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SOME FACTORS INFLUENCING THE RESPIRATION OF RHIZOBIUM

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In the first isolation of the bacteria infecting the roots of the Leguminosae, Beijerinck (2) found that the common meat-peptone gelatin substrate is not well adapted to their growth; but that a medium very suitable for their culture consists of a water extract of legume leaves, 0.25 per cent asparagin; 5 per cent sucrose and 7 per cent gelatin. Later he modified this medium by making a more concentrated legume extract from pea seedlings and omitting the asparagin. At about the same time Breal (3) found that a water extract of the roots of legumes also favors the growth of these organisms. Hiltner and Störmer (7) suggested a medium with asparagin as the only source of nitrogen. Later a neutral water extract of pressed yeast was advocated by Fred (5) and this is in general use at the present time. However, extracts of some non-leguminous plants have been used with apparently equally good results. Müller and Stapp (8) obtained excellent growth of the various cultures of the root nodule bacteria on an extract of carrots; and very recently Carroll (4) reports that an aqueous extract of asparagus promotes the growth of these organisms just as well as yeast extract.

Allison, Hoover, and Burk (1) have reported finding a coenzyme specific for the respiration of rhizobia. They state that it is essential for the normal respiration of these organisms and they attribute the apparent stimulating effects of these various extracts to the presence of this coenzyme. It is described as being soluble in water and absolute alcohol, but insoluble in ordinary fat solvents; not injured by continued autoclaving at 15 lbs. pressure, but destroyed by ashing. They obtained a concentrated preparation of the coenzyme by extracting commercial cane sugar with absolute alcohol. At a concentration of 5 p.p.m. they found that the extract definitely stimulated the respiration to half of the maximum in one hour.

This investigation was planned to study some of the factors which have been reported as having a beneficial influence on the activities of rhizobia and especially their relation to the respiration

of these organisms. Particularly has the work been directed toward the study of the question of a coenzyme of respiration. Some effort has been made to check the findings of Allison and his associates; and the properties of yeast and legume extracts in their relation to respiration have been studied.

For this study a strain of the alfalfa root nodule species, *Rhizobium meliloti,* was used because it grows readily on laboratory media and produces less gum than most other rapidly growing species. Because of the latter characteristic it is easier to separate a mass of the organisms from foreign material.

Simple uncompensated Barcroft manometers were used according to the Warburg technique for the measurement of the respiration. Oxygen consumption was assumed as the criteria of the rate of respiration; this greatly simplified the experimental measurements and the calculations.

The culture medium employed was a modification of that devised by Fred and Waksman (6). It includes

K_2HPO_4	0.5 Gms.
$CaCO_3$	3.0 "
$MgSO_4 \cdot 7H_2O$	0.2 "
NaCl	0.1 "
Dextrose	10.0 "
Yeast extract	1.0 "
Distilled H_2O	make up to 1000 cc.

The medium was heated and the excess insoluble $CaCO_3$ filtered off. One hundred cc. portions of this clear medium were sterilized in liter flasks, inoculated and incubated at 28° for 48 to 72 hours. The organisms were then separated from the medium by centrifuging, washed once or twice by suspending in a physiological salt solution and again centrifuged. As a result a small mass of the organisms comparatively free from foreign material was obtained. They were suspended in a physiological salt solution, $\frac{1}{2}$ concentration, and aerated with sterile air for about 15 minutes before being used for inoculation.

The manometer flasks were sterilized with chromic acid cleaning solution and thoroughly washed with distilled water. Three-tenths cc. of 2 N KOH was placed in the center cup of the flask; 1 cc. of doubly concentrated culture medium, including the mineral salts of the medium, usually a nitrogen source of $NaNO_3$ or NH_4Cl (100 p.p.m. nitrogen), dextrose and the factor to be tested, was placed in the flask and inoculated with 1 cc. of the bacterial suspension. The flask was immersed in a water bath with the temperature accurately controlled at 28°C. $\pm$ 0.01° and the manometer

shaken so that the flask moved with an amplitude of about 2 inches, making 100 complete cycles per minute. Readings were made every two hours or more often if necessary.

In the preliminary work an attempt was made to obtain a concentrated preparation of the reported coenzyme from various commercial sugars. Oxygen consumption was determined with the following substances used as the carbon source.

