LX. INVESTIGATIONS ON THE ROOT NODULE BACTERIA OF LEGUMINOUS PLANTS.

XVIII. BREAKDOWN OF PROTEINS BY THE ROOT NODULE BACTERIA.

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THE literature of the root nodule bacteria contains very little information regarding the ability of these organisms to decompose proteins. Several investigators have shown that the nodule bacteria slowly liquefy gelatin. However, this slow liquefaction takes place only in older cultures so that the nodule bacteria cannot be classed among the gelatin-liquefying bacteria proper. No further data are available concerning the changes in gelatin caused by these organisms.

The behaviour of the nodule bacteria towards milk has been studied by several workers. Löhnis and Hansen [1921] showed that these bacteria can be divided into two groups according to the changes they produce in milk. The organisms of the first group form a serum zone in milk, whilst those of the second group do not. The first group includes the nodule organisms from most cultivated legumes. However, even the bacteria forming a serum zone effect only very slight changes in milk proteins. Thus, for instance, Fred *et al.* [1932] found that in skimmed milk cultures with an initial content of 453.7 mg. of insoluble nitrogen (caseinogen, albumin) per 100 ml., the alfalfa organisms effected, during 450 days' incubation, an increase of 29.3 mg. in soluble nitrogen per 100 ml., whilst the corresponding figure for sweet clover bacteria was 19.1 mg. Virtanen [1928] has shown that in water suspensions in the presence of toluene the nodule organisms and crushed nodules cause a slow decomposition of their cell proteins. There is, however, no formation of ammonia in this process, but only of soluble organic nitrogen compounds, with an increase in amino-nitrogen.

The proteoclastic effect of the rhizobia upon different proteins is of particular interest in view of the much-discussed question, how the host plant receives its nitrogen from the root nodules. Virtanen [1928] earlier assumed that the enzymes contained in the bacterial cells caused a breakdown of the cell proteins and that the host plant utilised the amino-acids thus formed in the nodules. It was shown later in this laboratory [Virtanen *et al.*, 1931; 1933] that amino-acids are excreted from the nodules into solid media and that this excretion commences as soon as the nodules are formed, reaching a maximum rate at an early stage of growth (before the plant blooms). These results show that the formation of amino-acids in the nodules is not attributable to the action of the bacterial enzymes upon the cell proteins. Consequently, Virtanen assumed that the excreted amino-acids represent the primary products of nitrogen fixation and that the host plant also utilises these amino-acids. It is interesting to note that, following quite different lines of reasoning, Bond [1933] likewise concludes that the host plant receives from the nodules nitrogenous compounds excreted by the bacteria. According to current views the proteoclastic effect of the rhizobia apparently plays no decisive rôle in the process through which the nodules

supply their host plant with nitrogenous food. Nevertheless, it is interesting to obtain some information regarding the behaviour of these organisms towards different protein compounds.

EXPERIMENTAL.

The proteoclastic effect of the rhizobia was studied with the following substrates: milk, caseinogen, gelatin and autoclaved rhizobia suspensions. Soluble nitrogen was determined in the clear filtrate after the precipitation of proteins by acetic acid in a boiling solution. Amino-nitrogen and ammonia were determined according to Van Slyke, whilst the formaldehyde titration was carried out by Sørensen's method.

Decomposition of milk proteins.

200 ml. of skimmed milk were pipetted into 300 ml. Erlenmeyer flasks, which were then autoclaved at 120° for 20 min. and inoculated, in groups of four flasks, with the following different strains of *Rhizobium*:

<i>Rh. trifolii</i>	VI	(efficient strain)
<i>Rh. trifolii</i>	IV	(weak strain)
<i>Rh. leguminosarum</i>	X	(efficient strain)
<i>Rh. leguminosarum</i>	XVI	(weak strain)

The flasks were plugged with cotton-wool and provided with tight-fitting caps of vegetable parchment to prevent too rapid evaporation. The cultures were incubated at 29°. From each set of four flasks, one culture was analysed after 2, 4 and 6 weeks and again after 6 months (see Table I).

Table I. *Experiment with Rh. trifolii VI.*

	Total N mg./10 ml.	Soluble N % of total N	Amino-N % of soluble N	Formald. titration ml. 0.2 N NaOH/20 ml.	NH ₃ -N % of total N
Initial	36.2	13.3	7.0	4.3	1.0
After 2 weeks	36.3	13.8	8.7	4.0	2.8
After 4 weeks	36.8	14.3	8.7	3.7	2.4
After 6 weeks	37.0	14.3	8.7	3.8	2.2
After 6 months	43.0	22.3	17.0	6.7	1.0

The decomposition of the milk proteins has thus been exceedingly slow. A distinct rise occurred in the values of soluble and amino-nitrogen only after 6 months' incubation. Precisely similar results were obtained with other bacterial strains, so it is not necessary to record these experiments in detail.

