GROWTH FACTOR REQUIREMENTS OF THE ROOT NODULE BACTERIA[1]

P. M. WEST AND P. W. WILSON

College of Agriculture, University of Wisconsin, Madison

Received for publication August 3, 1938

It is known that yeast or plant extracts markedly stimulate the growth of the root nodule organism in media of purified ingredients. An essential growth factor or superior source of nitrogen has been invoked to explain the results, but neither theory has a broad experimental basis. The results of Allyn and Baldwin (1930) suggest that the extracts may affect favorably the oxidation-reduction potential of the medium. Allison, Hoover and Burk (1933) report that a specific co-enzyme for respiration of rhizobia can be extracted from commercial sucrose, azotobacter cultures and various plant or animal tissues. This extract markedly stimulates the growth of rhizobia in a medium of purified ingredients. Hoover and Allison (1935) state, "the growth of certain species, on the usual synthetic (sugar, inorganic salts, and nitrate) medium is negligible if pure ingredients are used, and that the addition of a small amount of growth factor is essential."

Thorne and Walker (1936) also found that an extract of azotobacter (or other substances) was able to stimulate growth of several species of rhizobia in a medium of highly purified materials but did not regard it as essential for growth. By substituting ammonium chloride or asparagin for potassium nitrate, they grew four species in repeated transfer for more than two months with no appreciable decrease in rate of growth of the organisms. Addition of reducing substances to Allison and

[1] Herman Frasch Foundation in Agricultural Chemistry, Paper No. 183. This work was supported in part by a grant from the Wisconsin Alumni Research Foundation.

Hoover's synthetic medium improved it, indicating that the potassium nitrate in this medium poised it at an oxidation-reduction potential too high for continued growth.

Clark (1936) was able to secure only very slight or no growth in the absence of added organic stimulants. Nilsson, Bjälfve and Burström (1938a) observed no growth in the synthetic medium of Allison and Hoover even if NH_4Cl or asparagine were used as the source of nitrogen. Likewise, the addition of reducing substances failed to favor growth; normal growth occurred only in the presence of yeast extract.

In view of the contradictory findings, it seemed desirable to investigate further the claims that certain unknown substances are necessary for growth of rhizobia, and to determine what factors are important for successful continuous transfer of the organisms in a synthetic medium.

EXPERIMENTAL

To avoid confusion in interpretation of results, the base medium adopted was that of Allison and Hoover (1934), consisting of mineral salts, one per cent mannitol, and 200 parts per million nitrogen as potassium nitrate. *Rhizobium trifolii*, Wisconsin strain 205 was used as the test organism.

The "coenzyme R" preparations employed were made from cultures of *Azotobacter vinelandii* according to Hoover and Allison's procedure (1935). In preliminary work a Petroff-Hauser direct count was made on fluid cultures of the organism to determine growth. Later, the growth response was measured by turbidity of fluid cultures as indicated by the Evelyn electrophotometer or development of giant colonies on agar.

Factors influencing rate of growth of R. trifolii in base medium

1. *Yeast extract and azotobacter extract.* A water extract of yeast (Fred, Baldwin and McCoy, 1932) and Allison's azotobacter extract were selected as representative of the numerous natural preparations known to stimulate the growth of *R. trifolii*. Tubes containing from 5 to 500 parts per million of these extracts were inoculated with a standard loop (approximately 500,000 cells per 10 ml. tube) from a 24-hour 10-per-cent yeast water

culture. The maximum stimulation occurred in the presence of 25 to 50 parts per million of either substance (table 1). It was noted that azotobacter extract consistently failed to replace, completely, yeast extract in fluid culture (fig. 1), while both were equally stimulative to growth on agar (Plate 1). In fluid culture, growth in the base medium was, in all cases, relatively slight, and failed completely after three or four transfers. From these data alone, one might conclude that certain stimulative organic enrichments are required for the prolonged growth of the organism in laboratory media. However, subsequent experiments demonstrate the need for modification of this opinion.

TABLE 1

Comparative stimulative effects of "coenzyme R" preparations and yeast extract
Counts in millions per ml.

MEDIA	2 DAYS	3 DAYS	4 DAYS
Coenzyme R, 5 ppm	92	206	285
Yeast extract, 5 ppm	160	385	586
Coenzyme R, 10 ppm	70	200	308
Yeast extract, 10 ppm	318	635	720
Coenzyme R, 25 ppm	75	280	341
Yeast extract, 25 ppm	485	856	840
Coenzyme R, 50 ppm	63	312	322
Yeast extract, 50 ppm	580	830	836
Control	<5	15	65

2. *Reducing substances.* Since the base medium was oxidizing in nature, the possibility of increased growth of the bacteria in the presence of reducing substances was investigated. Tubes containing sodium sulfite, sodium nitrite and sodium thioglycollate were inoculated from a twenty-four-hour culture as before. Table 2 indicates the amount of growth obtained in cultures supplied with optimal quantities of each compound. These substances all improved growth in fluid medium, but, as shown in Plate 1, they are without effect on agar. The use of mass inocula

apparently overcomes, within reasonable limits, effects of alteration in oxidation-reduction potential.

