AN ACCESSORY FACTOR FOR LEGUME NODULE BACTERIA

SOURCES AND ACTIVITY

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The medium commonly used for the growing of legume nodule bacteria consists of the usual mineral salts, a source of energy (usually sucrose, mannitol, or glucose), and a water extract of yeast. In many experimental studies, where the addition of a material containing various forms of nitrogen and miscellaneous unknown substances is undesirable, nitrate, ammonia, or asparagine is substituted for the yeast water. Results vary widely as to how satisfactory this latter type of medium is, some investigators having reported heavy growths while others have obtained none. In the earlier work at this laboratory just as widely varying results were sometimes obtained when working with the same strain of organism and under supposedly similar conditions, but on different dates. The writers have determined the primary cause of most of these discordant results, namely, that many species of these organisms are unable to make an appreciable growth on a synthetic (sugar-mineral-nitrate) medium prepared from highly purified chemicals, but require an additional factor, probably organic in nature. This factor, as pointed out in a previous paper (Allison, Hoover and Burk, 1933), is a coenzyme directly essential for respiration and growth and not identical with "bios." For convenience it was termed coenzyme R.

The purpose of this paper is to present some of the earlier observations with typical data which led to the finding of the co-

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enzyme, and then to consider sources and preparation of the material in more concentrated form. Since the observations were made over a period of years, and in the earlier work were merely chance observations, some of the results may appear to be somewhat disconnected. It was, however, the accumulation of these miscellaneous observations that served as a basis for the later experiments and determination of the nature of the active factor. The previous paper stressed respiration but in this paper the results are reported in terms of growth. It should, however, constantly be borne in mind that the coenzyme is necessarily a growth factor as well as a respiration factor since anything essential for respiration must necessarily be essential for growth.

METHODS

Medium. The basal medium used in nearly all of the work was similar to the one recommended by Burk, et al. (1932) for Azotobacter. The composition of the medium was as follows: K_2HPO_4, 0.7 gram, KH_2PO_4, 0.3 gram; NaCl, 0.2 gram; $MgSO_4 \cdot 7H_2O$, 0.2 gram; $CaSO_4 \cdot 2H_2O$, 0.1 gram; and H_2O, distilled, 1000 grams. The addition of 0.01 gram $Fe(SO_4)_3 \cdot 9H_2O$ will sometimes improve it but numerous tests showed that the constituents of the medium usually contain enough iron impurity to supply the limited demands of the legume bacteria. The above stock solution is allowed to stand for at least two days, filtered, or the clear solution siphoned off, 1 per cent sucrose (or mannitol or glucose) added and sufficient KNO_3 to give 5 mgm. N per 25 cc. of the medium. Since this medium is a saturated solution of certain salts, the addition of 10 per cent additional water is necessary before autoclaving in order that the medium may remain clear after cooling. While this medium is excellent for *Azotobacter*, little or no growth of most rhizobia will occur in it if the sugar used is very pure, except where a heavy inoculum is used. The addition of the necessary respiration coenzyme, as will be shown later, causes a heavy growth. This medium, due to its clearness, proper pH (about 6.9), adequate buffer capacity, and synthetic composition is the most satisfactory the authors

have found for general laboratory use for either rhizobia (with coenzyme) or Azotobacter (without coenzyme).

Culture method. The usual method of studying the growth in these investigations was to maintain the bacteria at 28°C. in 250-cc. Erlenmeyer flasks, plugged with cotton and containing 25 cc. of medium per flask. Unless otherwise stated, two drops of a two- to five-day culture of the organism, grown on the above basal medium containing 1 per cent commercial sucrose, were used for inoculation. Tests were usually made in duplicate.

Organisms used. In the experimental work reported in this paper the clover organism, *Rhizobium trifolii*, strain 205, obtained from Dr. E. B. Fred of the University of Wisconsin, was the usual test organism used. *Rhiz. leguminosarum* (pea 302) and *Rhiz. meliloti* (alfalfa) were used in two experiments in comparison with clover 205. While no data are reported here for *Rhiz. phaseoli* (kidney bean), this organism was used in several experiments and gave responses practically identical with those obtained with the clover organism. These cultures had been tested frequently for purity and for ability to produce nodules.

