BIOTIN AS A GROWTH FACTOR FOR RHIZOBIA[1]

JOE B. WILSON AND P. W. WILSON

Department of Agricultural Bacteriology, University of Wisconsin

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Although it is established that biotin is a growth factor for most rapidly-growing strains of the rhizobia, whether it is *essential* or *stimulatory* has not been definitely settled. Most investigators imply, though they do not always explicitly state, that they consider it impossible to grow the rhizobia in synthetic media of purified materials unless this factor is added (Allison and Minor, 1938; Clark, 1936; Bjälfve, Nilsson, and Burström, 1939; Steinberg, 1938). Thorne and Walker (1936) and West and Wilson (1939), however, believe that continuous transfer of the rhizobia in certain synthetic media is possible.

The resolution of the disagreement does not appear to be insurmountable as the difference of opinion apparently hinges to a great extent on defining "essential" and "stimulative." If by essential is meant indispensable for growth, few critical experiments are available for test of this point. Most investigators have merely recorded that "little or no growth" is obtained without the specific factor, but whether this means slight growth or actually no growth is not always evident. Unless growth is absent in the initial transfer, continuous culture is necessary in order to demonstrate that reproduction eventually ceases without the factor. On the other hand, if by essential is meant a factor which permits close to maximum development, then biotin must certainly be termed essential. In this paper the convention is adopted that a factor is essential only if it is indispensable for growth and reproduction. If continuous transfer is possible in its absence, then no matter how sparse the growth, the factor

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will not be called essential. If a factor is not essential but its addition results in increased growth, it will be regarded as a growth stimulant.[2]

The positive continuous transfer experiments have been criticized on the ground that a small quantity of biotin was present as an impurity in the ingredients of the alleged biotin-free synthetic media. To determine the validity of this claim, we have reinvestigated the ability of different strains of rhizobia to grow through continuous transfer under rigidly controlled conditions in media specifically treated to render them biotin-free.

EXPERIMENTAL

Methods

The base medium used throughout this work was: K_2HPO_4, 0.500 g.; NH_4Cl, 0.376 g.; $MgSO_4$, 0.200 g.; $CaSO_4$, 0.200 g.; NaCl, 0.100 g.; and a trace of $FeCl_3$; H_2O, 1000 ml.

Growth was measured by means of the Evelyn photoelectric photometer using a 540 filter. A standardization curve was prepared by plotting cell counts made with a Petroff-Hauser counting chamber against corrected galvanometer readings. As a check, such counts were made on at least two tubes following each transfer in order to insure the validity of the conversion curve.

Unless otherwise specified, the initial inoculum was from a culture grown in a liquid medium containing sucrose and 1 per cent yeast-extract. In the early work the inoculum was three drops from the preceding transplant; later this was reduced to one drop. Transfers were made weekly.

Purification of constituents

The magnesium sulfate, calcium sulfate, and sodium chloride were ignited for two hours in small crucibles over a Meeker burner. The dipotassium phosphate and the ammonium chloride

[2] It is emphasized that these particular definitions are used primarily to avoid confusion. Within reasonable limits it is immaterial what shall be defined as essential so long as in a given discussion a consistent terminology is used. See Mueller (1940) for pertinent observations on the dangers of using confused, ill-defined terms in discussing growth factor requirements.

were extracted in a Soxhlet with absolute ethyl alcohol for twenty-four hours; later, these two salts were prepared from their acid and base constituents after these had been thoroughly heated. The ferric chloride was sublimed. The water was distilled, treated with potassium permanganate and silver nitrate, then redistilled in an all-glass still which was thoroughly cleaned with an acid dichromate solution.

All glassware was kept in an acid dichromate solution for at least twelve hours before use. The effectiveness of this method in freeing glassware of biotin was demonstrated by the following experiment: A water solution containing 1.2 mγ of biotin was added to clean, dry culture tubes which were then dried in an oven at 96°C. for twelve hours. Sets of these tubes were treated with acid dichromate solution for twelve hours, or with *superoxol* for twelve hours, or were ignited; a set was kept without treatment as a control. After treatment, 10 ml. of a synthetic culture medium (purified salts plus 0.5 per cent sucrose which had been extracted with absolute ethyl alcohol) were added to each. Following sterilization, all tubes were inoculated with *Rhizobium trifolii* 205, which had been transferred continuously in a synthetic medium of purified constituents. After five days incubation only the untreated biotin tubes showed more growth than did the control tubes of the purified medium.

