7 of these tubes were amended with 1.3mg/l of each of the following seven vitamins: p-aminobenzoic acid, pyridoxine phosphate, inositol (meso), thiamine mononitrate, calcium pantothenate, riboflavin, and biothin. The growth factors were obtained from Nutritional Biochemicals Corp. .Cleveland, Ohio, U.S.A. Each tube was then inoculated with 0.1ml of a cell suspension derived from a 48-hour culture of each of cowpea *Rhizobium* KO4SRPR, *R. meliloti* 87TR, and *R. Phaseoli* 203CR, in separate experiments. The cell suspensions had earlier been washed three times with 0.*IM* phosphate buffer, pH7.0. In each case the cell density was 0.10 as measured by a Spectronic 20, spectrophotometer, at a wavelength of 540nm. Controls consisting of inoculated and uninoculated mineral salts-mannitol media were also prepared. Each treatment was in duplicate. All the tubes were incubated on a rotary shaker at 30C for 2 days, with a shaker speed of 180 rpm. Growth of bacteria in each tube was measured turbidimetrically at intervals of 3 hours using a spectrophotometer (Spectronic 20), and measuring optical density at 540nm.

The cowpea *Rhizobium* strain KO4SRPR was obtained from Dr. S. O. Keya, University of Nairobi, Nairobi, Kenya. *R. meliloti* 87TR and *R. phaseoli* 203CR came from Prof. M. Alexander, Cornell University, Ithaca, New York, U.S.A.

RESULTS AND DISCUSSION

As shown in Fig. 1, the cowpae *Rhizobium* strain KO4SRPR grew better in a mineral salts – mannitol broth amended with the growth factor calcium pantothenate, than in the same medium treated with any of the other six vitamins. The vitamins thiamine mononitrate, biotin, inositol (meso), riboflavin, and pyridoxine phosphate in that decreasing order of effectiveness, were also found to be stimulatory to the growth of the rhizobium. In a similar study, Graham (1963) also found that strains of *R. leguminosarum, R. phaseoli* and *R. trifolii* exhibited positive growth response to calcium pantothenate as well as thiamine and biotin. On the other hand, surprisingly, p-aminobenzoic acid (PABA) was found to lack the physiological or nutritional function of a growth factor as it retarded growth of the bacterium instead of enhancing it.

Table I depicts the general patterns of growth of the cowpea *Rhizobium* KO4SRPR, *R. phaseoli* 203CR, and *R. meliloti* 87TR in the 0.5% mannitol-mineral salts medium amended with each of the seven vitamins. It is noteworthy that calcium pantothenate stimulated the growth of all the strains while p-aminobenzoic acid did not enhance growth of any. Further studies revealed that the addition of different combinations of the growth factors to the mineral salts-mannitol broth enhanced growth of the root nodule bacteria, but none of the strains of rhizobia studied had a generation time less than 4.5 hours in the defined medium containing a combination of three or four of the different growth factors. The tentative conclusion is that calcium pantothenate is almost as effective as a combination of all the other vitamins in promoting growth of rhizobia.

It is evident from Table I that the different strains of rhizobia had an average generation time of 4.2 hours in YEM medium and 4.6, hours in mineral salts-mannitol broth amended with calcium pantothenate. Hence, the latter medium may conveniently substitute for the former, at least, as far as growth of the root nodule bacteria is concerned. It is not completely understood why calcium pantothenate readily stimulates proliferation of rhizobia. However, the favourable influence of this growth factor on the growth of rhizobia may be due to the important biochemical properties of this vitamin B_5. For instance, pantothenic acid is directly involved in the synthesis of coenzyme A, and acetyl CoA is an essential key intermediate in the metabolism of fatty acids, proteins and carbohydrates via the tricarboxylic acid cycle (Lehninger, 1970). Hence, this vitamin would directly enhance metabolic activities and growth of the bacteria. The reasons for the lack of stimulation of rhizobial growth by p-aminobenzoic acid are

not clear, but the vitamin is known to be incompatible with ferric salts and oxidizing agents (Stecher, 1968), hence it might have been inactivated by these compounds in the mineral salts medium. However, this finding agrees with that of Graham (1963) who reported that none of the several strains of *Rhizobium* he studied showed growth response to p-aminobenzoic acid.

In general, the growth of rhizobia in defined media is useful for the study of the metabolism and the physiology of the root nodule bacteria. For instance, Phillips and Torrey (1970) employed a mineral salts-mannitol medium amended with several vitamins to study the production of cytokinin by a strain of *R. japonicum*, and Odeyemi and Alexander (1977) used a similar synthetic broth to study the metabolism of some fungicides by rhizobia. Nutritional requirements have also been used in Adansonian classification of bacteria (Sneath and Cowan, 1958). Hence, this modified, defined medium which is simple in composition would facilitate the study of the root nodule bacteria by rhizobium geneticists, biochemists and ecologists.

LITERATURE CITED

Graham, P.H: 1963. Vitamin requirements of root nodule bacteria. J. Gen. Microbiol. 30: 245 – 248.

Lehninger, A. L. 1970. Biochemistry. Worth Publishers Inc. New York.

Odeyemi, O. and M. Alexander, 1977. Resistance of *Rhizobium* strains to phygon spergon, and thiram. Appl. Environ. Microbio. 33: 784 – 790.