Estimation of growth. All growth measurements reported here, except in table 1, represent bacterial numbers determined by direct microscopic count using a hemacytometer modified for bacterial counting. If the medium used is clear and the counts are made after a period of two to five days, while the organisms are growing rapidly and before clumping and film formation have become appreciable, the method is remarkably accurate. Successive counts, made on the same culture by a well-trained operator, seldom vary more than ± 5 per cent. The accuracy and speed are greater if the cultures are kept agitated during growth. This is not essential, however, but it is at least very desirable that all cultures be shaken a little every day during the growth period, as well as just prior to counting, in order to break up clumps. As many determinations as desired can be made on the same culture without interfering in the least with its growth. The work involved represents only a fraction of that required for plate counts, the accuracy is several times greater, and the result can be had at once instead of five days later. A comparison of

the method with certain other methods of measuring growth, such as by turbidity measurements, dry-weight determinations, and nitrogen analyses of the cells, only serves to emphasize the many advantages of the direct count method except for very special problems.

SOURCE AND QUANTITY OF INOCULUM

One of the first observations made at this laboratory, when investigations with legume nodule bacteria were begun a number of years ago, was that the quantity of growth obtained on a synthetic (sugar-mineral-nitrate) medium is markedly influenced by the source of inoculum, that is, the previous history of the organism, especially whether grown on a medium favoring a heavy or a very light growth. If cultures to be used later for inoculation were kept on a medium containing extracts of higher plants, yeast, or many other organic mixtures those organisms usually made at least a fair growth when transferred to a synthetic medium. In contrast, organisms that had previously grown under much less favorable conditions usually made little or no growth when placed in this medium. This observation was made so many times and the details so frequently checked that the earlier standard laboratory procedure followed for most experimental work was to transfer the stock cultures several times on yeast or other plant extract media just prior to their use. At the time it was believed that this procedure in some way modified the growth of the organism or possibly even affected its life cycle to such an extent that the effect continued for some time after being placed in an unfavorable medium. This explanation for the observed facts, as will be brought out more clearly later, was essentially wrong. The organisms grown on a rich medium contained such an abundance of the essential respiration and growth factor that when transferred, especially in large numbers, to a medium nearly free from this factor they were still able to grow. While the inoculation of such media with organisms taken from synthetic media resulted in little growth, the addition of a little yeast extract would cause excellent growth, showing that the organisms were alive and normal.

EGG ALBUMEN AS A GROWTH STIMULATOR

In the earlier stages of this investigation the effect on the growth of the addition of various materials, particularly plant extracts and other organic substances, to synthetic media was studied (Allison, 1927). Among the many materials tested was commercial egg albumen. The addition of 0.05 to 1.0 per cent of this

TABLE 1

Growth of legume bacteria on media containing egg albumen

MATERIAL ADDED	QUANTITY ALBUMEN ADDED	QUANTITY KNO_3 ADDED	GROWTH OBSERVATIONS AFTER 7 DAYS	
			Rhiz. trifolii	*Rhiz. leguminosarum*
	per cent	*mgm. N per 25 cc.*		
Check	0	4	+*	+*
Commercial egg albumen	0.1	4	++++	+++
	0.2	4	+++++	++++
	0.2	0	+	+
Pure egg albumen	0.1	4	+	+
	0.2	4	++	++
Commercial egg albumen, hydrolyzed	0.1	4	++++	+++
	0.2	4	+++++	++++
Pure egg albumen, hydrolyzed	0.1	4	+	+
	0.2	4	++	++
Commercial egg albumen, hydrolyzed	0.1	0	++++	+++
	0.2	0	+++++	++++
Pure egg albumen, hydrolyzed	0.1	0	+	+

* The number of +'s denotes relative growth.

material to a sugar-mineral-nitrate medium causes the formation of a heavy growth of the clover bacteria within a period as short as two or three days. Table 1 shows a typical series of results. In this experiment glucose was used as the source of energy and both the clover and pea bacteria as test organisms. This experiment was carried out before the system of direct microscopic

counting was in general use at this laboratory, hence the results are reported as relative growths as observed by the eye. Where a clear medium is used this method, as we have since determined, checks rather closely with actual bacterial counts and for many purposes is adequate.

The pure egg albumen had only a slight stimulating effect compared with that of the unpurified sample, thus furnishing additional proof that the impurities present and not the albumen itself are important in this connection. The sample of purified crystalline egg albumen was prepared by Dr. D. B. Dill of Harvard University.