Because of its biological origin the source of carbon is probably the most likely carrier of biotin. Also, if mere traces of this highly stimulative factor are present in the c.p. compounds used, the comparatively large quantity added might contain sufficient for minimum growth. Several means have been employed to control this crucial factor in the experiments, details of which will be supplied in the appropriate section.

Continuous transfer of cultures with alcohol-extracted sugars as the source of carbon

Kögl and Tönnis (1936) demonstrated that biotin is soluble in absolute ethyl alcohol; therefore, carbohydrates might be freed from this growth factor by use of this solvent. Even if biotin was still present in traces, such purified sugars supplied at differ-

ent levels should cause variation in counts because of difference in biotin content.

Sucrose and glucose (c.p. grades) were extracted with hot absolute ethyl alcohol for 24 hours in an all-glass Soxhlet intermittent extractor. Although these carbohydrates are slightly soluble in hot ethyl alcohol, relatively little sugar is lost during the extraction. After extraction, the sugars were removed from the cone, thoroughly dried on a porous plate, then stored in glassware cleaned with an acid-dichromate solution.

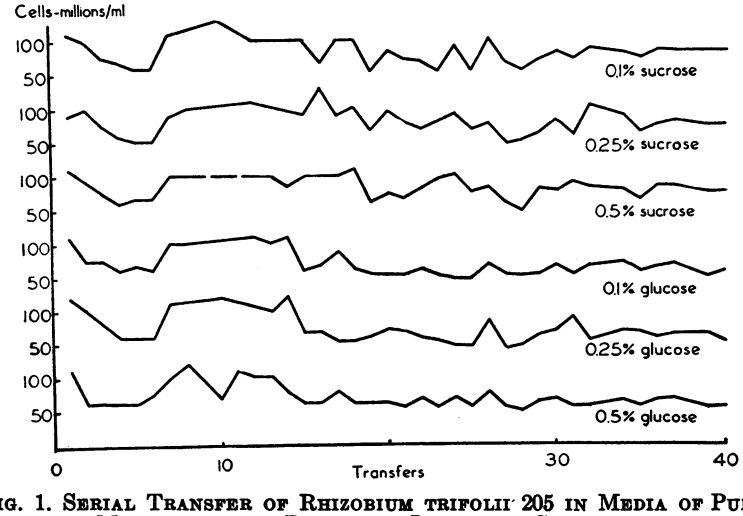

FIG. 1. SERIAL TRANSFER OF RHIZOBIUM TRIFOLII 205 IN MEDIA OF PURIFIED MATERIALS WITH DIFFERENT LEVELS OF CARBOHYDRATE

Series of weekly transfers of *Rhizobium trifolii* 205 and *Rhizobium meliloti* 131 were made in 6 media prepared from the base medium plus 0.1, 0.25, and 0.5 per cent extracted sucrose or glucose. Duplicate tubes of each medium without biotin and a single tube plus 0.0002 γ per 10 ml. of the biotin concentrate described by West and Wilson (1940) were taken.

The data obtained from the weekly transfers are shown graphically in figures 1 and 2. The number of cells in all the media without added biotin varied between 50 to 100 million per ml. and was independent of the level of carbohydrate. If the carbohydrate had contained even traces of biotin, then a significant

difference in the growth obtained in the medium of 0.5 per cent concentration of carbohydrate in comparison with that in 0.1 per cent concentration would be expected. The growth in the three media which contained different levels of sugar plus biotin was essentially the same regardless of the carbohydrate level as would be expected since the number of cells probably depends on the biotin added rather than variation in the concentration of carbohydrate. The cell concentrations varied from 400 to 750 millions per ml. in the glucose media and from 600 to 1000 millions per ml. in the sucrose media.