Sea Island cane sugar
Sea Island brown cane sugar
Domino cane sugar cubes
Sea Island powdered cane sugar
C & H powdered cane sugar
Cane sorghum (local mfg.)
Utah-Idaho beet sugar
Great Western beet sugar

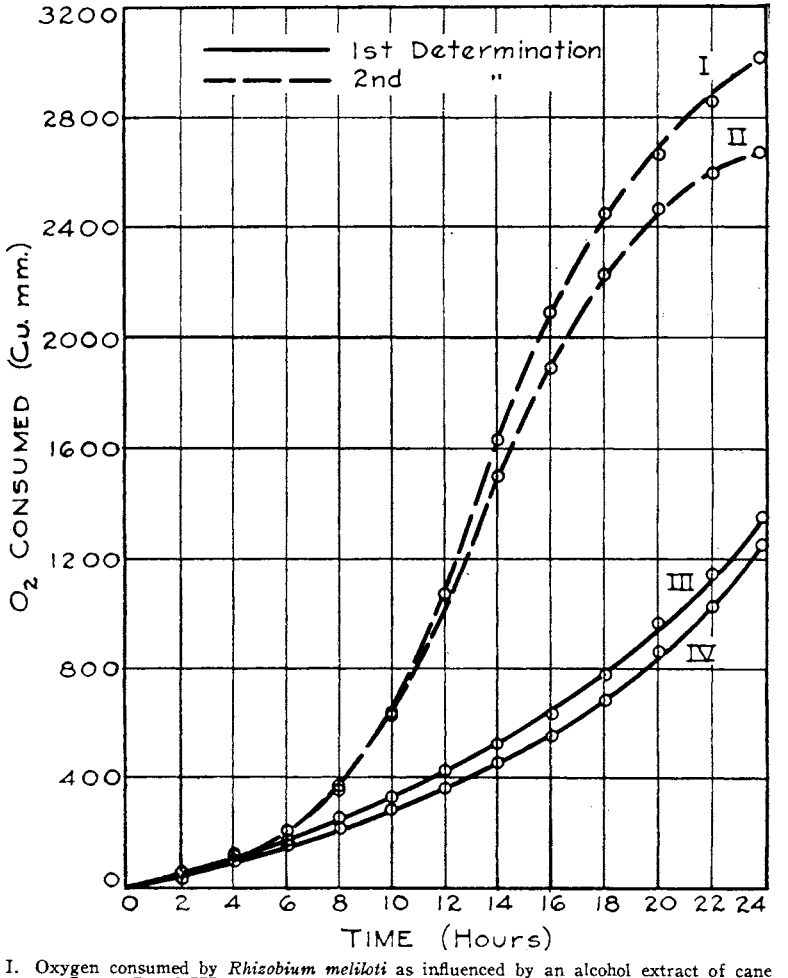
Sea Island white and brown sugar, cane sorghum and the Great Western beet sugar gave slightly higher O_2 consumption than the other sugars.

The cane sorghum was previously dried slowly in a partial vacuum at low heat and extracts were made of the four substances by extracting portions in Soxhlet condensors on six successive portions of the same sugar with the same sample of alcohol. The extract was placed in the refrigerator over night and the sugar which crystallized out was filtered off. The alcohol containing the extract was evaporated under reduced pressure and the residue taken up in water. Each extract of about 2 grams represented about 700-800 grams of the original substance.

The extracts were added to the medium of mineral salts, dextrose and $NaNO_3$ (100 p.p.m. of nitrogen) such that the final concentration was 5, 10 and 25 p.p.m. (dry weight of the extract) and the O_2 consumed in the manometers with equal inoculations measured over a 24 hour period. An increase of about 10% in the total O_2 consumed with the extract of white cane sugar was noted at 5 p.p.m. which was not increased with increasing concentrations. Brown sugar extract produced about the same increase at 10 p.p.m. Increases with the sorghum and beet sugar extracts were less and of doubtful significance. The white cane sugar extract giving the greatest activation was selected for further study. Two typical examples of the results with this extract are shown in fig. 1.

It is readily observed that at no period is there any sharp increase in the rate of respiration with those organisms receiving the extract over those not receiving it. This would indicate that such an extract could not be considered essential to respiration, although it might be classed as a mild activator.