The effect of hydrolysis is also shown in table 1. Samples of both the commercial and purified egg albumen were hydrolyzed by boiling in 25 per cent (by weight) of H_2SO_4 for twenty-six hours with reflux condenser attached. The stimulating factor was not appreciably affected by this drastic treatment. Furthermore, the hydrolyzed commercial albumen produced a heavy growth in the absence of KNO_3, the amino acids serving as excellent nitrogen sources. The hydrolyzed pure egg albumen produced only a fair growth.

At the time these experiments were carried out the nature of the growth stimulant was not known but the experiments left little doubt that such a factor was present in the egg albumen as an impurity. It was also established that its activity could not be attributed to its value as a nitrogen or energy source and that it was highly resistant to acids.

In recent quantitative experiments, carried out for the purpose of checking the results reported in table 1, hydrolyzed commercial egg albumen produced a slightly better growth than did the equivalent weight of unhydrolyzed material. Furthermore, the activity of extracts (described on a subsequent page), containing the factor in concentrated form, was not affected appreciably by the acid hydrolysis.

A COMPARISON OF ENERGY SOURCES

Another entirely different line of evidence was obtained from numerous studies with various carbohydrates. In the earlier

tests of energy sources in media containing plant extracts it was found that glucose, sucrose, and mannitol were about equally good. Glucose was chosen for general use. In subsequent experiments, using synthetic media, sucrose in many cases gave much better results. It was thought, at first, that the formation of toxic compounds from glucose during the sterilization process was responsible for the poor growth but sterilization at lower pressures did not result in as good growths as with the sucrose. Sterilization does, of course, break down glucose to acids as Smith (1932) has shown, and these acids may be harmful to growth but this was not the major factor involved in this case.

TABLE 2

The stimulating effect of commercial egg albumen and natural humic acid on Rhiz. trifolii, using different energy sources

MATERIAL ADDED	1 PER CENT GLUCOSE		1 PER CENT COMMERCIAL SUCROSE		
	5 days	7 days	2 days	5 days	8 days
	millions per cc.	*millions per cc.*	*millions per cc.*	*millions per cc.*	*millions per cc.*
Check	2	10	60	400	840
Natural humic acid, 10 p.p.m.			156	780	1,000
Natural humic acid, 25 p.p.m.	20	100			
Natural humic acid, 100 p.p.m.	180	300	320	940	1,380
Natural humic acid, 1,000 p.p.m.			500	2,000	2,500
Egg albumen, 200 p.p.m.			196	800	1,260
Egg albumen, 2,000 p.p.m.	1,000	2,000	420	2,100	2,000
Egg albumen, 20,000 p.p.m.			1,060	4,600	5,000

The data in table 2 show the importance of the sugar source. At the end of a growth period of five days in a glucose-nitrate medium there were 2 millions of clover bacteria per cubic centimeter while with commercial sucrose there were 400 millions present. The figures can not be considered as direct comparisons in this case because the experiment with sucrose was carried out a few days later than that with glucose but under the same experimental conditions. Such results have been obtained frequently and illustrate the point mentioned previously, that growth on a sugar-mineral-nitrate medium may vary from practically nothing to a very heavy one.

It is of interest to note that with glucose as the energy source heavy growths were obtained where either natural humic acid or egg albumen was present, showing that the glucose is actually a good energy source for clover bacteria and the failure to use it in the absence of the humic acid or egg albumen can be attributed to the absence of a necessary constituent in the medium. The growth with commercial sucrose was somewhat more rapid but both energy sources gave heavy growths where the medium was satisfactory.

Humic acid, prepared from soil, served as a good source of the growth factor although at the concentration used the growth was

TABLE 3

A study of various energy sources for Rhiz. trifolii

ENERGY SOURCE	BACTERIAL NUMBERS AFTER 4 DAYS WITH VARYING CARBOHYDRATE CONCENTRATIONS			
	0.5 per cent	1 per cent	2 per cent	4 per cent
	millions per cc.	*millions per cc.*	*millions per cc.*	*millions per cc.*
Glucose, C.P.	22	10	No growth	No growth
Sucrose no. 1, C.P.		60	60	60
Sucrose no. 2, C.P.		70	80	120
Sucrose no. 3, commercial		360	620	640
Mannitol, C.P.		30	30	36
Mannitol, C.P. with yeast water		840		

not as good as with egg albumen. A discussion of natural humic acid and other active iron compounds in relation to the growth of nodule bacteria will be reserved for inclusion in a later paper.

