

Studies on the Nodule Bacteria. XI.
Influence of Some Stimulating Chemicals with
Special Reference to the Alkaloids upon
the Fixation of Nitrogen.

By

Arao Itano and Akira Matsuura.

[December 14, 1937.]

In the preceding paper¹⁾, the influence of some stimulating chemicals with special reference to the alkaloids on the growth and morphology of the nodule bacteria was reported, and in this investigation, their influence on the nitrogen fixation by the nodule bacteria was tested. In order to clarify the question, the chemical composition of bacterial cells was determined at the same time.

Experimental.

I. Chemical composition of bacterial cells :

No collective report on the composition of nodule bacterial cells was available so far as we are aware. Consequently the cells of Genge nodule bacteria, strain A, B and C, bean and clover cultures were analysed for nitrogen, carbon, hydrogen and ash contents.

The cells were prepared from the culture grown on the yeast-mannit agar for seven days, separated by centrifuge as in the previous investigation²⁾ where the weight of cells was determined except no acetic acid was used but the distilled water, and then dried before they were subjected to the analyses :

a.) Determination of nitrogen :

After the cells were separated from the culture medium, filtered through a filter paper of known weight ; dried in an oven at 105—110°C. for five hours and when a constant weight was reached, the filter paper with the cells were transferred into a KJELDAHL flask and the nitrogen was determined as usual.

b.) Determination of carbon and hydrogen :

After the cells were separated by centrifuge, transferred into a platinum dish and evaporated first on the steam bath and then in an oven at 105—110°C. for five hours, and finally analysed by PREGL's micro-combustion method.

c.) Quantitative determination of ash :

The dried bacterial cells were burned in a platinum dish until the constant weight was reached which designates the ash content.

The results of analyses are given in Table 1.

Table 1.
Chemical Composition of Nodule Bacteria.

Constituents.	Genge nodule bacteria.				Bean bacteria.	Clover bacteria.
	A.	B.	C.	Average.		
Nitrogen.	(%) 9.056	(%) 9.313	(%) 9.374	(%) 9.248	(%) 8.631	(%) 5.384
Carbon.	44.586	44.726	45.618	44.977	50.347	51.402
Hydrogen.	9.325	10.980	9.569	9.958	10.643	10.651
Ash.	1.929	2.099	1.864	1.964	2.302	3.702

Note: The numerals in the table indicate the percentage on dry basis.

The composition of nodule bacteria seems to be influenced by various factors. HOPKINS and others³⁾ reported that *Rh. meliloti* contained 5 per cent nitrogen and less than 55 per cent carbon, and the nitrogen increases in the course of cultivation so that the carbon-nitrogen ratio becomes narrower. VIRTANEN and HAUSER⁴⁾ investigated the relation of nitrogen content to both P_H and phosphorus content, and reported that the nitrogen content was about 8–12 per cent.

According to our investigation, the Genge nodule bacteria contained 9.248 per cent which was the maximum while the carbon content was highest in the clover nodule bacteria being 51.402 per cent and smallest in Genge, 44.977 per cent. No appreciable difference was found in hydrogen content and the maximum was that of the clover and the minimum in the Genge nodule bacteria being about 10 per cent which is much higher than that of *Azotobacter chroococcum* reported by OMELIANSKY⁵⁾ as 6.41 per cent. Further OMELIANSKY noted that the ash content of *Azotobacter* was 4.16 per cent while the highest ash content of nodule bacteria was 3.702 per cent of clover and the lowest was 1.964 per cent of Genge. According to the general statement appears in BUCHANNAN'S book⁶⁾, the ash content of microorganisms in general is given as 2.0–30.0 per cent and a majority contain 5 per cent. Judging from these facts, it may be said that the ash content of the nodule bacteria is very small. As to the carbon and nitrogen contents, the nodule bacteria are about the average although the nitrogen in Genge nodule bacteria was slightly more than the average while the clover bacteria were less. Thus the composition of nodule bacteria differs by the strain.