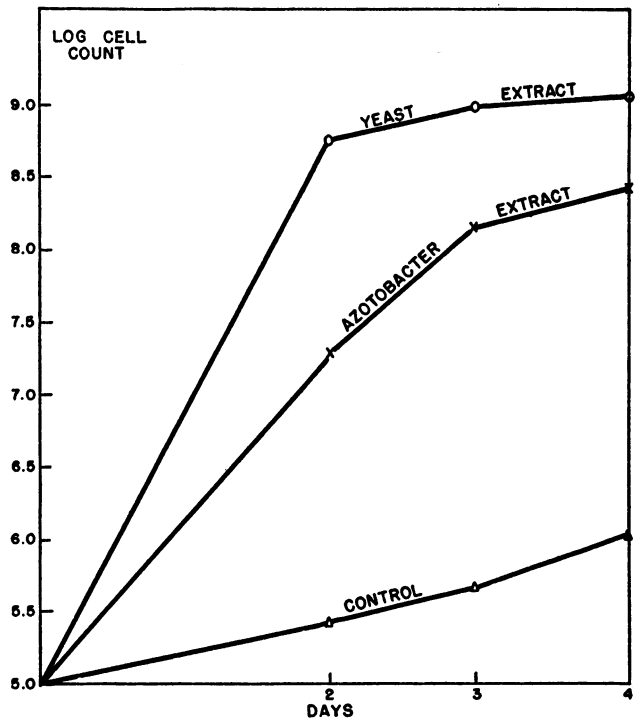


FIG. 1. Comparative stimulative effects of Azotobacter and Yeast extracts (50 ppm.) on growth of *Rhizobium trifolii* in fluid culture.

TABLE 2

Comparative stimulative effects of "coenzyme R" and reducing substances on growth of Rhizobium trifolii

Counts in millions per ml. Inoculum from 24-hr. culture

MEDIA	2 DAYS	3 DAYS	4 DAYS
Sodium nitrite, 25 ppm	72	245	318
Sodium sulfite, 25 ppm	75	188	345
Sodium thioglycollate, 10 ppm	85	390	416
Coenzyme R, 50 ppm	75	280	341
Control	<5	15	75

Figure 2 shows the relative stimulative abilities of thioglycollic acid and azotobacter extract, both at a concentration of 10 ppm.

At this concentration the stimulation of growth in fluid culture, due to azotobacter extracts, is of the same order as that effected by reducing substances. However, it is clear from Plate 1 that the former contains material of benefit to the organism for reasons other than its ability to alter the oxidation-reduction potential of the medium.

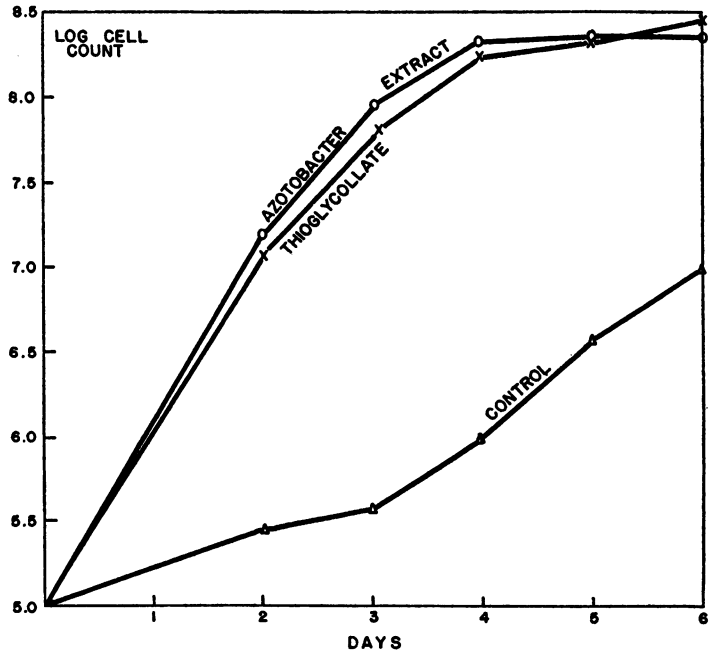


FIG. 2. Comparative stimulative effects of Azotobacter extract and thioglycollic acid (10 ppm.) on growth of *Rhizobium trifolii* in fluid culture.

Continuous growth of Rhizobium trifolii in synthetic media

Results of the foregoing experiments suggested the possibility of growing *R. trifolii* in fluid culture in the entire absence of unknown organic enrichments, over an indefinite period, providing the media were at a favorable oxidation-reduction potential. For the purpose of this experiment, the base medium enriched with 10 ppm. thioglycollic acid was selected. The original inoculation consisted of approximately 500,000 cells from a 24-hour 10-per-cent yeast water culture. Loop transfers were made every three days, and after thirty-four successive

transfers in this medium, the organisms were growing at the same rate as in the first transfer (table 3). After this number of transfers, it was concluded that any objection that growth could be due to activators from the original inoculum, was beyond consideration. Mannitol, which had been continuously extracted for 24 hours with absolute ethyl alcohol to remove any soluble activator, was used in a parallel experiment with identical results. Other reducing substances previously studied behaved similarly to thioglycollate. Purified glucose or sucrose could be substituted for mannitol with essentially the same results. Inoculation of a suitably reduced basal medium from either a yeast-extract enriched culture, or one grown in the absence of

TABLE 3

Growth of Rhizobium trifolii through thirty-four successive transfers in synthetic medium (base medium plus thioglycollic acid—10 ppm.)

Counts in millions per ml.

TRANSFER	2 DAYS	3 DAYS	4 DAYS
Original	86	295	382
1st transfer	64	170	284
2nd transfer	75	292	367
18th transfer	94	268	412
34th transfer	80	246	395

yeast extract for over thirty transfers, gave the same response. Evidently, then, the small amount of yeast extract carried in the inoculum from a 10-per-cent yeast-water culture does not influence the growth. In the presence of greater amounts of yeast extract, however, there is, as has been previously shown, a considerable stimulation beyond the growth induced by reducing substances.

It is concluded from these data, that *R. trifolii* synthesizes all the organic substances essential for its growth from the simple ingredients of the properly reduced base medium; and that although various tissue extracts are stimulative to growth, they are not required.

Growth factors synthesized by Rhizobium trifolii

Further study suggested that continuous transfer of *R. trifolii* in the base medium, properly reduced by thioglycollate, is dependent upon some highly active material, synthesized by the growing culture and transferred in sufficient amount with a loop inoculum (.005 ml.) to stimulate growth initiation in the new medium. Evidence for this view was the observation that cells removed from the medium in which they had grown, and washed free of their metabolic products, produced much less growth when inoculated into fresh media than did cells not washed. This separation of the organisms from the activators they produce is accomplished either by aseptic centrifugation of a fluid culture, or by suspending growth from the surface of an agar slope in fresh medium. An active culture filtrate, free from cells, can be obtained readily by ultra-filtration.