In table 3 are data giving a direct comparison of growth on a synthetic medium using glucose, mannitol, and three samples of sucrose as the energy sources. Samples of pure sucrose, as well as of glucose and mannitol, produced relatively small growths while commercial sucrose produced a heavy growth. Obviously, then, it is the impurity in the sugar that is important and not the particular energy source. Highly purified sucrose is probably little if any better as a source of energy than is pure glucose or mannitol. In this particular experiment the results

with glucose may be set aside since the flasks were sterilized at 15 pounds pressure for fifteen minutes and evidently toxic compounds were produced. The growth on the synthetic medium containing mannitol was very limited but with 10 per cent yeast water added a heavy growth was obtained, showing that mannitol is an excellent energy source but is used only if the bacterial growth factor is also present.

The data of table 3 also indicate that with the possible exception of the medium containing yeast extract in no case was sufficient of the growth essential present for maximum growth. Sucrose sample 1 and the mannitol were evidently quite free from the growth factor since increasing concentrations gave practically no increase in growth. Sucrose sample 2, although labelled C.P., contained a little of the factor because growth did increase with increase in quantity used. With such slight growths 0.1 per cent sugar would have been more than adequate to meet the energy requirements for a four-day growth period, as will be shown later in table 4, hence the increased growth can not be attributed to the sugar itself. One per cent commercial sucrose did not furnish enough of the growth factor for maximum growth since doubling the concentration nearly doubled the growth, but it is evident that it contained a high concentration compared with that in the C.P. sucrose. With 2 per cent commercial sucrose the growth was about 75 per cent as good as in the usual standard 1 per cent mannitol yeast extract medium.

It will be observed further that 60 millions of bacteria per cubic centimeter developed in four days in the cultures receiving sucrose sample 1 even though the sugar was practically free from the essential respiration and growth factor. The culture used for inoculation was grown on a commercial sucrose medium and the 2 drops of inoculum carried enough of the growth factor to permit the limited development observed. The same explanation holds for most of the results reported in subsequent tables.

Additional results, showing the growth of the clover nodule bacteria on various concentrations of pure and commercial sucrose, are given in table 4. It will be observed again that the growth was not appreciably affected by the concentration of pure

sucrose used, while with the commercial sample the growth increased markedly and rather uniformly with concentration.

Sugar analyses made by the method of Stiles, Peterson and Fred (1926), show that even with the lowest concentrations added the percentage present at the end of seventeen days was still greater than the quantity consumed. Lack of energy, therefore, was not the primary cause of limited growth in any of the flasks, although there was undoubtedly some effect due to concentration.

Table 5 shows the comparative activity of 4 samples of com-

TABLE 4

The effect of various concentrations of sugars on the growth of Rhiz. trifolii

SOURCE OF SUGAR	SUGAR ADDED	BACTERIAL NUMBERS AFTER			SUCROSE	
		3 days	6 days	14 days	Present after 17 days	Consumed in 17 days
	per cent	*millions per cc.*	*millions per cc.*	*millions per cc.*	*per cent*	*per cent*
Glucose, C.P.	1	20	60			
Sucrose no. 1, C.P.	0.2	20	56			
	0.5	44	80			
	1	48	100			
	2	44	72			
	5	34	76			
Sucrose, no. 3, commercial	0.2	90	160	220	0.12	0.08
	0.5	132	240	360	0.38	0.12
	1	152	400	600	0.76	0.24
	2	180	600	1,500	1.55	0.45
	5	240	720	1,600	3.57	1.43

mercial cane sugar, all being quite active in comparison with pure sucrose. None of the sugars, however, contained sufficient coenzyme to allow of near-maximum growth where used at the usual 1 per cent concentration. The rather heavy inoculum used is again the explanation for the amount of growth observed with the pure sucrose.

PURIFICATION OF SUCROSE BY RECRYSTALLIZATION

A sample of commercial sucrose 3, which gave excellent growths in the above experiments, was purified by crystallization from

hot 30 per cent alcohol followed by recrystallization from hot 70 per cent alcohol. These two samples were then tested in com-

TABLE 5

A comparison of various samples of commercial sucrose on the growth of Rhiz. trifolii

	PER CENT	BACTERIAL NUMBERS		
		3 days	4 days	7 days
		millions per cc.	*millions per cc.*	*millions per cc.*
Sucrose no. 1, C.P.	0.5	36	40	100
	1.0	30	46	80
Sucrose no. 3, commercial	0.5	80	175	180
	1.0	100	360	360
Sucrose no. 4, commercial	0.5		100	
	1.0		220	
Sucrose no. 5, commercial	0.5	100	150	200
	1.0	130		340
Sucrose no. 6, commercial	0.5	80	150	220
	1.0	106		300