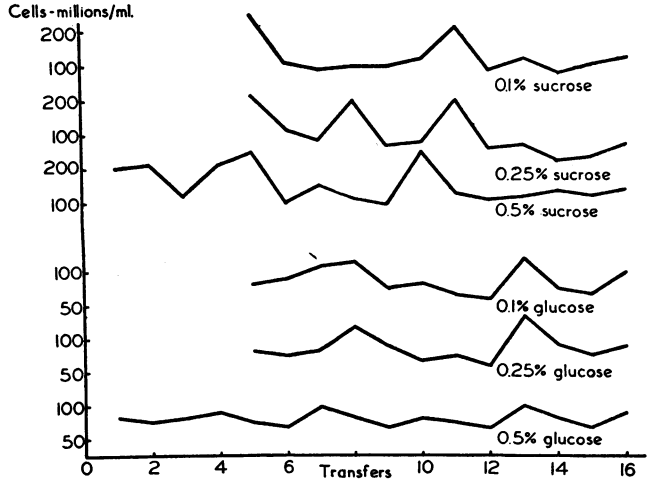

FIG. 2. SERIAL TRANSFER OF RHIZOBIUM MELILOTI 131 IN MEDIA OF PURIFIED MATERIALS

Analysis of the sugars for biotin by the yeast method of Snell, Eakin, and Williams (1940) showed that both the sucrose and glucose contained less than that detectable by the assay, i.e., less than 2×10^{-2} m γ biotin per gram. In the medium containing 0.5 per cent carbohydrate, then, there is less than 10^{-4} m γ of biotin per ml. and in the 0.1 per cent carbohydrate, less than 2×10^{-5} m γ per ml. Through the use of a biotin methyl ester of 75 per cent purity supplied by Kögl a definite response by *R. trifolii* 205 under our experimental conditions required approximately 10^{-3} m γ per ml. of this factor. Therefore, any im-

purities of biotin in the carbohydrate were present in smaller quantities than will stimulate the growth of this strain of rhizobia.

Continuous transfer of R. trifolii 205 using sucrose purified with norite as the source of carbon

Since Kögl and Tönnis (1936) demonstrated that biotin was adsorbed by activated charcoal, Allison and Minor (1939) suggested that traces of this factor may be removed from a solution of carbohydrate by this method. Sucrose extracted with absolute ethyl alcohol was made up to a 10 per cent solution in redistilled water to which was added 5 per cent by weight of norite activated by heating. This mixture was brought to 90°C. and filtered hot to remove the norite. The filtrate was concentrated to a thick syrup by vacuum distillation and transferred to a large beaker using hot absolute methyl alcohol to facilitate the transfer. After heating the alcohol-sugar solution to 64°C. in a water bath, it was kept at 10°C. At the end of 24 hours the alcohol was decanted from the crystallized sugar which was transferred to a porous plate on which it air-dried for several days. The yield of purified sugar was 90 per cent.

R. trifolii 205 was transferred continuously in media using the sucrose purified with norite. The source of the initial inoculum was a culture in its twenty-eighth transfer in the medium which contained sucrose extracted with absolute ethyl alcohol. The data for this series of transfers are presented in table 1. Although the carbohydrate concentration varied from 0.1 per cent to 1.0 per cent, the growth at all levels was not significantly different. This result should exclude the carbohydrate as a source of growth factors for the bacteria growing in the various media. The type of curve for growth in the media which contained sucrose purified with norite plus biotin is identical with that for the media to which no biotin was added; the difference is that growth in the former was ten times that in the latter. In this connection it should be noted that the variation in total numbers from transfer to transfer is independent of either content in carbohydrate or presence of added biotin. The exact amount of growth in the various media of purified materials probably depends on factors

not entirely constant from week to week, e.g., oxygen content of medium, viability of culture from last transfer, distribution of organism in medium (gum production alters this).

Continuous transfer of cultures with glycerol as the source of carbon

As added evidence that the growth in the synthetic media did not arise from a minute undetectable impurity of biotin, a source of carbon, synthetic glycerol, in which the presence of this growth factor would be highly improbable was used. Synthetic glycerol was prepared by the method of Wagner (1888) which depends on the oxidation of allyl alcohol with potassium permanganate.

TABLE 1

Growth in synthetic media using sucrose purified with norite as source of carbon

TRANSFER NUMBER	SUCROSE			PLUS BIOTIN
	0.1 per cent	0.4 per cent	1 per cent	
1	45.0	45.0	45.0	600
2	62.0	67.5	60.0	700
3	79.0	82.5	82.5	800
4	100.0	67.5	120.0	1000
5	72.0	75.0	73.0	800
6	60.0	56.5	60.0	800
7	75.0	75.0	76.0	800
8	100.0	95.0	82.5	1120
9	90.0	92.5	82.5	1000
10	95.0	87.5	87.5	1000

Counts in all tables represent millions of cells per ml.