II. Influence of stimulants on the nitrogen fixation.

In this experiment, the same strain of yeast and the same stimulants as in the preceding investigation were used by growing the yeasts on the yeast-mannit solution medium.

Experimental procedure :

Fifty cubic centimeter of the medium were placed in 150 cc. ERLIENMEYER flask to which the stimulant equivalent to 0.05 per cent was added and autoclaved; inoculated with 1 cc. of 4 days old culture grown at 28°C. and examined at different intervals; after 21 days, the cells were stained with carbol fuchsin and examined microscopically, and the nitrogen was determined by KJELDAHL method. Since LÖNNIS reported that often the nitrogen is lost during the incubation, the control was carried along uninoculated and placed in the incubator, and the difference of nitrogen between the inoculated and the control was determined.

Results :

The results are given in Table 2. (See Table 2 on page 72.)

As Table 2 indicates, the nitrogen contents differ greatly by the stimulant added and some nitrogen was found even in those non-alkaloids such as sodium succinate, anthraquinone and emodine which are non-nitrogenous. The largest amount of nitrogen was found in guanidine followed by caffeine, and the others were about the same.

Among the uninoculated series, the increase of nitrogen over the control was noted in eight cases, decreased in six and no change in one case. The maximum increase was 0.494 mg. of strychnine nitrate and the maximum decrease was 0.635 mg. of strychnine.

In the inoculated series, the nitrogen increased over the initial in seven cases and decreased in eight; the maximum increase was 0.353 mg. of morphine hydrochloride and the maximum decrease was 0.423 mg. of pyridine and emodine.

The increase of inoculated over the uninoculated took place in three cases, decreased in ten and no change in two cases; the maximum increase was 0.423 mg. of strychnine and that of decrease was 0.564 mg. of emodine.

The growth was better than that in the preceding investigation¹⁾ in all cases except the control which is the only one without the addition of yeast water. However no special effect of the stimulants was noted so far as the growth is concerned. Chinine compounds prohibit the growth completely while chinoline depressed the growth markedly. Morphologically caffeine, strychnine, strychnine nitrate had great influence and found many large bacteroids.

(See Table 3 on page 73.)

As shown in Table 3, the change of nitrogen content was similar to that of preceding case.

In the uninoculated series, the increase of nitrogen over the initial was noted in eight cases, decreased in six and no change in one case. The maximum increase was 0.846 mg. of chinoline followed by caffeine being 0.494 mg., and the maximum decrease was 0.564 mg. of the control which was followed by guanidine and strychnine, 0.282 mg.

In the inoculated series, the nitrogen increased over the initial in ten cases and decreased in five cases, the maximum increase was 0.634 mg. of strychnine nitrate, followed by caffeine and strychnine being 0.423 mg. The maximum decrease was 0.564 mg. of the control and very small in the rest.

Table 2.
Influence of Chemicals on Genge Nodule Bacteria A.