To determine the effect of culture products on the growth of *R. trifolii*, the following were used as inocula:

(a) A 48-hour culture growing in 10-per-cent yeast extract medium.
(b) A suspension of cells from a 10-per-cent yeast-extract agar slope in fresh 10 per cent yeast extract medium.
(c) A suspension of cells from a slope as in (b) in a sterile filtrate from culture (a).

All inocula were adjusted to contain the same number of cells. The results of this experiment (table 4) indicate that the bacteria during their growth synthesize some highly active material, important in growth initiation, which can be separated from the culture by Berkefeld filtration. That this effect is independent of the presence of yeast extract in the medium is shown in figure 3, which demonstrates rates of growth when the inocula used were:

(a) A growing culture, free of yeast or other extracts for 30 transfers.
(b) Cells from (a) centrifuged and resuspended in the fresh basal medium.

These examples, which are typical of a number of similar tests, illustrate the dependence of the organism on activators synthe-

TABLE 4

Influence of filtrate from growing culture on initiation of growth

Counts in millions per ml.

MEDIUM	INOCULUM	2 DAYS	3 DAYS	4 DAYS
Basal medium + 10 ppm. thioglycollate	*A*. 48-hour culture,	90	390	445
Basal medium + 15 ppm. thioglycollate	growing in 10 per	55	384	395
Basal medium + 20 ppm. thioglycollate	cent yeast ext.	72	341	380
	medium			
Basal medium + 10 ppm. thioglycollate	*B*. Suspension of	5	15	75
Basal medium + 15 ppm. thioglycollate	cells in sterile 10	5	5	83
Basal medium + 20 ppm. thioglycollate	per cent yeast	5	5	35
	ext. medium			
Basal medium + 10 ppm. thioglycollate	*C*. Suspension of	106	390	428
Basal medium + 15 ppm. thioglycollate	cells in sterile	93	374	410
Basal medium + 20 ppm. thioglycollate	filtrate from *A*.	50	240	325

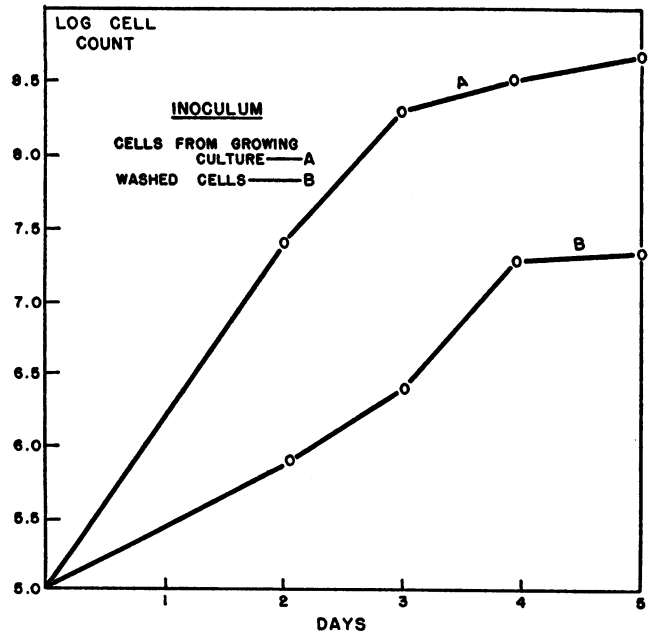


FIG. 3. Effect of type of inoculum on growth of *Rhizobium trifolii* in base medium plus thioglycollate.

sized during the period of active growth of the cells for growth initiation of small inocula. In the absence of this active material, growth is so slight that continued transfer is impossible.

Influence of heat on growth factor synthesized by R. trifolii

The heat stability of the stimulative factor liberated in growing cultures was determined by subjection of the Berkefeld filtrates obtained therefrom to a temperature of 100°C. Heating was carried out at neutrality and with the addition of 10 per cent N/1 sodium hydroxide or hydrochloric acid. After heating for periods of 30 minutes and one hour, the tubes were immediately cooled and adjusted to neutrality with sterile acid

TABLE 5

Influence of heat on stimulative properties of filtrates from growing cultures

Counts in millions per ml.

MEDIUM USED FOR SUSPENDING INOCULUM	2 DAYS	3 DAYS	4 DAYS
Filtrate untreated	105	340	385
Filtrate heated at pH 6.8, 30 min.	<5	20	74
Filtrate heated at pH 6.8, 60 min.	<5	17	65
Filtrate heated with N/10 NaOH, 30 min.	<5	32	86
Filtrate heated with N/10 NaOH, 60 min.	7	23	51
Filtrate heated with N/10 HCl, 30 min.	18	25	72
Filtrate heated with N/10 HCl, 60 min.	7	30	90
Control	<5	25	84

or alkali. One drop of a heavy suspension of cells was added to each differently treated filtrate, so that an inoculum of one loop taken from it carried approximately 200,000 cells. Results of this experiment showed that destruction of the growth-stimulating properties of the filtrate had occurred in every case (table 5). This is in marked contrast to the heat stability of the stimulative ingredients of yeast or azotobacter extract.

Activity of growth factor synthesized by R. trifolii

A quantitative study of the activity of *Rhizobium* culture filtrates in stimulating growth of the same organism was under-

taken, using a modified method for growth measurement. In this and subsequent experiments, twelve-ounce bottle plates were sown with giant colonies (see Plate 3). Usually, 11 or 12 colonies were sown to insure obtaining growth of at least 10. After 72 hours' incubation at 28°C., growth from ten colonies was removed and suspended in 10 ml. distilled water, and the turbidity read in an Evelyn electrophotometer. From a standardization curve the electrophotometric readings could be converted directly into numbers of cells. Parallel experiments were run, using fluid cultures as before, with the exception that growth was determined in the electrophotometer after 48 hours, instead of by direct count.