TABLE 6

Growth tests with sucrose purified by recrystallization from alcohol, using Rhiz. trifolii

SOURCE OF SUCROSE	BACTERIAL NUMBERS WITH VARYING SUCROSE CONCENTRATIONS									
	3 days					5 days				
	0.2 per cent	0.5 per cent	1 per cent	2 per cent	3 per cent	0.2 per cent	0.5 per cent	1 per cent	2 per cent	3 per cent
	millions per cc.	*millions per cc.*	*millions per cc.*	*millions per cc.*	*millions per cc.*	*millions per cc.*	*millions per cc.*	*millions per cc.*	*millions per cc.*	*millions per cc.*
Sucrose no. 1, C.P.	30	34	34	38	24	44	50	50	50	36
Sucrose no. 3, commercial	46	86	170	220	280	82	200	390	640	720
Sucrose no. 3, 1st recrystallization	34	50	48	48	20	54	90	92	94	170
Sucrose no. 3, 2nd recrystallization	14	*	*	18	20	26	*	*	60	124

* Lost.

parison with some of the original commercial sucrose as well as with C.P. sucrose. The results are shown in table 6.

It will be observed that one crystallization from alcohol removed most of the active material from the sugar; after the second crystallization the final product was nearly as free from activity as was the C.P. sucrose.

PREPARATION OF ACTIVE EXTRACTS FROM SUCROSE

Attention was next turned toward the preparation of the active factor in a concentrated form. A 50 gram sample of commercial sucrose no. 3 was extracted in a Soxhlet extractor with 100 cc. of absolute alcohol for about fifteen hours. The extract was evaporated to dryness under partial pressure at 50°C., taken

TABLE 7

Growth tests with an absolute alcohol extract prepared from commercial sucrose, using Rhiz. trifolii

TREATMENT	QUANTITY ADDED	BACTERIAL NUMBERS AFTER 4 DAYS
	per cent	*millions per cc.*
Sucrose no. 1, C.P.	1	84
Sucrose no. 3, commercial	1	480
Sucrose no. 1 (1 per cent) + alcohol extract	0.25*	140
	0.5*	300
	1*	420
	2*	660
Sucrose no. 3, alcohol extracted	1	120

* These figures refer to extract equivalent to quantity of sugar designated.

up with water and again evaporated to remove all traces of alcohol. The residue was taken up in 50 cc. of water, filtered to remove the small quantity of material which did not go into solution, and its activity tested as in previous experiments.

The data given in table 7 show that absolute alcohol removed nearly all of the growth factor and that the final aqueous extract exerted a stimulating effect only slightly less than that of the equivalent amount of original sucrose. This indicates that the impurity in the sugar is present largely on the surface of the crystals.

An improved method used in obtaining the active factor was as follows: Two 350-grams samples of commercial sucrose were

extracted in a large Soxhlet extractor for fifteen hours with absolute alcohol, the extracts combined, kept over night at about 0°C., filtered, and evaporated to dryness under partial pressure as previously. The residue weighed 1.72 mgm. per gram of sugar extracted. A portion of this alcohol extract was in turn extracted with benzene and the latter removed by evaporation. Table 8 gives the results of the tests of the alcohol and

TABLE 8

Growth tests on Rhiz. trifolii using absolute alcohol and benzene extracts prepared from commercial sucrose

TREATMENT	QUANTITY SUCROSE ADDED	QUANTITY EXTRACT ADDED		BACTERIAL NUMBERS 4 DAYS
		Dry matter	Sucrose no. 3 equivalent	
	per cent	*p.p.m.*	*grams*	*millions per cc.*
Sucrose no. 1, C.P.	1			36
	2			36
Sucrose no. 3, commercial	1			260
	2			480
Sucrose no. 3, extracted	1			44
	2			50
Sucrose no. 1 + alcohol extract	1	80	1.16	390
	1	160	2.33	620
	1	320	4.67	700
Sucrose no. 1 + benzene extract	1		1.16	36
	1		2.33	36
	1		4.67	36

benzene extracts, as well as of the original and extracted sugar, on the clover nodule bacteria.

