THE GROWTH OF *RHIZOBIUM* IN SYNTHETIC MEDIA

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Summary

A chemically defined medium for the growth of *Rhizobium* is described in which populations of up to 5×10^9 cells/ml were obtained. For the six strains of bacteria studied the complete medium supported exponential growth for two to five generations. The concentrations of biotin giving best growth varied with strain between 125 and 250 μg/l when the nitrogen source was sodium glutamate. NH_4^+, NO_3^-, and other amino acids, singly or in combination, did not support as good growth as did sodium glutamate.

The medium was used to demonstrate a calcium requirement for growth of all strains. When the concentration of this ion was reduced to 0·1 p.p.m. with four strains, lag was lengthened and the exponential phase was shortened but growth rate was not affected. With two other strains, calcium deficiency did not affect lag or the length of the exponential phase, but the growth rate was reduced. Calcium-deficient cultures attained final populations which were 20–60% lower than those grown in the complete medium, and with four strains deficient cells were enlarged.

I. Introduction

Monod (1949) stated "the study of the growth of bacterial cultures does not constitute a specialized subject or branch of research: it is the basic method of microbiology". This basic study of *Rhizobium* has been very largely neglected as most published work has been concerned with total populations and the ability to survive serial subculture rather than with the dynamics of growth.

West and Wilson (1940) and Wilson and Wilson (1942) used serial-subculture methods to show that biotin may be essential, stimulatory, or without effect for the growth of *Rhizobium*. More recently Norris (1959) was able to subculture the organisms for prolonged periods in media containing less than 0·1 p.p.m. calcium, and concluded that they were satisfied with minute traces of this element. In this work Norris used both a yeast extract–mannitol medium and a synthetic medium. Bergersen (1958) obtained linear growth of *Rh. japonicum* in yeast extract–mannitol medium; that is, the generation time of the bacteria became progressively longer and the steady state of growth was not attained. Preliminary use of Norris' (1959) synthetic medium in this Laboratory showed that this medium supported only a brief exponential phase of growth after a lag lasting in most cases several days. These results suggested that these two media used in Norris' (1959) work were severely growth restrictive and thus it seemed that the omission of the Ca^{++} ion could not be expected to have much effect when the growth of the bacteria was already restricted by the constitution of the media.

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The objective of the work described in this paper was, therefore, the development of a synthetic defined medium for the growth of *Rhizobium*, which would support a considerable period of exponential growth after as short a lag as possible and give a high density of total growth in as short a time as possible. The medium was then used to re-examine the effects of calcium deficiency upon the growth of *Rhizobium*.

II. Materials and Methods

(a) *Bacterial Strains*

Six strains were used throughout, four of them being among those used by Norris (1959): Rothamsted AH_2, CC146A (*Rh. meliloti*); Rothamsted ClF, SU297/3 (*Rh. trifolii*); CB170 (*Rhizobium* sp. isolated from *Phaseolus lathyroides*); CB362 (*Rhizobium* sp. isolated from *Psoralea eriantha*). These were maintained on yeast extract–mannitol agar in screw-capped bottles. For determination of growth curves the organisms were grown in tubes of the medium to be used so that, with the comparatively large inocula used, there would be no carry-over of nutrients not present in the test medium. The volumes of these tube cultures used to inoculate the growth flasks were adjusted from counting-chamber counts so that the same number of organisms was used in inoculating all flasks of an experiment.

(b) *Growth Media*

Yeast extract–mannitol medium was that used by Nutman (1946), omitting the agar when a liquid medium was required.

The salts of the synthetic medium were basically the same as those used by Norris (1959): $Na_2HPO_4.12H_2O$, 0·045%; $MgSO_4.7H_2O$, 0·01%; $FeCl_3$, 0·002%, and $CaCl_2$, 0·004%, were added to the medium as separately sterilized solutions after autoclaving. The energy source was 1% mannitol. All media were adjusted to pH 6·8.

In the determination of the best nitrogen source, KNO_3, $NH_4H_2PO_4$, glutamic acid (sodium salt), asparagine, aspartic acid, arginine, histidine, tyrosine, lysine, and serine were all used singly or in combination to give 8 mg nitrogen/100 ml medium. For this work 100 μg thiamine and 25 μg biotin were used per litre of medium.