It was found more convenient in these studies to employ autolyzed cultures of the organism (8 to 10 weeks old) as a source of the growth factor. After the cells had settled, the supernatant medium was removed and, by pasteurization at 80°C. for 5 minutes, was rendered sterile without any destruction of stimulative activity. These culture autolysates were prepared from cultures grown in purely synthetic media for over ten transfers.

To determine the potency of the culture autolysate as a stimulant for *R. trifolii* it was tested at concentrations of .0001, .001, .01, .1 and 1.0 ml. per 10 ml. of medium. Additions were made both before and after autoclaving, to compare the effects of heated and unheated autolysate at various concentrations. At the same time, an autolysate of *Azotobacter chroococcum* prepared in the same manner, was tested to determine whether or not this organism also synthesized the factor.

The results (fig. 4) indicated that the *Rhizobium* autolysate was most active when added to the medium in concentrations between .001 and .01 ml. per 10 ml. of medium. The optimum concentration of autolysate (which is approximately equal to the amount of medium carried from an old to a new culture in a loop inoculation) is the same whether determined by plate or fluid cultures. Of interest, is the observation that additions beyond 0.1 per cent resulted in loss of stimulative action; this is

a peculiarity which assisted in identifying the active material. Autoclaving inactivated the autolysate at all concentrations.

Azotobacter autolysate apparently contains very little, if any, of the heat-labile *Rhizobium* factor, but does cause response at higher concentrations due to the presence of a heat stable substance, presumably that described by Allison and Hoover.

Although *Azobacter* did not synthesize appreciable quantities of the *Rhizobium* factor, it was thought possible that a more

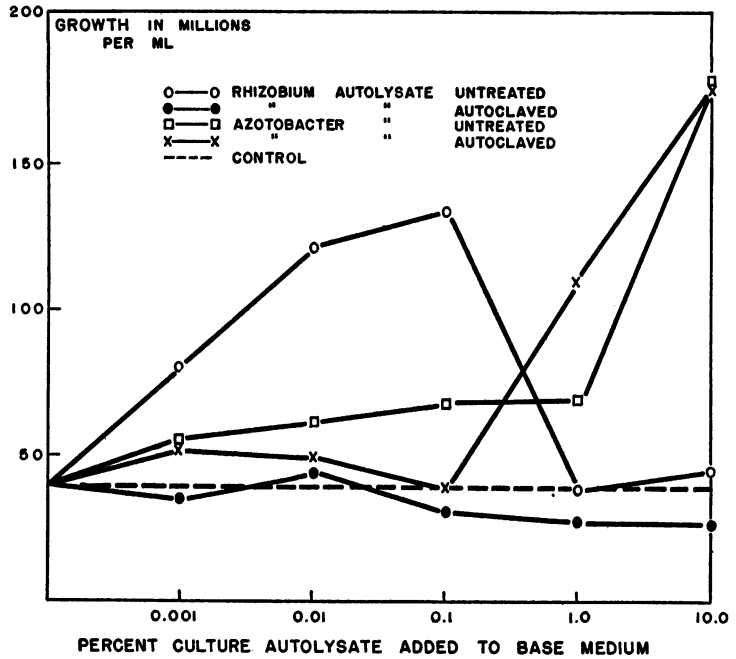


FIG. 4. Effect of heat on growth stimulants synthesized by *Rhizobium trifolii* and *Azotobacter chroococcum* determined by the giant colony method.

closely related species such as *Phytomonas tumefaciens* might do so, unless the phenomenon were strictly confined to the nodule organisms. The effects of *Phytomonas* autolysate on the growth of *R. trifolii* as determined by the giant colony technic are shown in figure 5. It appears that *Phytomonas tumefaciens* synthesizes the stimulative material in approximately the same quantity as do the rhizobia themselves.

Thiamin and riboflavin as growth factors for R. trifolii

From certain of the properties already discussed, it appeared possible that the growth activator synthesized by the rhizobia might be some known constituent of the vitamin B group, such as thiamin or riboflavin (or the unstable complexes which these substances form in the living cell). In order to test this hypothesis, inocula for both giant colony and fluid cultures were employed consisting of washed cells, which permitted detection of the factor or factors which could replace the material syn-

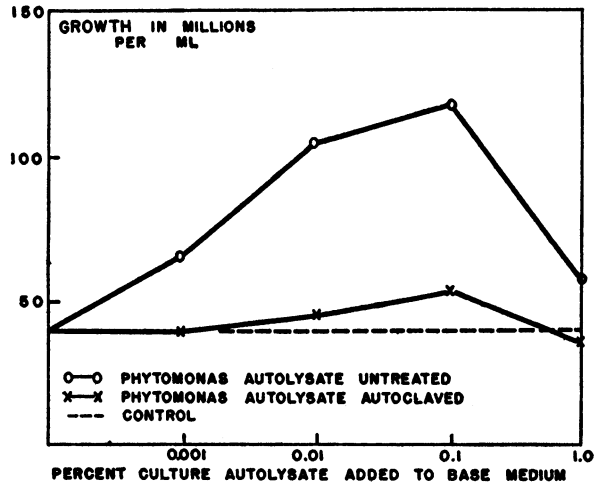


FIG. 5. Synthesis of *Rhizobium* factor by *Phytomonas tumefaciens*

thesized by the organisms. Pure thiamin and riboflavin were used in these experiments.

In preliminary studies, both of these substances were found to possess marked activity, but the organisms exhibited great sensitivity to only slight alterations in the concentration of the activator. The activity of these vitamins appeared to be limited to a narrow range of concentrations around 0.1 microgram per ml. This range of activity was studied more closely with the results, for thiamin, shown in Table 6. A similar experiment with both thiamin and flavin is shown in figure 6. In all cases it will be observed that as the concentration of either vitamin

increases beyond the optimum its stimulatory effect decreases. This is of interest in view of the fact that the factor or factors synthesized by the rhizobia possess the same peculiarity. This effect is shown clearly in the accompanying Plates 2 and 3.