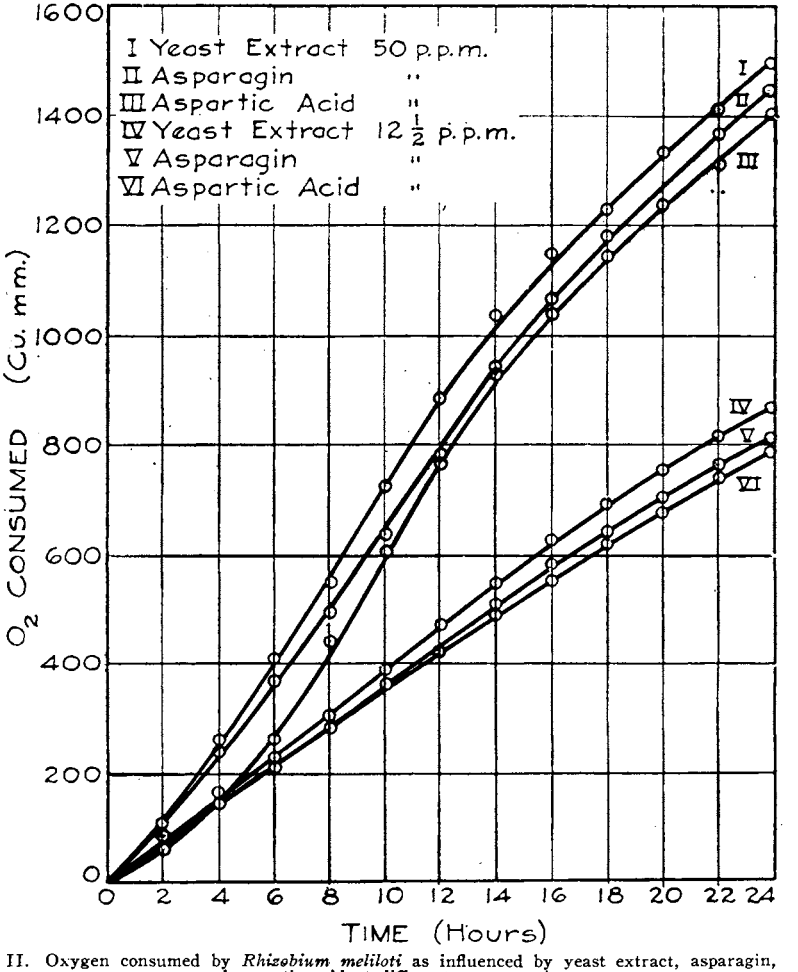
The yeast extract used was from a pound jar of a standardized preparation made by the Digestive Ferments Company. It induced a greater O_2 consumption when used as a nitrogen source than either $NaNO_3$ or NH_4Cl. Allison suggested that the reason for this activation was the presence of a coenzyme of respiration, in the yeast extract. However, c.p. asparagin and neutralized aspartic acid, on the basis of the same nitrogen content, produced comparable increases in the rate of respiration at concentrations of 100, 50, 25 and $12\frac{1}{2}$ parts of nitrogen per million parts of medium. These results are represented by plotting respiratory rate curves at two concentrations, 50 and $12\frac{1}{2}$ p.p.m. as shown in fig. II.



I. Oxygen consumed by *Rhizobium meliloti* as influenced by an alcohol extract of cane sugar. Curves I and III represent O_2 consumed in a medium containing 5 p.p.m. of the extract. Curves II and IV represent O_2 consumed in the medium without the extract.

The fact that chemically pure compounds can induce similar increases in the rate of respiration indicates that a stimulative or coenzyme property need not be attributed to yeast extract, but rather its action can be accounted for on the basis of a readily available nitrogen source.

Alcohol extracts were made of the yeast extract in an attempt to isolate a stronger activating preparation, but it failed to produce any activation beyond that which can be attributed to the nutritional value. Analysis showed the alcohol extract to contain 6.8% nitrogen and the residue 2.8%; the former induced a consumption of 1450 c.mm. O_2 and the latter 1350 c.mm. O_2 in 24 hours with each in 25 p.p.m. N concentration. The difference might be ac-



II. Oxygen consumed by *Rhizobium meliloti* as influenced by yeast extract, asparagin, and aspartic acid at different concentrations.

counted for on the basis of a more available form of nitrogen in the extract.

Extracts were made of the tops and roots of nodulated and non-nodulated alfalfa and red clover plants. The plants were grown in sand. After six weeks they were harvested, the tops and roots separated and each group macerated in a mortar, water was added and the mass autoclaved 30 minutes at 15 lbs. pressure. The liquids were filtered and pressed from the solid residue and the extracts used in place of the nitrogen source in the medium placed in the manometers. In all tests made the oxygen consumed correlated with the nitrogen content of the extracts as shown in table I which gives the N content and totals of O_2 consumed in 24 hours.

This work indicates that a very important factor in the rate of respiration of these organisms is the concentration and availability of the nitrogen source. Many of the activations which have been attributed to unknown factors can be reasonably accounted for by the nature of the nitrogen source present.