Peters, R. J. and M. Alexander. 1966. Effect of legume exudates on the root nodule bacteria. Soil Sci. 102: 380 – 387

Phillips, D. A. and J. G. Torrey. 1970. Cytokinin production by *R. japonicum*. Physiol. Plant. 23: 1057 – 1063.

Sneath, P.H.A. and S. T. Cowan. 1958. An electro-taxonomic survey of bacteria. J. Gen. Microbiol. 19: 551 – 565.

Stecher P. G. 1968. The Merck Index. Merck and Co. Inc. Rahway New Jersey. pp.53

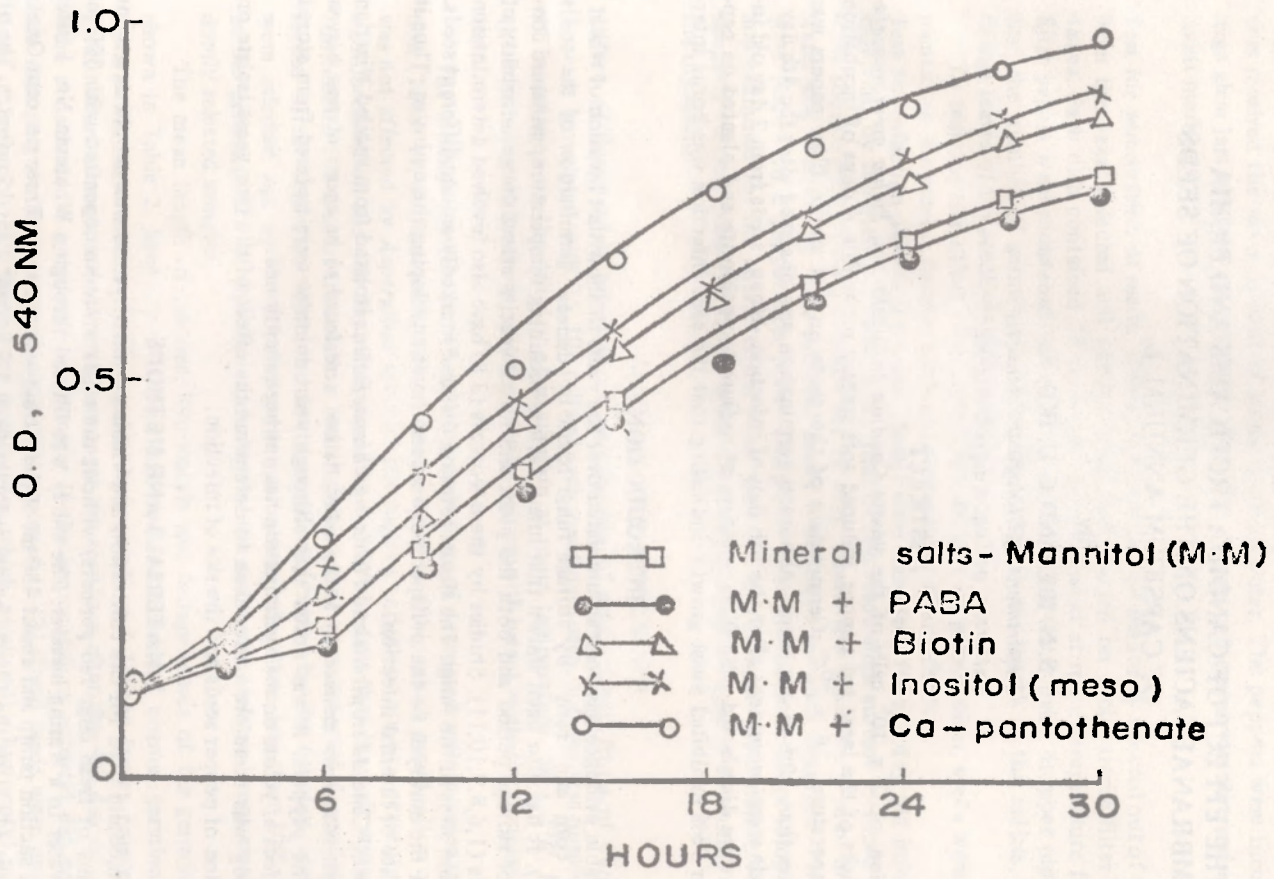
Valera, C. L. and M. Alexander, 1965. Nodulation factor for *Rhizobium* – legume symbiosis. J. Bacteriol. 89: 1134 – 1139.

Vincent, J. M. 1970. A manual for the practical study of root nodule bacteria. Blackwell Scientific Publications, Oxford. pp. 1 – 64.

TABLE I

Generation time of rhizobia in YEM and in mineral salts-mannitol broth amended with different vitamins.

Vitamin added	Generation time, hr		
	Cowpea Rhizobium	*R. phaseoli*	*R. meliloti*
Calcium pantothenate	4,5	4.6	4.7
Thiamine mononitrate	5.0	4.9	5.1
Biotin	5.3	5.2	5.4
Riboflavin	5.4	5.5	5.8
Inositol (meso)	5.4	5.8	4.6
Pyridoxine phosphate	6.0	6.1	5.0
p-Aminobenzoic acid	6.6	6.4	6.6
Mineral salts-mannitol alone	6.6	6.7	6.6
YEM	4.1	4.2	4.2



LEGEND

Fig. 1. Growth of cowpea Rhizobium KO4SRPR in 0.5% mannitol-mineral salts medium amended with different growth factors.