Chemicals.	N in 50 cc. medium.			Change in N.			Rate of growth by days.		Bacterial cells after 21 days.	
	Initial.	After 21 days incubation.		'Without' Initial.	'With' Initial.	'With' 'Without'.	7	21	Form.	Size. (μ)
		'Without'.	'With'.							
Control.	(mg.) 0.705	(mg.) 0.564	(mg.) 0.423	(mg.) -0.141	(mg.) -0.282	(mg.) -0.141	+	+	Rod.	0.3×0.8-0.5×2.0
Guanidine.	13.959	14.100	13.818	+0.141	-0.141	-0.282	###	###	Rod granule.	0.3×0.8-0.6×2.0
Pyridine.	5.499	5.288	5.076	-0.211	-0.423	-0.212	###	###	Rod.	0.3×0.5-0.6×1.5
Caffeine.	11.351	11.139	11.421	-0.282	+0.070	+0.282	###	###	Irregular.	0.3×0.7-1.0×3.5
Strychnine.	5.993	5.358	5.781	-0.635	-0.212	+0.423	##	##	Rod air bubble.	0.2×0.8-0.8×2.5
Strychnine nitrate. . . .	6.627	7.121	6.909	+0.494	+0.282	-0.212	##	##	"	0.3×0.8-0.9×3.0
Brucine.	5.922	6.275	5.852	-0.353	-0.070	-0.423	###	###	Rod coccic.	0.2×0.2-0.6×1.8
Chinoline.	5.640	5.851	5.781	+0.211	+0.141	-0.070	-	+	Rod.	0.3×0.8-0.5×2.0
Chinine sulfate.	6.063	5.781	5.781	-0.282	-0.282	0.000	-	-	-	-
Chinine hydrochloride. .	5.781	6.204	6.063	+0.423	+0.282	-0.141	-	-	-	-
Morphine hydrochloride.	5.499	5.499	5.852	0.000	+0.353	+0.353	###	###	Rod.	0.3×0.6-0.8×1.8
Sodium succinate. . . .	4.653	4.935	4.512	+0.282	-0.141	-0.423	###	###	"	0.2×0.6-0.6×1.8
Anthraquinone.	4.794	5.006	5.006	+0.212	+0.212	0.000	###	###	"	0.3×0.8-0.5×1.5
Emodine.	5.358	5.499	4.935	+0.141	-0.423	-0.564	##	##	"	0.2×0.6-0.6×1.5
Yeast water.	4.512	4.935	4.583	+0.423	+0.071	-0.352	###	###	"	0.2×0.8-0.6×2.0

Notes: 1.) The increase and decrease of nitrogen are denoted by + and -- respectively.
3.) 'With' = inoculated; 'Without' = not inoculated.

2.) The number of + indicates the rate of growth.

Table 3.
Influence of Chemicals on Genge Nodule Bacteria B.

Chemicals.	N in 50 cc. medium.			Change in N.			Rate of growth by days.		Bacterial cells after 21 days.	
	Initial.	After 21 days incubation.		'Without' Initial.	'With' Initial.	'With' Without'.	7	21	Form.	Size. (μ)
		'Without'.	'With'.							
Control.	(mg.) 0.705	(mg.) 0.141	(mg.) 0.141	(mg.) -0.564	(mg.) -0.564	(mg.) 0.000	+	+	Short rod.	0.3 x 0.5 - 0.6 x 0.8
Guanidine.	13.818	13.536	13.959	-0.282	+0.141	-0.423	###	###	Short rod coccic.	0.3 x 0.3 - 0.5 x 0.8
Pyridine.	5.217	5.358	5.076	+0.141	-0.141	-0.282	###	###	Short rod.	0.3 x 0.4 - 0.5 x 1.0
Caffeine.	11.703	12.197	12.126	+0.494	+0.423	-0.071	##	###	Rod air bubble.	0.2 x 0.3 - 0.8 x 2.5
Strychnine.	4.935	4.653	5.358	-0.282	+0.423	+0.705	###	###	"	0.2 x 0.4 - 0.8 x 2.0
Strychnine nitrate. . . .	6.698	7.050	7.332	+0.352	+0.634	+0.282	##	##	Rod.	0.3 x 0.4 - 0.6 x 1.2
Brucine.	5.499	5.512	5.781	+0.013	+0.282	+0.269	###	###	"	0.3 x 0.5 - 0.5 x 1.5
Chinoline.	5.781	6.627	6.345	+0.846	+0.564	-0.282	-	+	Rod air bubble.	0.4 x 0.7 - 1.0 x 1.8
Chinine sulfate.	6.204	6.063	6.486	-0.141	+0.282	+0.423	-	-	-	-
Chinine hydrochloride. .	6.063	6.345	6.345	+0.282	+0.282	0.000	-	-	-	-
Morphine hydrochloride.	5.640	5.781	5.358	+0.141	+0.282	-0.423	###	###	Rod.	0.2 x 0.5 - 0.5 x 1.2
Sodium succinate. . . .	5.076	4.935	5.006	-0.141	-0.070	-0.071	###	###	Short rod coccic.	0.3 x 0.3 - 0.5 x 1.0
Anthraquinone.	5.217	5.076	4.935	-0.141	-0.282	-0.141	###	###	Rod.	0.3 x 0.5 - 0.5 x 1.5
Emodine.	5.499	5.499	5.570	0.000	-0.071	+0.071	##	##	"	0.3 x 0.5 - 0.6 x 2.0
Yeast water.	4.935	5.076	5.358	+0.141	+0.423	+0.282	###	###	"	0.3 x 0.4 - 0.6 x 1.2

