SYNTHESIS OF COENZYME R BY CERTAIN RHIZOBIA AND BY AZOTOBACTER CHROOCOCCUM

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A recent paper (Allison and Minor, 1938) from this laboratory pointed out that an occasional strain of *Rhizobium* grows well in a medium not supplied with the essential growth substance, coenzyme R, although most of the rapidly growing strains tested required its addition. Only one of 27 strains of *R. trifolii*, *R. leguminosarum*, *R. meliloti*, and *R. phaseoli* grew appreciably without this material, and even this strain responded to it. Simultaneous experiments with additional species showed, however, that a few fast-growing strains make practically no response to coenzyme R. In earlier work (Allison, Hoover and Burk, 1933; Hoover and Allison, 1935) it had been pointed out that *Azotobacter vinelandii* and certain other bacteria synthesize the substance. Tests (Allison and Minor, 1938, p. 481) with rhizobia that grow without coenzyme R additions also showed synthesis. This paper presents quantitative data and other pertinent information relating to this synthesis and liberation of coenzyme R from the cells of rhizobia and *Azotobacter chroococcum*.

EXPERIMENTAL METHODS

Growth of organisms

The organisms were grown at 28°C. on our basal medium, described elsewhere (Allison and Minor, 1938), using 250 ml. cotton-stoppered Erlenmeyer flasks, containing 25 ml. of medium. After varying intervals of time the bacteria were separated from the medium, and the content of growth factor in the latter determined by tests of graduated concentrations on the growth of *R. trifolii*.

The cultures used and their sources were as follows: *R. meliloti* 131, U. of Wisconsin; *R. meliloti* 6, from our own stock cultures; *R.* from *Dalea alopecuroides* 900 and 901, U. of Wisconsin; *R.* from *Coronilla varia* 81, from our own stock cultures; *R. trifolii* 205 and 209, U. of Wisconsin; *A. chroococcum*, from our own stock cultures.

The organisms were tested for purity, and with the exception of one of the *Dalea* cultures the rhizobia had been tested recently for nodule production.

Preparation of culture medium for assay

Two methods were used for removing the bacteria and gum from the culture medium prior to determining the content of growth substance. The first method consisted of drying the cultures at about 100°C., adding warm water without stirring, and decanting after several hours through filter paper. In the second method, most of the water was removed by evaporation, ethyl alcohol added, the liquid decanted through a filter, and the gummy precipitate washed thoroughly with 80 per cent alcohol. After removing the alcohol, the preparations were adjusted to volume, autoclaved, and stored until a convenient time for assay. The two methods gave similar results, but since the second was easier and removed the gum more effectively, it was usually employed.

Method of assay

The populations of 4-day cultures of *R. trifolii*, grown on our basal medium, are roughly proportional (within a 2-fold range) to the quantity of growth factor added, when the population density is between 50 and 500 millions per cubic centimeter. An assay unit was therefore adopted based on the population produced by one unit of coenzyme R in a definite quantity of medium. The tentative definition is:

One assay unit = quantity of coenzyme R which in 1 ml. of our usual culture medium produces 400 million *R. trifolii* 209 in 4 days at 28°C. with an inoculum of about 400,000 bacteria per ml.

A population of 400 millions per ml. is slightly below half maximum growth under our experimental conditions.

In practice, the unknowns are tested at several concentrations against an active preparation considered as a standard. The use of a standard is not necessary, of course, but facilitates assay and is, in fact a positive control guarding against errors due to differences in condition and quantity of inoculum, cultural conditions, etc. It is advisable to reassay the unknowns, using concentrations which will produce the desired population density. The material used as a standard was an aqueous-acetone extract

TABLE 1
Effect of coenzyme R on growth of various rhizobia
Millions of bacteria per cubic centimeter on 4th day

ORGANISMS	EXPERIMENT 1*		EXPERIMENT 2*	
	No coenzyme R	Coenzyme R concentrate 1 p.p.m.	No coenzyme R	Coenzyme R concentrate 1 p.p.m.
<i>R. trifolii</i> 205.....	4	800		
<i>R. trifolii</i> 209.....	4	800		
<i>R. meliloti</i> 131.....	10	600	30	540
<i>R. meliloti</i> 6.....	800	800	540	1,000
<i>R. from Coronilla</i> 81.....	800	800		
<i>R. from Dalea</i> 900.....			1,080	1,980
<i>R. from Dalea</i> 901.....			1,080	1,100

* The figures given for experiment 1 are estimates, based upon turbidity, whereas those for experiment 2 are cell counts.

of molasses. It was not our most active preparation, but was available in quantity.