Biotin requirements were determined in media containing the above salts and mannitol with sodium glutamate as the nitrogen source and 100 μg thiamine/l. Biotin was obtained from Hoffmann la Roche and Company, Basle, and was made up in pH 7·0 phosphate buffer and stored at 4°C.

In the experiments to determine the effects of calcium deficiency the constituents were purified. Mannitol was three times recrystallized from supersaturated solutions. $MgSO_4$ was recrystallized from a filtered supersaturated solution and the crystals washed with alcohol. Sodium glutamate was dissolved in water and precipitated as the acid by the addition of conc. HCl; the acid was washed with cold water and then suspended in water and titrated to neutrality with high purity NaOH. The solution was then poured into six volumes of ethanol and crystallized at 4°C overnight. The crystals were washed with cold ethanol and dried. Na_2HPO_4 was used without further purification and glass-distilled water was used throughout. The calcium levels, determined by an atomic absorption spectrophotometric method, are given for the various constituents in Table 1, which shows that although they contain

a total of less than 0·088 p.p.m. calcium in the concentrations used in the medium, calcium leached from the glass during autoclaving raised the level to 0·11 p.p.m. All glassware was soaked in 25% HCl overnight and then exhaustively washed with glass-distilled water before use; glassware used for low-calcium cultures was not used for plus-calcium cultures. Cotton wool and gauze plugs were prepared from materials washed in 2% ethylenediaminetetraacetic acid (EDTA) and distilled water.

Table 1

RESIDUAL CALCIUM IN PURIFIED CONSTITUENTS OF SYNTHETIC MEDIUM

Purified Constituent	Concentration of Solution Analysed	Calcium Content (p.p.m.)	Calculated Calcium in Medium Used (p.p.m.)
Mannitol	10%	<0·05	<0·005
$MgSO_4.7H_2O$	0·1%	0·09	0·009
$Na_2HPO_4.12H_2O$	0·45%	<0·05	<0·005
Sodium glutamate	1·1%	0·62	0·062
$FeCl_3$	0·02%	0·05	0·005
Biotin	25 μg/ml	<0·05	<0·005
Thiamine	1 mg/ml	0·14	0·0014
Total			<0·0879
Low-calcium medium analysed after autoclaving			0·11

(c) *Growth Conditions*

Cultures were grown in 250-ml Erlenmyer flasks which were equipped with ½-in. diameter side-arms for turbidity measurement: 40 ml of medium was used per flask. Inocula were 1–3 ml of young tube cultures and the flasks were incubated at 25°C on a rotary shaker running at 160 c/min.

(d) *Measurement of Growth*

Growth was measured turbidimetrically in an "EEL" photoelectric nephelometer. Total numbers of bacteria were determined from calibrations prepared for each strain of bacteria in each medium used, by measurement of the turbidity of suspensions which had been counted in a Petroff–Hauser counting chamber.

The various growth characteristics were determined graphically by plotting $\log_2$ bacterial numbers against time (see Fig. 1); this facilitated determination of growth rate, since an increase of 1 unit was equivalent to a doubling of the population (Monod 1949). These growth characteristics were:

(i) Lag time (L), the time between inoculation and the commencement of growth had all cells commenced to divide together at the maximum rate (Hinshelwood 1946);

(ii) The generation lag (L_g), the time for the first doubling of the initial population (Lockhart 1960);

(iii) The growth rate in the steady (exponential) state in generations per hour (R);
(iv) The duration of the exponential phase (E);
(v) The total population increase during the growth period (G).

One strain, SU297/3, grew in small uniform clumps and the characteristics of growth were determined from turbidity alone.