The data show that the absolute alcohol extracted practically all of the active material from the cane sugar and that the extract, when added to C.P. sucrose, produced essentially the same effect as did the original sucrose. The addition of 80 p.p.m. dry weight of the extract to the medium containing C.P. sucrose produced nearly an 11-fold increase in bacterial numbers within a

growth period of four days, while larger additions produced even greater growth increases. The data also show that the factor is not soluble in benzene. An ether extract tested subsequently was also inactive.

A considerably more concentrated extract was then prepared by extracting about 4 kgm. of commercial sucrose (sample 5) in successive 350-gram portions in a large Soxhlet extractor for about five hours each, using the same alcohol for all extractions. This, of course, decreased the quantity of sugar per unit of active material present in the final extract. Table 9 gives the results of two separate tests of the active extract.

TABLE 9

Growth tests with active extract prepared from commercial sucrose, using Rhiz. trifolii

TREATMENT	BACTERIAL NUMBERS AFTER 4 DAYS	
	First experiment	Second experiment
	millions per cc.	*millions per cc.*
Sucrose no. 1, C.P. 1 per cent	26	18
Sucrose no. 1 + 7 p.p.m. dry matter in extract	106	200
Sucrose no. 1 + 18 p.p.m. dry matter in extract	320	450
Sucrose no. 1 + 36 p.p.m. dry matter in extract	580	
Sucrose no. 1 + 72 p.p.m. dry matter in extract	580	

As little as 7 p.p.m. of dry matter in the extract was sufficient to produce a 4-fold stimulation of bacterial growth after four days in one experiment and 11-fold in the other. Using 18 p.p.m. the corresponding figures were 12-fold and 25-fold. This extract, on analysis by the method of Stiles, Peterson and Fred (1926), was found to consist of 75 per cent sugar.

CANE MOLASSES AS A SOURCE OF GROWTH FACTOR

The above experiments show that the bacterial growth factor can be almost quantitatively removed from commercial sucrose by extraction with absolute alcohol. Since the factor is presumably present chiefly on the surface of the sucrose crystals the logical assumption is that it is contained in the film of molasses

always present in traces in commercial cane sugar. In table 10 are given data showing the activity of ordinary cane molasses.

Approximately 70 p.p.m. of the molasses, about one-third of which was water, gave a growth of half-maximum. In table 9, 18 p.p.m. of the most concentrated extract of sucrose produced a somewhat similar effect. The alcohol extract of sucrose was, therefore, approximately three times as concentrated in growth

TABLE 10

The growth-promoting properties of varying concentrations of cane molasses, using Rhiz. trifolii

TREATMENT	QUANTITY MOLASSES ADDED*	BACTERIAL NUMBERS AFTER 4 DAYS		
		Experiment 1	Experiment 2	Experiment 3
	p.p.m.	*millions per cc.*	*millions per cc.*	*millions per cc.*
Check (sucrose no. 1, C.P.)		50	28	26
Cane molasses	10,000	900		
	8,000	760		
	4,000	800		
	2,000	860	920	
	1,000	860	960	
	800		920	
	400		960	1,000
	200		700	800
	80		480	640
	40		320	280
	20			160
	8			80
	4			42
	2			30
	0.8			26

* These quantities refer to ordinary molasses of which approximately one-third consists of water.

factor per unit of dry matter as was the molasses. The crude molasses per unit dry weight, was nearly 200 times as active as commercial sucrose.

PREPARATION OF ACTIVE EXTRACTS FROM CANE MOLASSES

In order to prepare a concentrated extract from molasses it must first be thoroughly dried and ground. The most satis-

factory method found for accomplishing this was as follows: 25 grams of cane molasses were thoroughly mixed with 60 grams of sand (passing 30-mesh) which had previously been calcined, and dried under reduced pressure at 90°C., with a current of heated dried air passing through the system. The dried sand-molasses mixture was quickly ground in a mortar and extracted with absolute alcohol in a Soxhlet extractor for about six hours in the usual manner. The alcohol was then distilled off under reduced pressure and the activity of the dried residue compared with that of the extract previously prepared from sucrose (see table 9).