If thiamin and flavin are the actual substances synthesized by *R. trifolii*, it would be expected that they would not give further stimulation of growth if the inoculum were taken direct from a growing culture. This was found to be the case (see table 6). In the presence of sufficient of the activator, further additions

TABLE 6

Influence of inoculum on response of Rhizobium trifolii to vitamin B_1 (thiamin)

Counts (millions per ml.) on 48-hour fluid cultures calculated from electrophotometer turbidity measurements

MEDIA, VITAMIN B_1	INOCULUM	
	Washed cells	Growing culture
micrograms / ml.		
0.2	65	161
0.18	81	149
0.16	140	114
0.14	141	127
0.12	150	123
0.10	155	110
0.08	147	116
0.06	106	116
0.04	64	155
0.02	50	147
0.00 (control)	43	145

had either very little stimulative influence, or actually depressed growth. The fact that vitamin B_1 has very little effect when inocula are from actively growing fluid cultures has already been reported by Laird and West (1938).

From these and other experiments which gave similar results, it was concluded that either thiamin or flavin alone can replace, at least in part, the culture autolysate, thus enabling washed cell suspensions to grow nearly as readily in the reduced medium as cells inoculated directly from an actively growing culture.

Synthesis of vitamin B_1 and flavin by R. trifolii

Since thiamin and flavin were strongly suspected of being identical with the *Rhizobium* factor, it became necessary to determine the ability of the organism to synthesize these vitamins. For this purpose, a very sensitive quantitative method for assaying vitamin B_1 was developed, based on Knight's finding (1937) that in appropriate media, the growth of *Staphylococcus aureus* is proportional to the amount of vitamin B_1 present.

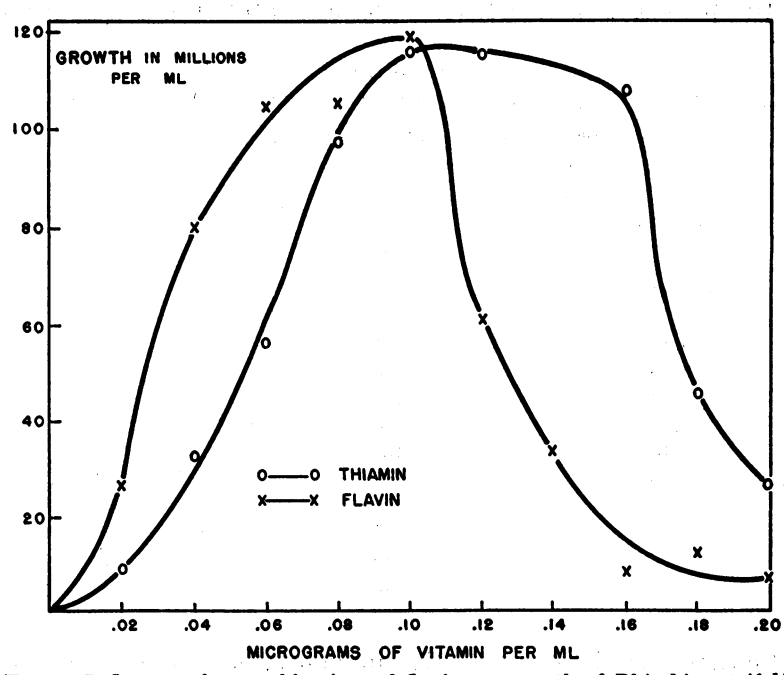


FIG. 6. Influence of pure thiamin and flavin on growth of *Rhizobium trifolii*

Details of this procedure are reported elsewhere (West and Wilson, 1938). Results of analyses of *R. trifolii* cultures grown on a synthetic, vitamin-free medium indicated an average of 19.6 micrograms of vitamin B_1 per gram of dry cells. This relatively high value indicates that the vitamin B_1 content of *R. trifolii* cells closely approximate that of yeasts.

The flavin content of *R. trifolii* was estimated by the method developed by Snell (unpublished data) involving growth re-

sponse and acid production by *Lactobacillus casei*. An average from four separate determinations showed the flavin content of *Rhizobium* cells to be 0.370 microgram per milligram dry cells. These cells are therefore rich in flavin, since the *Clostridium butyricum*, reported by Warburg and Christian (1933) to be high in flavin, contained only .09 microgram per milligram.

Since *R. trifolii* synthesizes thiamin and flavin in such appreciable amounts, it would appear that these substances must be of great importance in the metabolism of the organism. Possibly, one of the reasons for the existence of the lag phase in *Rhizobium* cultures is the necessity for synthesis of sufficient of these stimulants before multiplication can occur. Therefore, if the inoculum be washed, and the cells deprived of most of these growth factors which would ordinarily be carried from the previous culture, the lag is prolonged, in many cases indefinitely. The addition of vitamin B_1, flavin or culture filtrate overcomes this inactivity.

Nature of the growth factor synthesized by Rhizobium trifolii

From a comparison of the thiamin and flavin contents of the autolysates from *Rhizobium trifolii* cultures and the amounts of these vitamins which must be added in pure form to produce stimulation, it was found that more of either vitamin is required for growth response when added singly than occurs in the bacterial autolysates. There are two possibilities to account for this apparent discrepancy: (*a*) thiamin and flavin in combination may have a greater than additive effect; (*b*) these vitamins may exist as protein or other complexes with greater activity than they possess in the free state. Evidence has been obtained that both of these possibilities may be involved.

Experiments on the combined effects of thiamin and flavin showed that if either vitamin is present in optimum concentration, addition of the other reduces activity. However, if both are present in amounts too small to be effective alone, the combination is stimulative. This interaction of flavin and thiamin is shown in figure 7.