Notes: 1.) The increase and decrease of nitrogen are denoted by + and - respectively. 2.) The number of + indicates the rate of growth.
3.) 'With' = inoculated; 'Without' = not inoculated.

The increase of inoculated over the uninoculated took place in seven cases, decreased in six and no change in two cases, the maximum increase was 0.705 mg. of strychnine and that decrease was 0.423 mg. of morphine hydrochloride.

The growth was very similar to that of the preceding experiment and comparatively large cells were found in caffeine, strychnine and chinoline.

The results obtained with Genge nodule bacteria, strain C are presented in Table 4. (See Table 4 on page 75.)

Table 4 indicates that in the uninoculated series, the increase of nitrogen over the initial was only one of yeast which was 0.141 mg. while the decrease took place in nine cases, 0.564 mg. of caffeine was the highest ; in four cases no change took place.

The increase of nitrogen in the inoculated over the initial took place in five cases, the decrease in eight cases and no change in two cases ; the maximum of increase was 0.846 mg. in strychnine and that of decrease was 0.705 mg. in brucine.

The increase of nitrogen in the inoculated over uninoculated took place in seven cases, the decrease in seven and no change in one case ; the maximum increase was 0.950 mg. of caffeine, followed by 0.846 mg. in strychnine and that of decrease was 0.282 mg. in brucine.

As to the growth rate, nothing is marked but somewhat large cells were found in morphine hydrochloride and strychnine nitrate.

The results obtained with the bean nodule bacteria are given in Table 5.

(See Table 5 on page 76.)

As noted in Table 5, the nitrogen was increased in three of uninoculated over the initial cases, decrease in one ; the maximum increase was 0.353 mg. of strychnine nitrate and that of decrease was 0.705 mg. in pyridine.

In the inoculated series, the increase of nitrogen over the initial was found in three cases, and the decrease in twelve cases ; the maximum increase was 0.282 mg. and that of the decrease was 1.128 mg. of guanidine.

The increase of nitrogen of inoculated over uninoculated was in four cases, the decrease in eight cases and no change in two ; the maximum increase was 0.846 mg. of strychnine nitrate and that of decrease was 0.634 mg. in chinine sulfate.

The growth was poor in chinine and the large sized cells were found in chinoline and emodine.

The results obtained with clover nodule bacteria are presented in Table 6.

(See Table 6 on page 77.)

Table 6 indicates that the increase of nitrogen of the uninoculated over the initial took place in two cases, decreased in eleven and no change in two cases ; the maximum increase was 0.536 mg. of strychnine and that of the decrease was 0.846 mg. of anthraquinone followed by 0.809 mg. of brucine.

The increase of nitrogen in the inoculated over the initial was one case of strychnine which was 0.606 mg. while the decrease took place in eleven cases among which 0.846 mg. of both caffeine and anthraquinone, and no change in other three cases.

Table 4.
Influence of Chemicals on Genge Nodule Bacteria C.