TABLE 11

A comparison of the growth-promoting properties of alcoholic extracts of sucrose and molasses on Rhiz. trifolii

QUANTITY OF EXTRACT ADDED	BACTERIAL NUMBERS AFTER 4 DAYS			
	First experiment		Second experiment	
	Sucrose extract	Molasses extract	Sucrose extract	Molasses extract
p.p.m.	*millions per cc.*	*millions per cc.*	*millions per cc.*	*millions per cc.*
0	10	10	24	24
1	16			
2	20			
4	30			
8	80			
16	140		320	380
32	300	440	460	560
64	500			
128	640			
160		800		

The activity per unit of dry matter of the molasses extract, as shown in table 11, was only approximately 30 per cent greater than that of a similar extract of sucrose even though the original molasses was nearly two hundred times as active as was the commercial sucrose. This is not surprising since the source of the material and method of extraction are essentially the same in both cases. A comparison of the data given in table 11 with those in table 10 also shows that the alcoholic extract of molasses was about four times as active as molasses itself, 16 p.p.m. of the extract giving a 16-fold increase in growth within four days or

nearly half the usual maximum for such an experiment. This extract still contained 78 per cent sugar. Further work on the preparation of more active extracts is now in progress.

COMPARISON OF YEAST EXTRACT WITH SUCROSE EXTRACT

It was pointed out previously that a sugar-mineral-yeast medium is used extensively for growing rhizobia. As a result of the studies of this laboratory it is now quite evident that the chief function of the yeast is to supply the necessary respiration factor to permit growth. If some other source of the coenzyme and nitrogen are supplied, then the yeast water may be omitted

TABLE 12

A comparison of the growths obtained with yeast water and the alcoholic extract of commercial sucrose using different nitrogen sources

TREATMENT (ADDITIONS TO BASAL MEDIUM)	BACTERIAL NUMBERS AFTER 3 DAYS	
	Rhiz. trifolii	*Rhiz. meliloti*
	millions per cc.	*millions per cc.*
Check (with KNO_3)	20	20
Yeast water 10 per cent + KNO_3	540	740
Yeast water 10 per cent + urea	420	400
Sucrose extract 40 p.p.m. + KNO_3	360	280
Sucrose extract 40 p.p.m. + urea	14	440
Sucrose extract 40 p.p.m. + NH_4Cl	500	360
Sucrose extract 40 p.p.m. + asparagine	340	600

from the medium without greatly affecting its value. Table 12 gives typical data, obtained many times, illustrating this point.

It will be observed that in most cases the yeast water gave a slightly better growth than did the alcoholic extract of sucrose but both preparations were excellent growth promoters and usually gave increases of 15- to 35-fold over the check. The actual increases in growth depended to a large extent upon the nitrogen source used. All four sources of nitrogen tested were utilized satisfactorily by the two legume organisms except in the case of urea which was toxic to the clover, but not to the alfalfa organism. A determination of the pH of the cultures on the

third day showed that where urea was added the pH was high, usually near 8. It was probably even higher on the first day. If smaller amounts of urea had been added it is probable that it would have been utilized satisfactorily by the clover as well as by the alfalfa organism. The results of this experiment check closely with the data reported in table 3 where a 2 per cent commercial sucrose-mineral-nitrate medium gave approximately 75 per cent as good growth in four days as the usual mannitol yeast water medium.

Direct comparisons of yeast water and sugar extracts almost invariably show that the yeast water produces a somewhat more rapid initial growth stimulation than does the sugar extract but at the end of four or five days the differences are much less marked. The fact that yeast extract is slightly more stimulating is not surprising since the yeast water contains in addition to the respiration factor a variety of sources of nitrogen, traces of mineral elements, and miscellaneous factors. The fact that the sucrose extract is ordinarily 60 to 90 per cent as good as the yeast water, in spite of the many other materials present in the latter, only serves to emphasize that it is the essential respiration factor in the yeast that is of primary importance.

DISCUSSION

The experiments reported here have shown rather conclusively that legume nodule bacteria, at least the clover, pea, and alfalfa species, require an accessory growth factor not found in appreciable quantities in highly purified sugars but present in commercial sucrose, cane molasses, commercial egg albumen and yeast. This factor, as already shown (Allison, Hoover and Burk, 1933), is a respiration coenzyme and exerts its effect on growth indirectly through its effect upon respiration. In the light of previous work of this laboratory, already published (Allison, 1927), it may also be stated that the respiration coenzyme is widely distributed in the plant kingdom, and probably also in the animal kingdom.