The formation of ammonia was very slight. However, in older cultures the content of ammonia must have been higher than is indicated by the figures in Table I, since part of the ammonia escaped by evaporation. This is also evidenced by the fact that the 6 months' culture shows a decreased ammonia content.

Decomposition of caseinogen.

The decomposition of caseinogen was studied both with living cultures and with heavy suspensions in the presence of toluene. The following medium was used in the experiments with living bacteria:

Caseinogen	...	...	12 g.	FeCl ₃	...	...	...	Traces
Glucose	...	...	6 "	Yeast extract	...	...	...	50 ml.
K ₂ HPO ₄	...	...	0.25 "	Tap water	...	...	...	500 "
MgSO ₄ , 7H ₂ O	...	...	0.1 "	Agar	...	...	...	2 g.
NaCl	...	...	0.1 "	pH	...	...	...	7.4

100 ml. of the semi-solid medium were pipetted into 4 flasks. The cultures were inoculated with *Rh. trifolii* VI and incubated at 29°. The flasks were vigorously shaken daily so that the semi-solid agar medium broke and fresh surfaces were formed for the bacteria to grow on. The growth of the bacteria could thereby be greatly improved.

The initial content of ammonia-nitrogen (per 100 ml. of the medium) was 2.0 mg. The corresponding values after 3 and 6 weeks' incubation were 2.0 and 2.8 mg., respectively. The formation of ammonia was thus quite negligible.

The experiment with a heavy bacterial suspension was likewise made with *Rh. trifolii* VI, grown on 400 gelatin slopes. The gelatin medium was prepared as follows:

Sucrose	...	...	5.0 g.	Lupin extract	...	...	200 ml.
Asparagine	...	...	2.5 „	Tap water	...	...	200 „
Gelatin	...	...	130.0 „	p _H	...	...	7

The gelatin slope cultures were allowed to grow at room temperature for 7 days, after which the bacterial mass was washed off with sterile water and the suspension centrifuged clear. 400 tubes produced about 5 g. of moist bacterial mass, which was suspended in 100 ml. of sterile water; 15 ml. of toluene were added and the flask was thoroughly shaken and allowed to stand for 24 hours. 15 ml. of the well-shaken suspension were then pipetted into 150 ml. of 2% caseinogen solution. This solution contained 10 ml. of toluene and had p_H 6.9. The caseinogen was prepared according to Hammarsten and dissolved in dilute NaOH whereupon the solution was neutralised with acetic acid to p_H 6.9. The rubber-stoppered flasks were incubated at 29°. At various intervals, one culture was taken for analysis (see Table II).

Table II.

	Total N mg./10 ml.	Soluble N % of total N	Amino-N % of soluble N	Formald. titration ml. 0.2 N NaOH/20 ml.	NH ₃ -N % of total N
Initial	28.8	15.8	5.3	1.7	1.0
After 5 days	29.3	21.1	5.4	1.7	1.0
After 24 days	28.7	27.8	8.9	1.9	1.1
After 30 days	30.0	28.5	8.8	1.9	1.1
After 42 days	30.5	29.8	9.8	2.1	0.9
After 60 days	30.7	36.1	11.7	2.3	0.9

The results in Table II show clearly that there has been no production of ammonia. On the other hand, the values of soluble and amino-nitrogen have slowly risen.

Decomposition of gelatin.

The decomposition of gelatin was also studied both with living bacteria and with heavy suspensions in the presence of toluene. In the former case, two different methods were employed. In preliminary experiments, 0.4% of agar was added to the lupin extract-gelatin medium, and 100 ml. of this medium were pipetted into Erlenmeyer flasks. The flasks were plugged with cotton-wool, sterilised and inoculated with *Rh. trifolii* VI. The flasks were then fitted with caps of vegetable parchment and incubated at 29°. On the third day, a distinct

Table III.

	Total N mg./10 ml.	NH ₃ -N mg./50 ml.	NH ₃ -N % of total N	Formald. titra- tion ml. 0.1N NaOH/20 ml.
Initial	139.7	3.2	0.5	8.9
After 12 days	139.7	10.4	1.5	10.4

growth had appeared on the agar surface, and the flasks were thereafter shaken daily in order to produce fresh surfaces for the bacterial growth (see Table III).

Another experiment on the decomposition of gelatin by living nodule organisms was made with a suspension of *Rh. leguminosarum* X. The bacterial mass was grown for 7 days in a Roux flask, whereupon it was washed off with sterile water and glass beads. The volume of the suspension so obtained was 100 ml.