Chemicals.	N in 50 cc. medium.			Change in N.			Rate of growth by days.		Bacterial cells after 21 days.	
	Initial.	After 21 days incubation.		'Without' Initial.	'With' Initial.	'With' 'Without'.	7	21	Form.	Size. (μ)
		'Without'.	'With'.							
Control.	(mg.) 0.141	(mg.) 0.141	(mg.) 0.000	(mg.) 0.000	(mg.) -0.141	(mg.) -0.141	+	+	Short rod coccie.	0.3×0.3-0.5×1.0
Guanidine.	13.959	13.818	13.677	-0.141	-0.282	-0.141	≡	≡	Rod.	0.3×0.5-0.5×1.5
Pyridine.	5.076	5.076	4.935	0.000	-0.141	-0.141	≡	≡	"	0.3×0.8-0.4×1.2
Caffeine.	11.139	10.575	11.525	-0.564	+0.386	+0.950	≡	≡	"	0.3×0.5-0.6×1.5
Strychnine.	4.935	4.935	5.781	0.000	+0.846	+0.846	≡	≡	Short rod.	0.2×0.5-0.5×1.0
Strychnine nitrate. . . .	6.486	6.063	6.204	-0.423	-0.282	+0.141	≡	≡	Rod.	0.3×0.5-0.8×2.0
Brucine.	6.204	5.781	5.499	-0.423	-0.705	-0.282	++	++	"	"
Chinoline.	4.724	4.724	5.217	0.000	+0.493	+0.493	+	++	"	0.2×0.5-0.5×1.8
Chinine sulfate.	6.204	5.922	6.345	-0.282	+0.141	-0.423	-	-	-	-
Chinine hydrochloride. .	5.640	5.640	5.499	0.000	-0.191	-0.141	-	-	-	-
Morphine hydrochloride.	5.358	4.935	4.935	-0.423	-0.423	0.000	≡	≡	Rod air bubble.	0.3×1.0-0.8×2.5
Sodium succinate. . . .	4.230	4.089	3.948	-0.141	-0.282	-0.141	≡	≡	Rod.	0.3×0.8-0.6×1.5
Anthraquinone.	4.512	4.371	4.512	-0.141	0.000	+0.141	≡	≡	"	0.4×0.5-0.6×1.5
Emodine.	4.935	4.794	4.935	-0.141	0.000	+0.141	≡	≡	"	0.3×0.5-0.6×1.2
Yeast water.	4.371	4.512	4.486	+0.141	+0.115	-0.026	≡	≡	Short rod.	0.3×0.4-0.4×0.8

Notes: 1.) The increase and decrease of nitrogen are denoted by + and - respectively. 2.) The number of + indicates the rate of growth.
3.) 'With'=inoculated; 'Without'=not inoculated.

Table 5.
Influence of Chemicals on Bean Nodule Bacteria.

Chemicals.	N in 50 cc. medium.			Change in N.			Rate of growth by days.		Bacterial cells after 21 days.	
	Initial.	After 21 days incubation.		'Without' Initial.	'With' Initial.	'With' Without'.	7	21	Form.	Size. (μ)
		'Without'.	'With'.							
Control.	(mg.) 0.141	(mg.) 0.000	(mg.) 0.000	(mg.) -0.141	(mg.) -0.141	(mg.) 0.000	+	+	Short rod.	0.3×0.4-0.5×0.6
Guanidine.	13.959	13.395	12.831	-0.564	-0.128	-0.564	≡	≡	"	0.2×0.5-0.5×1.0
Pyridine.	5.076	4.371	4.512	-0.705	-0.564	+0.141	≡	≡	Rod.	0.3×0.5-0.8×1.8
Caffeine.	10.998	10.857	10.787	-0.141	-0.211	-0.070	≡	≡	Rod coccic.	0.3×0.4-0.6×1.5
Strychnine.	5.922	5.358	6.204	-0.564	+0.282	+0.846	++	++	Short rod.	0.3×0.5-0.5×1.0
Strychnine nitrate. . . .	6.063	6.416	6.204	+0.353	+0.141	-0.212	≡	≡	Rod.	0.3×0.5-0.6×1.5
Brucine.	5.076	4.794	4.794	-0.282	-0.282	0.000	≡	≡	"	"
Chinoline.	5.006	4.935	5.288	-0.071	+0.282	+0.353	++	≡	Rod air bubble.	0.3×0.5-0.8×2.5
Chinine sulfate.	5.640	5.922	5.288	+0.282	-0.352	-0.634	-	+	Short rod.	0.2×0.4-0.3×0.8
Chinine hydrochloride. .	5.570	5.640	5.499	-0.070	-0.071	-0.141	+	+	Rod.	0.2×0.5-0.6×1.2
Morphine hydrochloride.	4.935	4.653	4.512	-0.282	-0.423	-0.141	≡	≡	"	0.2×0.5-0.7×1.3
Sodium succinate. . . .	4.371	3.948	4.089	-0.423	-0.282	+0.141	≡	≡	"	0.3×0.5-0.8×1.8
Anthraquinone.	4.301	3.948	4.089	-0.353	-0.212	+0.141	≡	≡	"	0.3×0.5-0.6×1.6
Emodine.	4.653	4.371	4.301	-0.282	-0.352	-0.070	≡	≡	Rod air bubble.	0.3×0.5-0.7×2.5
Yeast water.	4.089	4.230	3.807	+0.141	-0.282	-0.423	≡	≡	Rod.	0.3×0.8-0.8×1.2