While the necessity for adding yeast extract or similar material to legume culture media in order to obtain abundant

growths has long been appreciated, practically no work has previously been reported dealing directly with the nature of the growth factor. Where the subject has been discussed the greatest emphasis has usually been placed upon the nitrogen compounds present in the yeast extract. Since it has been shown here that about 16 to 20 p.p.m. of an alcoholic extract of sucrose or molasses, still consisting chiefly of sugar, is sufficient to produce a heavy growth when added to a sugar-mineral-nitrate medium it is quite obvious that the nitrogen source is of secondary importance. It was pointed out above that legume bacteria, at least the species studied, are able to utilize readily the common simple nitrogenous compounds ordinarily supplied in bacterial culture media. There is, likewise, no evidence, so far as the writers have observed, that the carbohydrate or mineral requirements of these species are highly specialized.

In the light of the findings reported here it is interesting to search through the literature and note how many previously reported observations, heretofore inexplicable, can now be quite logically explained. Fred, Baldwin and McCoy (1932) mention several of these which need not be listed here. Many references have been made to the fact that legume bacteria grow more profusely on agar than in liquid culture media. This statement is not true for the clover, alfalfa, bean or pea bacteria if the medium is satisfactory, for certainly a growth of nearly one billion bacteria per cubic centimeter within four days, as commonly obtained with an adequate growth factor, is about as profuse a growth as most common soil organisms, such as Azotobacter, give. In the past, agar media were sometimes found to be superior to liquid synthetic media primarily for the reason that the latter frequently contained little coenzyme. Experiments at this laboratory have shown that agar commonly contains a fair amount of the essential factor. The observations of Allyn and Baldwin (1930) that not only dried agar but silica sand and ground filter paper favor the initiation of growth is also interesting. We have tested samples of quartz sand and found that even though supposedly rather pure it was possible in all cases to extract from the sand some of the growth factor which upon

addition to the culture medium produced a considerably increased growth, but, of course, only a fraction of the maximum. Extracts of soil, added in sufficient quantity, will give excellent growths.

SUMMARY

1. Experiments with legume nodule bacteria, *Rhiz. trifolii*, *Rhiz. leguminosarum*, and *Rhiz. meliloti*, are reported showing that these organisms are unable to make an appreciable growth in an ordinary synthetic medium containing highly purified sugars as the only energy source. Experiments, not reported in detail, showed that *Rhiz. phaseoli* behaves similarly.

2. The failure to grow in such a medium is due to the absence of a necessary factor, which, as previously pointed out, is a respiration coenzyme, essential primarily for respiration and indirectly for growth.

3. Studies reported here show that this respiration coenzyme is present in relatively high concentrations in yeast, cane molasses, humic acid and commercial egg albumen; commercial sucrose contains a smaller percentage. In fact, it is quite widely distributed in the plant kingdom and probably also in animal products.

4. The essential respiration and growth factor can be obtained readily in fairly concentrated form by extraction of commercial sucrose or dried cane molasses with absolute alcohol. Such extracts, even though containing about 75 per cent sugar, are of such activity that 16 to 20 p.p.m. added to a synthetic medium will commonly produce a half maximum growth.

5. Neither the nitrogen nor sugar requirements of *Rhiz. trifolii* are highly specific. The organism grows readily upon most of the common laboratory media used in soil bacteriological studies provided the pH is satisfactory and a source of the essential factor is supplied.

6. Direct comparisons of the growth of *Rhiz. trifolii* and *Rhiz. meliloti* in the commonly used sugar-mineral-yeast medium with that in a sugar-mineral-nitrate-coenzyme medium show that the growth in the latter is usually 60 to 90 per cent as good as in the former in spite of the miscellaneous substances in the yeast, par-

ticularly nitrogen and traces of various minerals in readi available form, known to favor growth.

REFERENCES

ALLISON, F. E. 1927 Jour. Agr. Res., **35**, 915–924.

ALLISON, FRANKLIN E., HOOVER, SAM R., AND BURK, DEAN 1933 Science, **78**, 217–218.

ALLYN, W. P., AND BALDWIN, I. L. 1930 Jour. Bact., **20**, 417-439.

BURK, DEAN, LINEWEAVER, HANS, AND HORNER, C. KENNETH 1932 Soil Sci., **33**, 413–453.

FRED, E. B., BALDWIN, I. L., AND MCCOY, E. 1932 Univ. of Wis. Studies in Sci., **5**, 1–343.

SMITH, MARGARET L. 1932 Biochem. Jour., **26**, 1467–72.

STILES, H. R., PETERSON, W. H., AND FRED, E. B. 1926 Jour. Bact., **12**, 427-439.