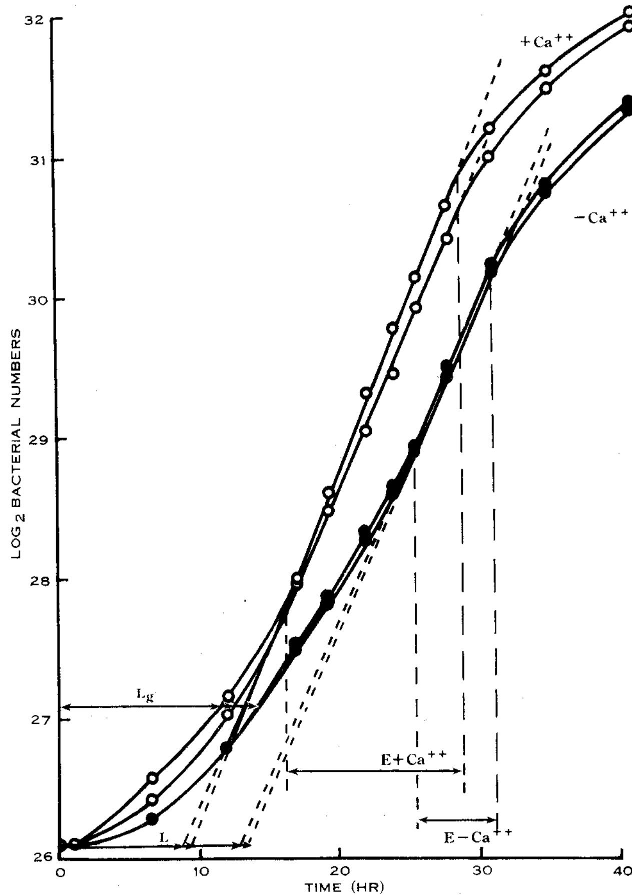

Fig. 1.—Determination of the growth characteristics of *Rhizobium*. Data for duplicate flasks are plotted for strain AH_2.

(e) *Plant Tests*

Tests of infectiveness and symbiotic effectiveness were done as follows in order to compare the synthetic medium with the commonly used yeast extract–mannitol medium. Cultures were carried through two subcultures in tubes followed by growth in shake flasks after which single colonies from the same medium, solidified with agar and spread with the flask culture, were used to inoculate appropriate host plants. After growth in a glass-house for 5 weeks the plants were harvested, nodulation examined, and dry weights obtained.

III. Experimental Results

(a) *Nitrogen Source*

Representative growth curves for strain ClF grown in the salts–mannitol medium with 25 μg biotin and 100 μg thiamine per litre and using various nitrogen sources are illustrated in Figure 2. The results of these studies clearly showed that glutamate was the best source of nitrogen for growth of all strains used. In addition

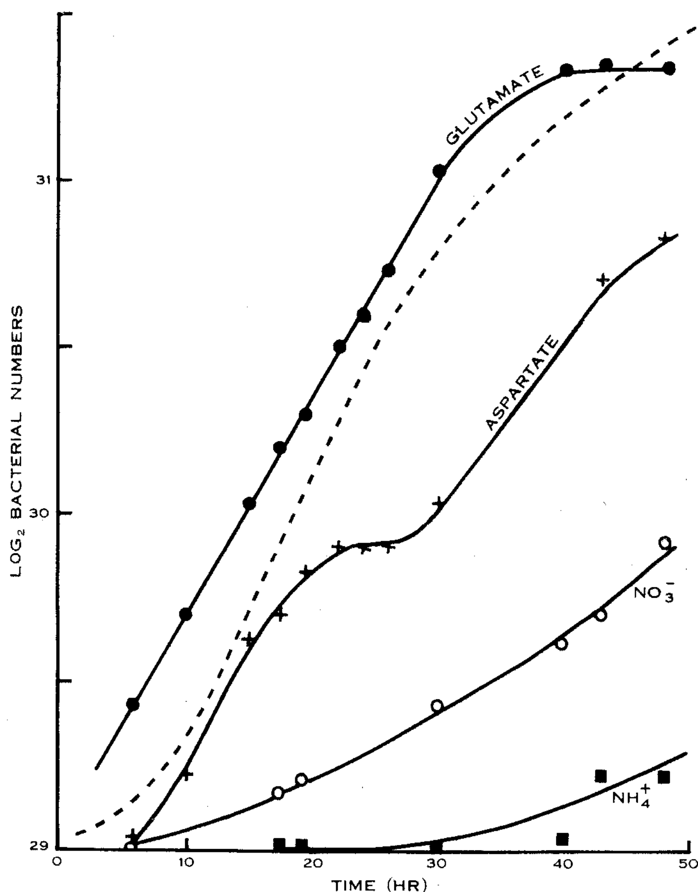

Fig. 2.—Effect of various nitrogen sources upon the growth of strain ClF (*Rhizobium trifolii*) in shake culture. The broken line shows the growth of this strain in yeast extract–mannitol medium from a separate experiment. The growth curve labelled NO_3^- is the curve for the medium used by Norris (1959).

to the results illustrated, it was found that no combination of amino acids or amino acids and inorganic nitrogen was as good as glutamate. Amino acid mixtures frequently gave multi-step growth curves of the type shown for aspartate, but with several steps. The reasons for this are not known and although high total numbers were often attained with these cultures the forms of the growth curves were quite unsuited to growth studies.