75 ml. of a medium, containing 30 g. of gelatin and 3 g. of K_2HPO_4 per l. of water (p_H 7.1), were pipetted into nine 150 ml. Erlenmeyer flasks. The flasks were sterilised at 100° on two successive days, whereupon 10 ml. of the well-shaken bacterial suspension were added to each flask. Three of the cultures were used as controls and analysed immediately; three cultures were analysed after 12 days' incubation at 37° and the remaining three after 21 days. Before analysis, the volume of each culture was made up to 100 ml. with distilled water. The mean results of each set of three cultures are given in Table IV.

Table IV.

	Total N mg./10 ml.	NH_3 -N mg./50 ml.	NH_3 -N % of total N	Formald. titra- tion ml. 0.1 N NaOH/20 ml.
Initial	31.3	0.42	0.27	2.5
After 12 days	30.7	0.80	0.52	2.9
After 21 days	31.2	1.13	0.72	3.1

The results show that very small amounts of ammonia are formed from gelatin. The ratio of ammonia-nitrogen to total nitrogen is, even after 21 days, only in the neighbourhood of 1 %. It is not known which particular constituent of gelatin is the source of this insignificant ammonia production.

The decomposition of gelatin in the presence of toluene was studied by pipetting 100 ml. of 5 % gelatin solution (p_H 6.5) into Erlenmeyer flasks, whereupon 10 ml. of toluene were added and the flasks were vigorously shaken and allowed to stand for 24 hours. 10 ml. of the bacterial suspension (see decomposition of caseinogen in the presence of toluene) were then added and the flasks were fitted with rubber stoppers and incubated at 29° (see Table V).

Table V.

	Total N mg./10 ml.	NH_3 -N mg./50 ml.	NH_3 -N % of total N
Initial	61.6	1.6	0.41
After 5 days	61.6	1.7	0.45
After 17 days	61.6	1.6	0.41
After 30 days	61.3	1.6	0.41
After 36 days	61.6	1.7	0.43
After 48 days	61.9	1.6	0.40

There is thus no formation of ammonia in the presence of toluene.

Decomposition of the cell proteins of rhizobia.

The bacterial mass was obtained from 500 gelatin slope tubes in the manner previously described. After centrifuging, the cell mass was suspended in 500 ml. of sterile water, and the suspension was autoclaved at 120° for 20 min. The following medium was then prepared:

Autoclaved suspension	...	...	500	ml.	$MgSO_4, 7H_2O$	...	...	0.1 g.
Yeast extract	...	...	50	"	$FeCl_3$	...	...	Traces
Glucose	...	...	5.0	g.	Agar	...	...	2.0 g.
K_2HPO_4	...	...	0.25	"	p_H	...	...	7.10
NaCl	...	...	0.1	"				

150 ml. of this medium were pipetted into three 150 ml. Erlenmeyer flasks, which were then plugged with cotton-wool and autoclaved at 115° for 20 min. After cooling, the flasks were inoculated with *Rh. trifolii* VI, covered with parchment caps and inoculated at 29°. When a distinct growth was visible the agar surface was broken by shaking the flasks. This was subsequently done every day (see Table VI).

Table VI.

	Total N mg./150 ml.	Soluble N mg./150 ml.	Soluble N % of total N	NH ₃ -N mg./150 ml.
Initial	504*	294	58.3	1.5
After 2 weeks	504	399	79.1	—
After 6 weeks	504	420	83.0	1.5

* Including 7.2 mg. N contained in the yeast extract.

Table VI shows that over 50 % of the nitrogen contained in the bacterial mass was in a soluble form at the start of the experiment. There is a distinct rise in the soluble nitrogen during incubation. On the other hand, no ammonia has been formed.

SUMMARY.

The decomposition of milk proteins, pure caseinogen, gelatin and the cell proteins of the nodule bacteria was studied both with living cultures of different strains of *Rhizobium* and with heavy suspensions in the presence of toluene. The breakdown of the different protein materials was found to be very slow and no differences could be noted between strains of different efficiency. There was a slow increase in the values for soluble and amino-nitrogen. Living rhizobia formed very small amounts of ammonia from milk proteins and from gelatin, whereas no formation of ammonia took place in the presence of toluene. No formation of ammonia could be demonstrated when the cell proteins of the rhizobia were used as substrates for living nodule organisms.

REFERENCES.

- Bond (1933). *Nature*, **132**, 748.
 Fred, Baldwin and McCoy (1932). Root nodule bacteria and leguminous plants, p. 85. (Madison.)
 Löhnis and Hansen (1921). *J. Agric. Res.* **20**, 543.
 Virtanen (1928). *Contrib. from the laboratory of Valio*, p. 12.
 ——— and v. Hausen (1931). *Biochem. Z.* **232**, 1.
 ——— and Karström (1933). *Biochem. Z.* **258**, 106.