R. trifolii 205 was transferred weekly for eight weeks in a medium containing one per cent c.p. glycerol. The initial inoculum was from a culture in its twenty-sixth transfer in the medium which contained sucrose extracted with ethyl alcohol. The amount of growth was about the same as when the carbohydrates were used, although growth in the glycerol was more stringy than in the media containing sugar. The ninth transfer in this series was made into a medium containing synthetic glycerol as the source of carbon; six transfers were made therein. As is shown in figure 3, the level of growth was essentially the same as when c.p. glycerol (or purified sugars) was used in the media. Cultures

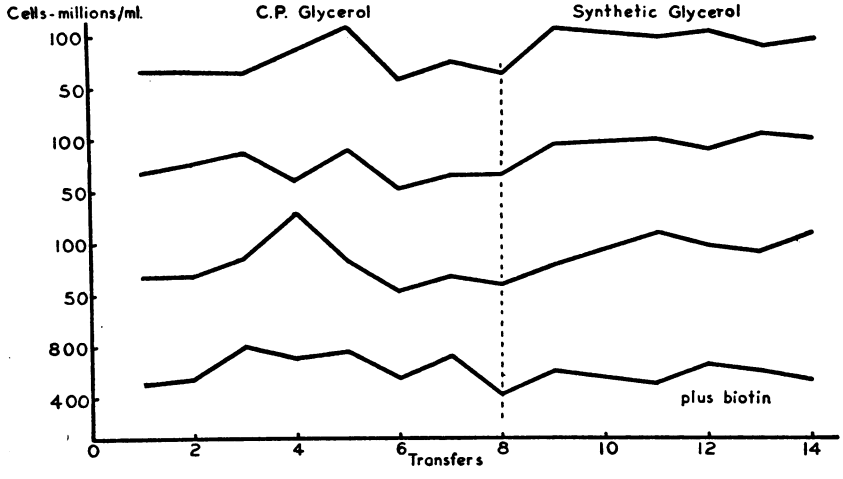

FIG. 3. SERIAL TRANSFER OF RHIZOBIUM TRIFOLII 205 IN MEDIA OF PURIFIED MATERIAL CONTAINING GLYCEROL AS THE SOURCE OF CARBON
The three upper curves are the values for triplicate cultures without added biotin

TABLE 2

Growth in media using synthetic glycerol as source of carbon

	TRANSFER NUMBER					
	1	2	3	4	5	6
R. meliloti 131						
Synthetic medium	40	100	75	100	110	90
	100	120	50	105	90	110
	100	120	95	90	120	85
Plus biotin	650	750	500	600	550	600
R. trifolii 209						
Synthetic medium	40	45	70	50	70	90
	70	60	50	70	45	50
	60	110	60	55	*	77
Plus biotin	650	500	550	500	500	750
R. leguminosarum 311						
Synthetic medium	80	60	80	85	90	80
	40	*	50	40	*	*
	50	70	52	52	50	110
Plus biotin	500	500	550	550	500	600

* Too stringy to count.

of *R. meliloti* 131, *R. trifolii* 209, and *Rhizobium leguminosarum* 311 were carried through six weekly transfers in media with synthetic glycerol as the carbon source. The inocula for transfer number one were from cultures of the various organisms after five transfers in media containing sucrose extracted with absolute ethyl alcohol. The data in table 2 show that the amount of growth was essentially the same for each strain during these transfers.

DISCUSSION

The chief criticisms directed toward the claims of continuous transfer of rhizobia in the absence of biotin have been: (1) biotin is present as an impurity in the constituents of the medium; (2) the growth factor is transferred in the inoculum. Allison and Minor (1938), for example, state, "We have not attempted to determine whether these organisms can be kept alive and growing slowly for a large number of successive transfers on media containing no accessory growth substance. It would, in fact, be difficult to perform this experiment because of the difficulty in eliminating all traces of the growth factor from the medium, and because the inoculum itself contains the substance."

With respect to the first objection, we believe that the experiments reported in this paper demonstrate that certain strains of the rhizobia[3] are capable of growth and continuous transfer in biotin-free media. If traces of biotin are present in these media, its concentration is less than 10^{-4} m γ per ml. which is less than the quantity of this factor necessary for detectable stimulation of the growth of these strains under the experimental conditions used. It is not clear how the second objection affects the conclusions. Obviously, the biotin carried in the first transfer from the yeast-extract medium plays a role, but it would be diluted some 60-fold with each transfer so that its effect would be limited to the first few sub-cultures. Aside from this transient initial

[3] Our experience with different strains of the rhizobia indicates that a few give practically normal growth in the absence of added biotin, a few will not develop, but the majority respond as do the strains described in this paper.

effect, carrying over of growth factors through use of a large inoculum is not objectionable if these have been synthesized by the organism itself. Such a procedure may possess definite technical advantage in that it favors initiation of growth in an unfavorable medium.