The use of the sodium salt of glutamic acid as a nitrogen source resulted in general in a greater rise in pH after growth in the synthetic medium than after growth in yeast extract–mannitol medium (Table 2).

(b) *Biotin Requirements*

Preliminary results indicated that the 0·5 μg/l used by Norris (1959) was sub-optimal and hence 25 μg/l was used in the tests of nitrogen sources. A more extensive investigation was then carried out using glutamate as nitrogen source. The results of this are summarized in Table 3, which shows that strain CC146A had no exponential

Table 2

pH of the cultures, measured with a glass electrode, after 72 hr. initial pH 6·8

Bacterial Strain	Synthetic Medium	Yeast Extract–Mannitol Medium	Bacterial Strain	Synthetic Medium	Yeast Extract–Mannitol Medium
AH_2	7·75	7·1	CB362	7·1	7·05
CC146A	7·6	6·9	ClF	8·1	7·4
CB170	7·7	7·2	SU297/3	7·6	6·6

phase with only 12·5 μg/l biotin and strain SU297/3 required more than 25 μg/l for exponential growth. All other strains grew exponentially at all biotin concentrations used, but total growth increased with increased biotin up to 125–250 μg/l according to strain. Biotin did not affect growth rate (R) but the length of the exponential phase (E) was increased with raised biotin levels for most of the strains. Both L and L_g were either unaffected or reduced with increased biotin except for strain AH_2 for which levels higher than 125 μg/l were inhibitory with respect to L, E, and G. With all strains raised biotin levels prolonged growth after the end of the exponential phase and the rate of retardation was greatest at the lowest biotin concentrations.

(c) *Effects of Low Calcium*

The effects on growth of the omission of calcium from the synthetic medium, using glutamate and supplying the best biotin concentration for each strain, are summarized in Table 4. Preliminary tests showed that 0·02% $CaCl_2$ as used by Norris (1959), was slightly inhibitory for strain AH_2 so the plus-calcium series contained 0·004% $CaCl_2$ or 14 p.p.m. calcium, while the low-calcium series contained about 0·1 p.p.m. calcium.

For four strains, AH_2, CC146A, CB170, and ClF, both L and L_g were increased in calcium-deficient cultures and the exponential phase (E) was shortened, although R was not affected. With the other two strains there was little effect on L or L_g and E was not affected by low calcium but the growth rate (R) was reduced. With all

strains calcium deficiency was expressed as a reduction in total growth, G varying from 40–80% of that attained with added calcium.

Determination of the level of calcium required proved difficult firstly because it was not possible to reduce the calcium content of the unautoclaved medium below

TABLE 3

EFFECTS OF BIOTIN CONCENTRATION ON GROWTH OF RHIZOBIUM IN SHAKE CULTURE

L = lag phase (Hinshelwood 1946) in hr; L_g = generation lag in hr; R = growth rate in generations per hr in exponential phase; E = length of exponential phase in hr; G = bacterial increase $\times 10^7$

Bacterial Strain	Biotin (μg/l)	L	L_g	R	E	G*	Growth Period (hr)
AH_2	25	4·5	8·3	0·26	13·5	236	41
	125	5·0	8·8	0·26	18·25	532	41
	250	6·0	9·5	0·26	13·0	492	41
	375	7·5	9·8	0·26	10·5	470	41
CC146A	12·5	—†	4·0	—†	0	72	54·5
	25	0·75	5·5	0·21	23·0	122	54·5
	125	0	4·5	0·21	24·0	308	54·5
	250	0	4·5	0·21	24·0	320	54·5
CB170	12·5	34·0	45·0	0·05	33·0	122	127
	25	29·0	44·0	0·05	49·0	153	127
	250	21·0	41·0	0·05	58·0	169	127
CB362	12·5	3·3	9·5	0·16	24·0	86	54·5
	25	7·0	13·0	0·17	27·6	154	54·5
	125	7·0	13·0	0·17	27·6	233	54·5
	250	7·0	13·0	0·17	27·6	228	54·5
ClF	12·5	4·5	12·0	0·13	19·0	60	53·5
	25	4·5	12·0	0·13	20·6	74	53·5
	125	4·5	12·0	0·13	23·5	88	53·5
	250	4·5	12·0	0·13	23·5	85	53·5
SU297/3	25	—†	3·8	—†	0	c. 400‡	41
	125	3·5	8·5	0·20	19·5	c. 600	41
	250	2·5	7·5	0·20	19·5	c. 600	41
	375	1·5	6·5	0·20	19·5	c. 600	41

* Includes growth in the phase of retardation: stationary phase was reached only in the biotin concentrations 12·5 and 25 μg/l.