It would seem that thiamin is present in the cell as an unstable

complex since, in assaying autolysates from *Rhizobium* culture for vitamin B_1, it was necessary to heat for 5 minutes (N/10 hydrochloric acid) at 100°C., a treatment liberating the free vitamin but not destroying it. On assaying the unheated autolysate, only 0.3 microgram of vitamin B_1, per gram of dry cells was found, whereas after a short heating the vitamin B_1, was freed (19.6 micrograms per gram).[2] In assaying for flavin it was likewise necessary first to liberate the free substance by heat treatment.

In combined form the vitamins are apparently more stimulative since, following the splitting of these complexes by heat, a

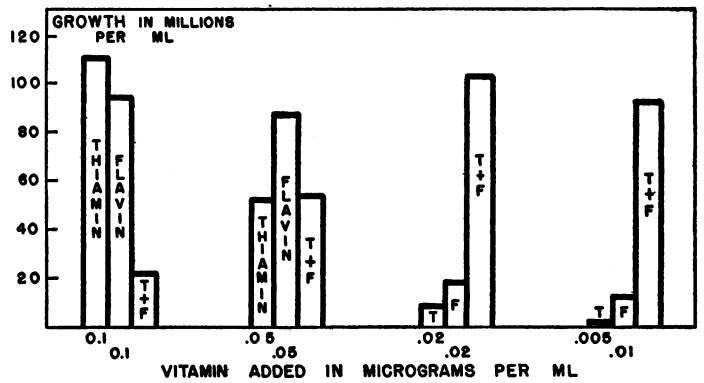


FIG. 7. Effect of combinations of thiamin and flavin on *Rhizobium trifolii* as determined by growth in fluid culture.

treatment which is sufficient to liberate the free vitamin but insufficient to destroy it, the activity of the *Rhizobium* autolysate is largely lost.

For these reasons it can be well understood that either flavin or thiamin alone does not possess the same degree of activity as the culture autolysate which may contain both of these vitamins in correct combination and in more suitable form.

[2] Recent work by Sinclair (1938) indicates that the vitamin B_1 of blood is also in combined form (not co-carboxylase). In assaying B_1 of blood by Meikeljohn's method it is necessary to subject it to a short heating capable of liberating the vitamin but insufficient to cause any destruction of the vitamin itself.

DISCUSSION

The more important results of this investigation may be summarized as follows:

1. Heat- and alkali-labile substances are synthesized by *R. trifolii* itself, which, when present in sufficient amount in the inoculum, permit considerable growth of the organism in a medium of purified ingredients. Even small inocula (200,000 to 500,000 per 10 ml.), washed free of the metabolic products of the surrounding medium, initiate growth satisfactorily if they are suspended in a filtrate from a growing culture. In fluid culture, the total population in this case may reach as high as 250–400 million per ml. in 72 hours. If the culture filtrate is merely added to the synthetic medium, the total population reaches only 100–150 million organisms per ml. in 72 hours. These essential factors occurring in the filtrate from an actively growing culture on synthetic medium can be replaced, at least partially, by the direct addition of riboflavin and thiamin in suitable concentration to the synthetic medium. Washed cells in the presence of the vitamin-enriched medium reach a density of 75 to 150 millions per ml. in 3 days. In the absence of culture filtrate, thiamin, or riboflavin, little or no growth occurs with such an inoculum.

The factor essential in growth initiation of *R. trifolii*, which is synthesized by actively growing cultures of the same organism has been termed the *Rhizobium* factor in order to distinguish it from the heat-stable substance of Allison and Hoover. The *Rhizobium* factor presumably consists of thiamin and riboflavin and possibly some other unidentified compounds, all of which the organism is able to synthesize, *once growth has been initiated.*

The use of the term "essential" as applied to the *Rhizobium* factor may appear to be somewhat arbitrary. It is used in the sense that the factor (or factors) must be present in order that growth may be initiated in a properly poised synthetic medium. Ordinarily, sufficient is carried over in a loop inoculum directly from an actively growing culture, and in this case the essential factor is furnished by the organisms themselves. If the organism is separated from its previously synthesized growth factor, it must be added to the medium, else little or no growth occurs.

But for initiation of growth in the synthetic medium the "essential" factor must be present, whether supplied directly in preparation of the medium or indirectly in the inoculum. In either case, once growth is initiated, the organism can synthesize sufficient of the factor to enable it to be transferred continuously even though maximum populations are not reached.

2. A second factor described by Allison and Hoover is also concerned with the nutrition of *R. trifolii*. This factor, termed "Coenzyme R," stimulates the growth of these bacteria, but does not appear to be essential for their continuous transfer. In the presence of optimum quantities of the factor of Allison and Hoover, the total population may reach 300–750 millions of organisms per ml. in 3 days. "Coenzyme R" differs markedly from the *Rhizobium* factor, and the two can be readily distinguished on the basis of stability and physiological effects.

It is possible that the heat-stable and heat-labile factors may be related as, for example, the factor described by Allison and Hoover may provide an organic nucleus which the organism converts readily into its necessary growth factors. According to Allison and Hoover "Coenzyme R" is concerned with respiration; the established importance of both thiamin and riboflavin to respiratory processes might be used as supporting this suggestion of relationship. However, much more experimentation will be required to investigate this phase of the subject.

3. In the light of these studies on the importance of condition of inoculum in determining whether or not growth will occur in a synthetic medium, it is believed that many of the discrepancies occurring in the literature on this point can be explained. For example, Thorne and Walker were able to obtain continuous transfer in synthetic medium starting with an inoculum from a yeast-extract slant and subsequently transferring one ml. of the 4 day old culture to 25 ml. of fresh liquid medium. Objection to their work on the grounds that they were transferring "Coenzyme R" from the original yeast extract culture can hardly be sustained, since they carried the organisms through 10 to 20 transfers. Under even more rigorous conditions with respect to size of inoculum, we have maintained continuous culture for over 30 transfers with no evidence of loss in reproductive power.