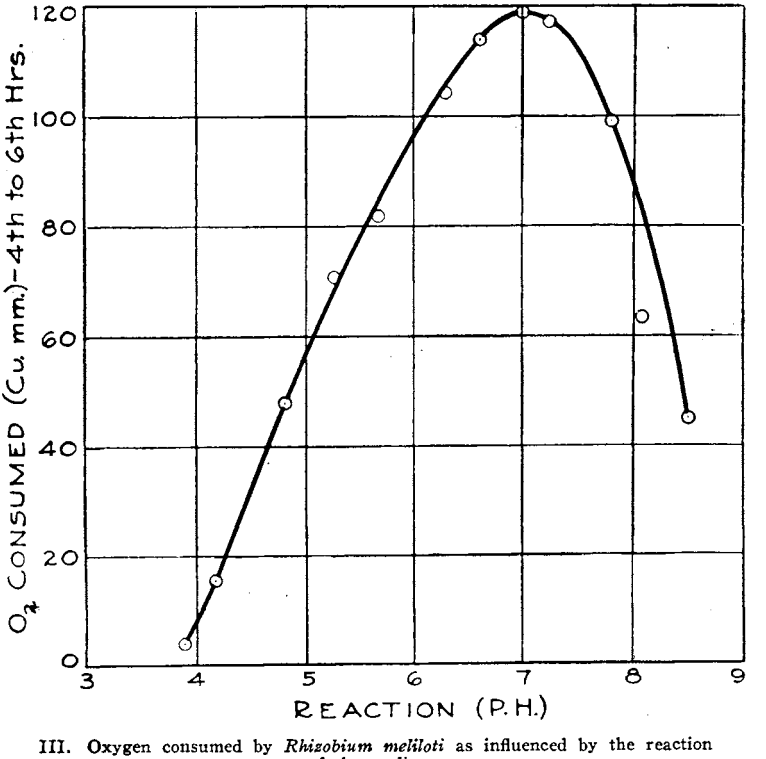
Table I. Effects of Aqueous Extracts of Alfalfa and Red Clover on Oxygen Consumption

Plant extracts employed	Nitrogen content of plant extract		O_2 consumed (c.mm.) in 24 hrs.			
	Percent	p.p.m.	1st trial	2nd trial	3rd trial	Ave.
Alfalfa tops; nodulated plants	.00863	86.3		1931.3	1074.7	1503.0
Alfalfa tops; non-nodulated plants	.0120	120.0		2358.2	1267.9	1813.0
Red clover tops; nodulated plants	.00871	87.1		2081.3	1988.6	1534.9
Red clover tops; non-nodulated plants	.00644	64.4		1670.4	912.7	1291.5
Alfalfa roots; nodulated plants	.00512	51.2	1470.4	1503.8		1487.1
Alfalfa roots; non-nodulated plants	.00376	37.6	1327.0	882.5		1104.7
Red clover roots; nodulated plants	.00512	51.2	1672.8	1384.5		1528.6
Red clover roots; non-nodulated plants	.00412	41.2	1441.4	1316.2		1378.8

Several workers have attributed the effect of many extracts upon the activities of microorganisms to changes produced in the reaction of the medium. Especially has the reaction been found to affect the rate of respiration. To study this effect upon the organisms concerned, a series of phosphate buffers was made up from varying proportions of 0.1 molar solutions of KH_2PO_4 and K_3PO_4 ranging in acidity from pH 4 to 9. The rate of respiration was determined in these solutions simultaneously. The final composition of the medium used was as follows:

Phosphate buffer	0.025 molar
$CaSO_4$	0.4 gm./liter
$MgSO_4 . 7H_2O$	0.1 gm./liter
NaCl	0.1 gm./liter
Dextrose	10.0 gms./liter
NH_4Cl	100 p.p.m. nitrogen

The phosphate buffer was sterilized separately from the other constituents of the medium. Respiration was measured over a period of 6 hours. The O_2 consumed between the fourth and sixth hours was taken for comparative results. This allowed time for overcoming the initial shock incident to the sudden change in reaction at inoculation, yet was not sufficiently long to allow any great change in pH of the medium as a result of the activities of the organisms. pH measurements were made of the medium prior to inoculation and after the test was completed. The quinhydrone electrode was used on the acid side and the glass electrode for the media on the basic side. The organisms were found to be fairly tolerant to changes in pH. The differences between O_2 consumed were not great between the range of pH 6.2 and 7.8 and the max-



III. Oxygen consumed by *Rhizobium meliloti* as influenced by the reaction of the medium.

imum was between 6.8 and 7.3 with the limits about pH 4 and 9. The results are shown with the O_2 consumed between the fourth and sixth hours as ordinates and the reaction as the abscissas (fig. III).

The reaction of the medium used throughout this series of experiments was about pH 7. The medium was well buffered so that changes produced in the reaction by the small concentrations of most materials added would not cause any great change in the rate of respiration.

The present investigation was limited in scope and much further study is needed to clear up the questions of activators and coenzymes of respiration of rhizobia. However, the data do indicate that the stimulation induced by certain materials may be satisfactorily accounted for on the basis of their nutritional value. No proof is presented to show that there is no coenzyme of respiration, but the results of this work fail to substantiate such an assumption.

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