The results of these experiments were in a certain sense unexpected since, as far as we know, analogous findings with other microorganisms have not been reported. Most species either synthesize a necessary growth requirement or will not grow unless it is in the medium. Some species will synthesize a requirement (and may even provide an excess) if a small quantity of the factor is furnished to allow initiation of growth; others may be "trained" to synthesize a factor, e.g., thiamin by the propionic acid bacteria (Silverman and Werkman, 1939). Most strains of the rhizobia resemble the latter two groups but differ in the important respect that the growth in the absence of added biotin never exceeds about 10 per cent of that attained when it is available in optimum concentration. In spite of continuous transfer on purified synthetic media for over a year, *R. trifolii* 205 never varied markedly in its rate or extent of growth.[4]

Two explanations are suggested for this rather surprising result: (1) the organism synthesizes biotin to a limited extent, but the ability is so feeble that the maximum population is only a fraction of that on a favorable medium; (2) growth and metabolism proceed at a greatly reduced level in the complete absence of biotin. Attempts to demonstrate synthesis of biotin by *R. trifolii* 205 have so far been unsuccessful. The assay method used is based on the observation of West and Wilson (1940) that the growth of *R. trifolii* 205 is proportional to the level of biotin.

[4] In this connection the "normality" of the strains after cultivation on the biotin-free media is of interest. *R. trifolii* 205 which had been transferred continuously in media containing synthetic glycerol or sucrose purified with absolute alcohol gave a typical reaction when transferred to litmus milk. Likewise its capacity for infecting red clover plants and fixing nitrogen in association with this host was unaltered as judged by greenhouse experiments. A suspension of resting cells of this strain grown in the purified synthetic medium with alcohol-extracted sucrose as a source of carbon (inoculum in 37th transfer on this same medium) had a $Q_{O_2}(N)$ of 421 on glucose, a value in good agreement with those reported by Burris and Wilson (1939) for this species.

Essentially, it consists of washing 6 colonies into an Evelyn tube and reading the turbidity in the photoelectric colorimeter. A standardization curve prepared from a sample of Kögl's biotin indicated that under the conditions of the test the log of the biotin concentration was a linear function of the log of the turbidity reading. Estimation of the constants of this line by the usual statistical procedure furnished a convenient equation for calculation of biotin content from the turbidity.

In the experiments with *R. trifolii*, cells from only 500 ml. of culture medium were used. Recently an assay was undertaken of biotin synthesis by *R. leguminosarum* HX, a strain which is not stimulated by this factor. Eight liters of the purified medium (2 per cent sucrose) was inoculated with HX, aerated for 8 days, then the entire culture hydrolyzed with H_2SO_4 and evaporated to 1500 ml. *in vacuo*. Biotin was adsorbed on activated charcoal, eluted with ammonia-acetone, and assayed by the *R. trifolii* method. One microgram was found in the entire culture, a surprisingly low value which may have been due to the methods used for recovery. Nevertheless, such a quantity would be sufficient to meet the needs of the rhizobia for maximum growth. Obviously, if biotin synthesis is to be detected by strains such as *R. trifolii* 205, large volumes of culture and more satisfactory methods of recovery will be necessary.

SUMMARY

1. The preparation of synthetic media containing salts which have been purified by special techniques is described.

2. Continuous growth in serial transfer of *Rhizobium trifolii* 205 and *Rhizobium meliloti* 131 in media containing the purified salts plus either glucose or sucrose extracted with absolute ethyl alcohol was demonstrated. The growth in these media was independent of the level of sugar or the number of transfers. The same was true for *R. trifolii* 205 transferred in a medium in which the alcohol-extracted sucrose was further purified by treatment with norite.

3. Continuous transfer of *R. trifolii* 205, *R. meliloti* 131, *Rhizobium leguminosarum* 311, and *R. trifolii* 209 without decrease in

growth was obtained in a medium containing the purified salts plus synthetic glycerol. In all the continuous transfer experiments on the purified media, the maximum growth was only about one-tenth that obtained with a complete medium, i.e., one to which the optimum quantity of biotin was added.

4. The continuous transfers on the purified media did not affect the ability of *R. trifolii* 205 to infect and fix nitrogen in association with the clover plant. Also, respiratory activity of this culture as measured by the $Q_{O_2}(N)$ on glucose was unchanged.

5. It is concluded that strains of the rhizobia can be divided into three groups based on their biotin requirements: (1) in the absence of biotin most strains grow very poorly and reach a population of about one-tenth of the maximum, but continuous serial transfer is possible; (2) a few strains attain practically maximum growth in its absence; (3) a few strains are unable to grow unless biotin is supplied.

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