† All growth in phase of retardation.

‡ Approximate numbers only because of clumpy growth of this strain.

0·08 p.p.m., and secondly because of variable amounts of calcium leached from the growth flasks during autoclaving, no satisfactory concentration series was obtained. However, for the strains of *Rhizobium* tested a response to 5 p.p.m. added calcium

could be detected and the maximum response occurred between 10 and 14 p.p.m. No treatment of the glassware prevented calcium leaching during autoclaving, although boiling and autoclaving with EDTA solutions, detergent, and acid treatments were tried. The 25% HCl soaking was slightly better than other treatments. Another

TABLE 4

EFFECTS OF CALCIUM UPON GROWTH OF RHIZOBIUM IN SHAKE CULTURE

Bacterial Strain	Calcium*	L†	L_g†	R†	E†	G†	Growth Period (hr)
AH_2	+	9·0	11·75	0·25	12·5	437	41
	+	8·75	12·5	0·24	12·5	413	41
	−	12·0	17·0	0·24	5·5	275	41
	−	12·0	17·0	0·22	5·5	269	41
CC146A	+	0	6·0	0·19	25·0	263	41
	+	0·6	6·6	0·16	28·0	288	41
	−	4·0	8·0	0·18	7·0	220	41
	−	6·0	8·0	0·18	7·0	228	41
CB170	+	10·0	27·0	0·05	40·0	180	96
	+	14·0	32·0	0·05	42·0	165	96
	−	29·0	44·0	0·05	17·5	108	96
	−	29·0	47·0	0·05	17·5	105	96
CB362	+	8·0	14·6	0·15	27·0	253	54·5
	+	8·0	15·5	0·13	25·0	218	54·5
	−	7·0	17·0	0·10	24·0	140	54·5
	−	9·6	19·0	0·11	26·0	145	54·5
ClF	+	5·3	12·6	0·14	20·6	85	50·5
	+	5·3	12·6	0·14	20·6	82	50·5
	−	8·5	16·5	0·13	12·3	32	50·5
	−	8·6	17·0	0·13	12·3	33	50·5
SU297/3	+	2·0	8·0	0·17	23·0	c. 450‡	41
	+	2·5	8·5	0·17	23·0	c. 450	41
	−	1·0	8·25	0·13	24·0	c. 250	41
	−	2·0	8·5	0·13	23·0	c. 250	41

* + = medium contains 14 p.p.m. calcium; − = medium contains 0·1 p.p.m. calcium.

† L, L_g, R, E, and G as in Table 3.

‡ Approximate numbers only, because of the clumpy growth of this strain.

difficulty encountered here was that of the analysis of low levels of the ion. Concentration of the medium was required and this also introduced the possibility of calcium being introduced from the glassware. With care, however, it was possible to demonstrate depletion of calcium from plus-calcium medium analysed before and after growth of the bacteria.

Another effect of calcium deficiency appeared when the growth flasks were calibrated for turbidity with suspensions of known numbers of cells from plus- and minus-calcium media. Figure 3 shows one such calibration. The difference in slope is attributable to the increased size of the deficient cells, which was readily seen when