Growth of various rhizobia in presence and absence of coenzyme R

In previous papers (Allison and Hoover, 1934; Allison and Minor, 1938) data have been presented showing the marked response of various species and strains of *Rhizobium* to coenzyme R. In table 1 are presented data for 4 cultures of *Rhizobium*, representing 3 strains, which do not require its addition. Two cultures of *R. trifolii* and one of *R. meliloti* that require additions of the growth substance are included for comparison.

In these experiments the medium contained 0.3 per cent c.p. sucrose. The nitrogen source in experiment 1 was ammonium chloride, and in experiment 2 potassium nitrate.

The data show that under conditions such that two strains of *R. trifolii* and one of *R. meliloti* made little growth without added coenzyme R, good growths were obtained with two strains of *Rhizobium* from *Dalea*, one from *Coronilla*, and one strain of *R. meliloti*. Some of these that grew well without additions of coenzyme R showed a considerable early response to it. This was especially true with *R. meliloti* 6. The effect noted with *Dalea* 900 was usually less marked than that shown in table 1. It is not to be inferred from table 1 that these organisms do not need coenzyme R; they synthesize it, as shown below.

*Synthesis and liberation into the medium of coenzyme R by
various rhizobia*

A typical experiment, showing the quantity of coenzyme R synthesized and liberated into the medium by rhizobia, is reported as assay I in table 2. Two 25 ml. cultures of *Dalea* organisms, grown on the usual medium for 30 days, synthesized considerable quantities of this substance. One ml. of the *Dalea* culture contained enough growth substance for half maximum growth of *R. trifolii* in 100 to 200 ml. of medium. The populations obtained in this assay were not in many cases as near 400 millions per milliliter as is desirable, but the error in evaluation is not great except with very small populations.

The results of another experiment, using 60-day cultures, is reported as assay IV, table 2. The quantity of growth substance synthesized by the two *Dalea* cultures was 5 to 10 times as great as in the previous experiment. *Rhizobium* from *Coronilla* also synthesized the substance but the amount was only about 7 per cent of that in the *Dalea* cultures.

Inoculations of similar media with cultures of *R. trifolii* 205 and 209 and with *R. meliloti* 131 were made, but, as expected, no appreciable growth occurred. It may be concluded that there was no synthesis of coenzyme R. This failure of *R. trifolii* 205 to synthesize coenzyme R emphasizes the marked difference be-

tween this essential heat-stable growth substance and the thermolabile growth factor of West and Wilson (1939), which they found to be synthesized by this strain of bacteria.

Assay studies were also made on *R. meliloti* 6, which, as shown in table 1, makes a good growth on such a medium. Of 4 assays of cultures varying in age between 11 and 60 days, three indicated a slight but definitely positive synthesis whereas the fourth was negative. Further data are needed to establish the conditions

TABLE 2
Quantities of coenzyme R synthesized by rhizobia

ASSAY NUMBER	SOURCE OF COENZYME R	QUANTITY ADDED PER 25 ML. MEDIUM		NUMBERS OF R TRIFOLIUM PRODUCED IN 4 DAYS	ASSAY UNITS IN 1 ML. OF ORIGINAL CULTURE
		milliliters	per cent		
I	Medium only	0	0	32	
I	Standard, 1 p.p.m.			500	
I	<i>Dalea</i> 900	0.25	1.0	800	192
I	<i>Dalea</i> 900	0.025	0.1	106	185
I	<i>Dalea</i> 900	0.0025	0.01	52	500*
I	<i>Dalea</i> 901	0.25	1.0	480	112
I	<i>Dalea</i> 901	0.025	0.1	68	90
I	<i>Dalea</i> 901	0.0025	0.01	56	600*
IV	Medium only	0	0	19	
IV	Standard 1 p.p.m.			400	
IV	<i>Dalea</i> 900	0.025	0.1	420	1,002
IV	<i>Dalea</i> 901	0.0125	0.05	240	1,104
IV	<i>Coronilla</i> 81	0.125	0.5	170	75

* These figures are too high, as explained in the text.

most favorable for synthesis and recovery. *R. meliloti* 6 probably synthesizes only as much coenzyme R as it needs and retains most of it in the cell.