Nilsson, *et al.* (1938a), on the other hand, have recently reported complete inability to obtain growth on a synthetic medium even in the presence of reducing substances; they attribute the success of other workers to faulty technique. Although they do not give much detail regarding the preparation of their inoculum, they do state that they used few cells, obtained by dilution of a culture. This probably corresponds to our inoculum of cells suspended in fresh medium and therefore deficient in *Rhizobium* factor. We are in complete agreement with them that under such conditions no growth results even on inoculation into an adequately reduced medium. And it is also understandable that under such conditions they find vitamin B_1 stimulative (Nilsson, Bjälfve and Burström (1938b), since it is only when an inoculum deficient in the *Rhizobium* factor is employed, that vitamin B_1 induces any marked response.

SUMMARY

1. *Rhizobium trifolii* synthesizes all the organic substances essential for its growth from the simple ingredients of a properly reduced carbohydrate mineral-salts medium.

2. Although various tissue and microbial extracts contain a heat-stable substance (or substances) stimulative to growth, these are not required for successful continued transfer of the organism in a synthetic medium.

3. Continuous transfer of *Rhizobium trifolii* in a synthetic medium is dependent on a factor synthesized by the growing culture which is ordinarily transferred in sufficient amount in the inoculum to stimulate growth initiation.

4. This factor can be separated from cultures of *Rhizobium* by ultrafiltration. It is readily destroyed by heat.

5. Small inocula, washed free of metabolic products of the culture from which they were taken, produce little or no growth in the absence of the *Rhizobium* factor.

6. In the presence of certain specific amounts of thiamin or flavin, either vitamin alone is capable of replacing to some extent the stimulative material synthesized by *R. trifolii*. However, certain combinations of both vitamins are more active than either one used singly.

7. *Rhizobium trifolii* synthesizes appreciable amounts of both vitamin B_1 and riboflavin. These vitamins are present in culture autolysates or filtrates as complexes which are readily destroyed by heat, resulting in decreased activity.

8. Further evidence is presented which indicates that the activity of the metabolic products of *R. trifolii*, in stimulating growth initiation of the same organism, is due, at least in part, to the presence of thiamin and flavin in those products.

REFERENCES

ALLISON, F. E., HOOVER, S. R., AND BURK, D. 1933 A respiration coenzyme. Science **78**, 217-218.

ALLISON, F. E., AND HOOVER, S. R. 1934 An accessory factor for legume nodule bacteria. Jour. Bact. **27**, 561-581.

ALLYN, W. P., AND BALDWIN, I. L. 1930 Effect of the oxidation-reduction character of the medium on the growth of an aerobic form of bacteria. Jour. Bact. **20**, 417-440.

CLARK, D. G. 1936 Physiological studies on *Rhizobium* species. N. Y. Agr. Expt. Sta. (Cornell) Mem. **196**, 3-30.

FRED, E. B., BALDWIN, I. L., AND MCCOY, E. 1932 Root nodule bacteria and leguminous plants. Univ. of Wis. Studies in Sci. No. 5, 1-343.

HOOVER, S. R., AND ALLISON, F. E. 1935 A growth and respiratory factor for certain Rhizobia. Trans. 3rd Internatl. Cong. Soil Sci., **1**, 158-160.

KNIGHT, B. C. J. G. 1937 The nutrition of *Staphylococcus aureus*; nicotinic acid and vitamin B_1. Biochem. Jour. **31**, 731-737.

LAIRD, D. G., AND WEST, P. M. 1938 The influence of bios on *Rhizobium trifolii*. Canad. Jour. Research **16C**, 347-353.

NILSSON, R., BJÄLFVE, G., AND BURSTRÖM, D. 1938a. Über Zuwachsfaktoren bei *Bact. radicicola*. Lantbruks-Hogskol. Ann., **5**, 291-322.

NILSSON, R., BJÄLFVE, G., AND BURSTRÖM, D. 1938b. Vitamin B_1 als Zuwachsfaktor für *Bact. radicicola*. Naturwissensch. **26**, 284.

SINCLAIR, H. M. 1938 The state of vitamin B_1 in blood. Chem. and Indus. **57**, 471.

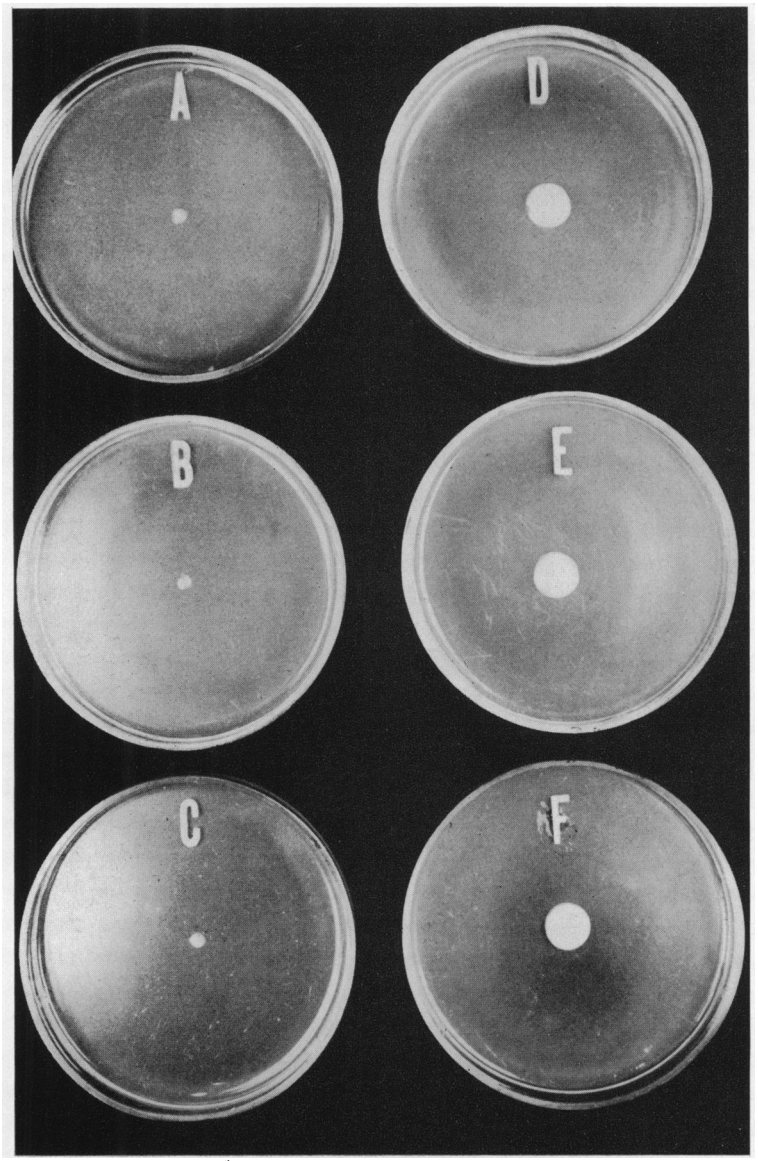
THORNE, D. W., AND WALKER, R. H. 1936 Physiological studies on Rhizobium. VI. Accessory factors. Soil Sci. **42**, 231-240.