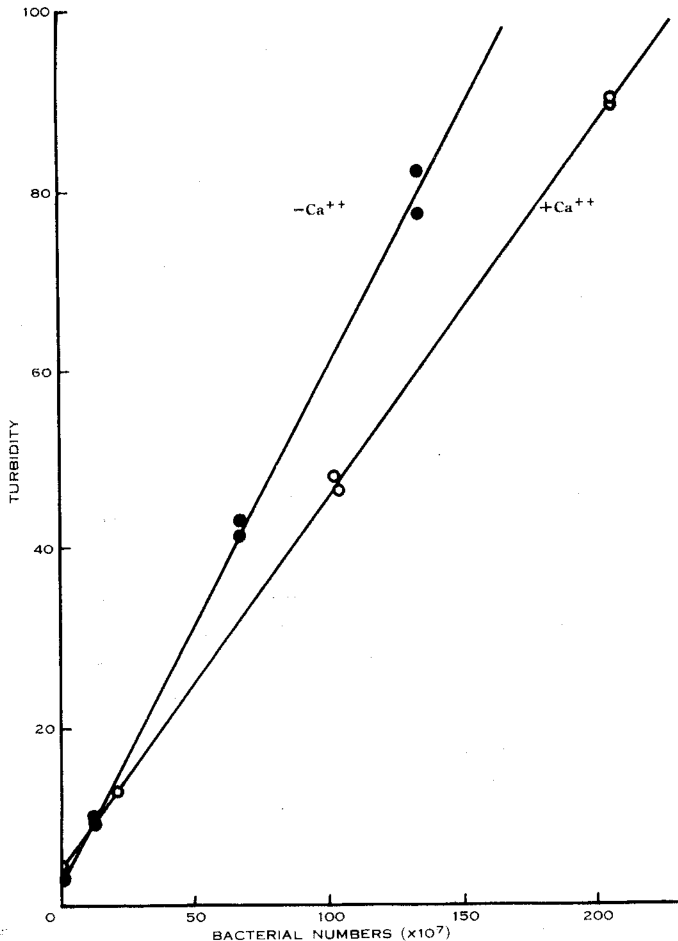

Fig. 3.—An example of the turbidity *v.* cell numbers calibration in which the different slope is a measure of difference in cell size. Strain CB362.

counting the suspensions in the Petroff–Hauser chamber. Strain CC146A did not show this difference in size and strain SU297/3, because of the clumpy growth, could not be assessed for cell size differences.

(*d*) *Synthetic Medium Compared with Yeast Extract–Mannitol Medium*

The growth of the six strains in yeast extract–mannitol medium and in the synthetic medium was compared. Strain CB170 grew better in the complex medium but the others grew as well in terms of L, R, and G in the synthetic medium as they

did in yeast extract–mannitol medium: E was longer for growth in the synthetic medium.

Strains AH_2, SU297/3, CC146A, and ClF grew better on synthetic medium solidified with 2% agar than on yeast extract–mannitol agar when these media were used for the isolation of single colonies for the plant tests.

The growth of ClF in yeast extract–mannitol medium is shown for comparison with the synthetic medium as a broken line in Figure 2.

(e) *Symbiotic Properties after Growth in Synthetic Medium*

With the exception of strain ClF, single colonies of all strains did not differ in infective properties or in symbiotic effectiveness, whether grown in the synthetic medium or in yeast extract–mannitol. Strain ClF, however, lost some degree of infectiveness, only 2 colonies out of 10 from the synthetic medium producing nodules on white clover while 6 out of 10 from the yeast extract–mannitol medium nodulated the host. With the other strains, all colonies tested from both media produced effective nodules on appropriate hosts.

(*f*) *Conclusions*

A synthetic growth medium for *Rhizobium*, which gives high total populations and in which growth characteristics are suitable for dynamic studies, is constituted as follows: mannitol 1%; $Na_2HPO_4.12H_2O$, 0·045%; $MgSO_4.7H_2O$, 0·01%; $FeCl_3$, 0·002%, and $CaCl_2$, 0·004%, added after autoclaving; sodium glutamate 0·11%; thiamine 100 μg/l; biotin 125–250 μg/l, depending on bacterial strain.

With the exception of strain CB170, the synthetic medium produced as good growth, in terms of maximum populations, as yeast extract–mannitol medium, but in much shorter growth periods and with relatively longer phases of exponential growth.

The synthetic medium, solidified with 2% agar, was superior to yeast extract–mannitol for strains AH_2, SU297/3, CC146A, and ClF.

All strains of *Rhizobium* studied exhibited a response to added calcium when the concentration of that ion in the medium was about 0·1 p.p.m. This response was manifested in the following ways:

(i) Low-calcium media gave longer lag (L or L_g) and a shortened exponential phase, or

(ii) Lag was not affected but growth rate in the exponential phase was reduced.

(iii) Low-calcium media produced only 40–80% of the growth with added calcium.