Effect of age of culture on the quantity of coenzyme R liberated

In the earlier experiments it was observed that older *Dalea* cultures showed a larger quantity of coenzyme R than did younger cultures. In one of these earlier experiments the amount of coenzyme R present in the medium during the first 7 days was near zero but on the 8th day was definitely positive.

FIG. 1. COENZYME R CONTENT OF CULTURES OF *AZOTOBACTER CHROOCOCCUM* AND *RHIZOBIUM* FROM DALEA AFTER VARYING INCUBATION PERIODS

FIG. 2. COENZYME R CONTENT OF CULTURES OF *RHIZOBIUM* FROM DALEA AFTER VARYING INCUBATION PERIODS

The results of a second experiment, continued for a longer time and including a series of assay values for cultures of *A. chroococcum*, are shown in figure 1. The coenzyme R content of the *Dalea* medium remained low for six days and then showed a marked increase until the cultures were a month old. At this time the organisms grown in 1 ml. of medium had liberated enough growth factor for half maximum growth of *R. trifolii* in 258 ml. of culture medium. The delay in appearance of the growth substance in the *Dalea* medium was not observed with *A. chroococcum*. At the end of 2 days this latter organism had given off 5 assay units of coenzyme R per milliliter and the quantity liberated showed a rapid increase to a maximum on the tenth day. Both *A. chroococcum* and the *Dalea* organism showed marked ability to synthesize the growth substance, the *Dalea* organism being slightly more active than *Azotobacter*. Loss in activity, indicated by the results with *Azotobacter*, is probably only apparent and possibly attributable to incomplete extraction.

A third experiment, showing the effect of age of culture (*Dalea* 900) upon the liberation of synthesized growth substance into the medium, is reported as figure 2. The delay in liberation was less marked in this experiment and the quantity synthesized by *Dalea* bacteria was nearly 3 times as large. At the end of 60 days 1 ml. of *Dalea* culture had produced enough coenzyme R for half maximum growth in 744 ml. of a *R. trifolii* culture. There was no evidence of decomposition on standing, since the maximum value was observed in the oldest culture. Our previous experience has indicated marked stability of the material and decomposition on standing would be quite unexpected.

DISCUSSION

The results reported here definitely establish that two strains of *Dalea* bacteria and *A. chroococcum* actively synthesized coenzyme R, whereas *Rhizobium* from *Coronilla* synthesized it in small quantities. Only one culture of *R. meliloti*, out of several tested, grew well without its addition, and this strain apparently synthesized it in small quantities.

These studies also show that coenzyme R is not liberated from

the *Dalea* organism as readily as from *A. chroococcum*. This is in agreement with our common observations that rhizobia retain their cell substances much more tenaciously than does *Azotobacter*, and autolyze less readily. The amount of coenzyme R synthesized was often greater than would have been expected on the basis of the assumption that the organisms synthesize only the quantity needed for growth.

In closing, it should be emphasized again that the rhizobia studied here represent the unusual so far as response to, and synthesis of, coenzyme R is concerned. All other fast-growing rhizobia studied make good growths only when the growth substance is supplied.

SUMMARY AND CONCLUSIONS

The results of laboratory studies on synthesis of the growth substance, coenzyme R, by a few unusual strains of *Rhizobium* and by *Azotobacter chroococcum* may be summarized as follows:

1. Two strains of *Rhizobium* from *Dalea*, which grow rapidly without additions of coenzyme R, synthesized and liberated it into the medium in comparatively large quantities. Similar results were obtained with a culture from the nodules of *Coronilla varia* except that the quantity synthesized was considerably less.
2. One culture of *Rhizobium meliloti*, which grows on a medium free from coenzyme R, showed a slight synthesis of this material.
3. *Azotobacter chroococcum* was an active synthesizer of the growth substance but the quantity formed was not quite as large as with the *Dalea* organism. The latter liberated it into the medium less rapidly than did *Azotobacter*.
4. Coenzyme R is clearly distinguished by its properties from the thermolabile factor that West and Wilson found to be synthesized by a strain of rhizobia that does not produce coenzyme R.
5. In general, most rapidly-growing rhizobia require additions of coenzyme R to the medium. Those tested that grow well without it synthesize it, and often in amounts far beyond the expected needs of such cells. The present work, therefore, in no sense contradicts our previously reported conclusions concerning the essentiality of coenzyme R.

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