WARBURG, O., AND CHRISTIAN, W. 1933 Über das gelbe Ferment und seine Wirkungen. Biochem. Ztschr. **266**, 377-411.

WEST, P. M., AND WILSON, P. W. 1938 Biological determination of vitamin B_1 (thiamin) in *Rhizobium trifolii*. Science **88**, 334-335.

PLATE 1

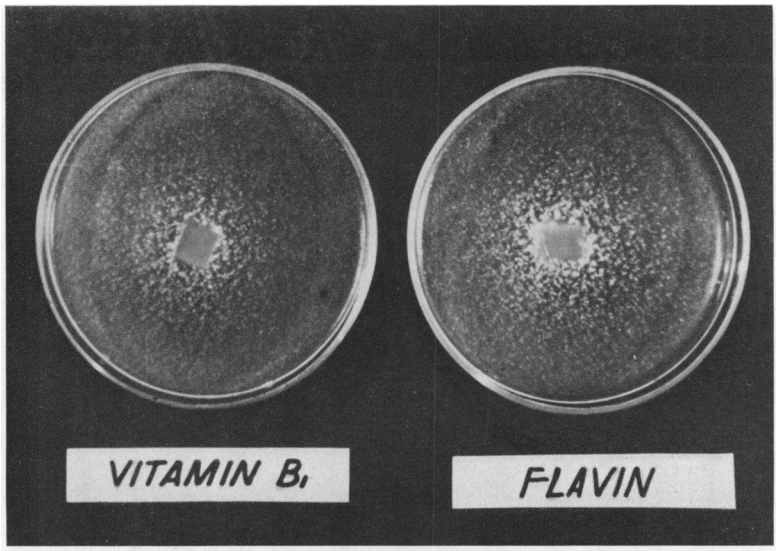
Influence of various supplements (25 parts per million) on growth of giant colonies of *Rhizobium trifolii*. A: Control. B: Casein hydrolysate. C: Thioglycollic acid. D and E: Allison's Azotobacter extract. F: Yeast extract.



(P. M. West and P. W. Wilson: Growth factor requirements of bacteria)

PLATE 2

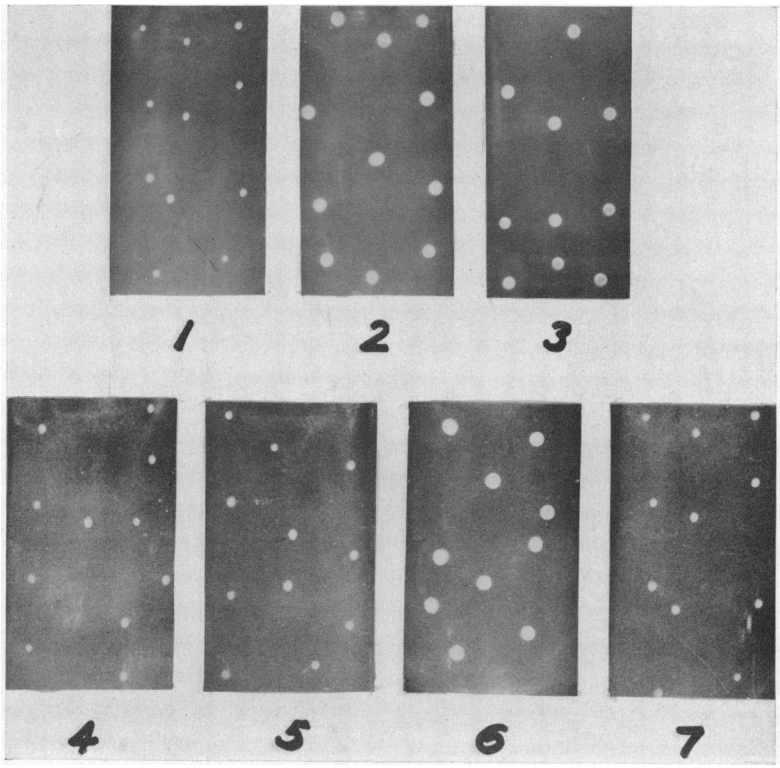
Stimulation of growth of *Rhizobium trifolii* in base medium around blocks containing 0.2 microgram per ml. vitamin B_1 and flavin.



(P. M. West and P. W. Wilson: Growth factor requirements of bacteria)

PLATE 3

Effect of varying concentration of vitamins on growth of *Rhizobium trifolii*. 1 and 4: Controls. 2 and 3: 0.1 and 0.2 microgram vitamin B_1 per ml. 5, 6, and 7: 0.05, 0.1 and 0.5 microgram flavin per ml.



(P. M. West and P. W. Wilson: Growth factor requirements of bacteria)