(iv) Calcium-deficient cells of four strains were larger than those from media with added calcium.

IV. Discussion

In discussing the results presented in this paper, attention is first of all drawn to the use of glutamate as a nitrogen source for the growth of *Rhizobium*. Yeast extract, as used in non-defined media, supplies both growth factors and nitrogen as amino acids. Glutamate is known to stimulate vigorous respiration of *Rhizobium* (Bergersen 1957). Other workers have chosen an inorganic ion such as nitrate as a

source of nitrogen for a synthetic medium (Norris 1959). In its natural location in the soil *Rhizobium* grows predominantly in plant rhizospheres where root exudates, especially of legumes, provide relatively large amounts of amino acids (Rovira 1956).

The unusually high biotin requirements which have been demonstrated may be linked with the use of glutamate as nitrogen source, which permits so much more rapid growth than occurs with nitrate. Alternatively, the biotin may be a source of some other growth factor present as an impurity or may be serving as a source of a fatty acid from the side-chain of the molecule. Thiamine was stimulatory for only two strains and its inclusion in all media was a step towards uniformity. Once again it may be stated that biotin is a significant constituent of root exudates of legumes although absolute amounts may be small, and is thus of importance for growth of *Rhizobium* in the rhizosphere of host plants (Rovira and Harris 1961).

It is not suggested that the synthetic medium used in this work is the best possible but the main objective has been to emphasize the principles which should be observed in such studies. The salts used by Norris (1959) are apparently satisfactory but nitrate is not a good nitrogen source. Glutamate is shown to be better for the six strains studied, but with other strains other amino acids may be better and give more satisfactory growth. Similarly, biotin requirements are likely to be different for other strains and the level of this vitamin in the medium should be that best suited to the strain being studied. A good growth medium should fulfil the following conditions:

(i) The lag should be as short as possible so that the original character of the culture is not altered by the selections of mutants.*

(ii) The exponential phase should be as long as possible with the best growth rate attainable. This is the critical stage of growth since it is in the exponential phase that the steady state is reached and thus it is the phase in which experimental deficiencies are most likely to be expressed.

(iii) The maximum population should be as high as possible if the medium is to be of any practical importance.

The growth characteristic L_g was included in this study for the reasons stated by Lockhart (1960), viz. it seems to be a better measure of lag when different growth rates are involved. With the six strains used it has been possible to test Lockhart's (1960) hypothesis that L_g is very close to the time that lag actually ends; in other words, when all the cells of the initial culture have divided once, all cells should be growing at the maximum rate. Strains which had a lag phase varied in their relation between L_g and onset of exponential growth, but in most cases with the complete synthetic medium, L_g was quite close to the time at which the steady state commenced. Calcium deficiency increased the discrepancy by delaying the onset of exponential growth for up to three generations in four of the six strains studied.

The other effects of calcium deficiency shown in this work may be assessed as follows. In the cultures in which low calcium lengthened lag (L or L_g), an alternative metabolic pathway may have developed during this phase and this alternative pathway was not able to support as long an exponential phase as the normal pathway in

* This apparently happened in the work of Norris (1959) when many strains, after maintenance in synthetic medium, had their symbiotic characters altered.

cultures in which calcium was not limiting. The shortened exponential phase may perhaps be caused by increased excretion into the medium of toxic metabolites from calcium-deficient cells. This type of response to reduced calcium levels may also be explained by the deficient cells slowly accumulating a factor or factors at a rate determined by the calcium level of the medium; when a threshold value was attained, exponential growth started but was soon inhibited by the low calcium level. With the two strains whose growth rate in the exponential phase was reduced by low calcium, the effect may be attributed directly to the deficiency, since in this phase, growth is at a maximum for the conditions of temperature, aeration, and energy level provided.

Norris (1959) concluded that *Rhizobium* is not a calcium-sensitive organism and any requirement which it has for this element is of trace magnitude only. The work reported in this paper confirms this since quite good growth occurred with all strains with 0·1 p.p.m. calcium. However, the results also show that at this low level of Ca^{++} a deficiency was expressed in the form of the growth curves, in the total population attained and in a cell abnormality, when compared with growth in 10–14 p.p.m. calcium. For ordinary culture media, at least 10–14 p.p.m. calcium would be supplied as impurities in the constituents especially if agar is employed, but with purified materials, calcium should be added.

V. Acknowledgments

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