Notes: 1.) The increase and decrease of nitrogen are denoted by + and - respectively.

2.) The number of + indicates the rate of growth.

3.) 'With'=inoculated; 'Without'=not inoculated.

Table 6.
Influence of Chemicals on Clover Nodule Bacteria.

Chemicals.	N in 50 cc. medium.			Change in N.			Rate of growth by days.		Bacterial cells after 21 days.	
	Initial.	After 21 days incubation.		'Without' Initial.	'With' Initial.	'With' Without'.	7	21	Form.	Size. (μ)
		'Without'.	'With'.							
Control.	(mg.) 0.141	(mg.) 0.000	(mg.) 0.000	(mg.) -0.141	(mg.) -0.141	(mg.) 0.000	+	+	Short rod.	0.2×0.5-0.5×1.0
Guanidine.	14.241	14.100	13.999	-0.141	-0.282	-0.101	≡	≡	Rod.	0.3×0.7-0.5×1.5
Pyridine.	5.781	5.217	5.217	-0.564	-0.564	0.000	≡	≡	"	0.4×0.8-0.5×1.2
Caffeine.	11.633	11.069	10.787	-0.564	-0.846	-0.282	≡	≡	Rod air bubble.	0.4×1.0-1.0×3.0
Strychnine.	6.275	6.811	6.881	+0.536	+0.606	+0.070	≡	≡	"	0.3×0.8-0.9×2.5
Strychnine nitrate. . . .	6.909	6.486	6.909	-0.423	0.000	+0.423	≡	≡	Rod.	0.3×0.5-0.6×1.2
Brucine.	6.204	5.395	6.063	-0.809	-0.141	+0.668	≡	≡	"	0.3×0.5-0.7×1.5
Chinoline.	5.922	5.922	5.922	0.000	0.000	0.000	-	+	Short rod.	0.3×0.5-0.5×0.8
Chinine sulfate.	6.345	5.781	5.781	-0.564	-0.564	0.000	-	-	-	-
Chinine hydrochloride. .	6.063	6.415	6.415	+0.352	-0.352	0.000	-	-	-	-
Morphine hydrochloride.	5.781	5.781	5.640	0.000	-0.141	-0.141	≡	≡	Rod air bubble.	0.3×0.9-1.0×2.8
Sodium succinate. . . .	4.935	4.512	4.512	-0.423	-0.423	0.000	≡	≡	Rod.	0.3×0.5-0.6×1.2
Anthraquinone.	5.076	4.230	4.230	-0.846	-0.846	0.000	≡	≡	"	"
Emodine.	5.640	5.499	5.640	-0.141	0.000	+0.141	≡	≡	"	0.3×0.6-0.7×1.5
Yeast water.	4.794	4.442	4.160	-0.352	-0.634	-0.282	≡	≡	Short rod.	0.3×0.5-0.6×0.8

Notes: 1.) The increase and decrease of nitrogen are denoted by + and - respectively. 2.) The number of + indicates the rate of growth.
3.) 'With'=inoculated, 'Without'=not inoculated.

The increase of nitrogen of inoculated over uninoculated was noted in four cases, decreased also in four cases, and no change in seven cases. The greatest increase was 0.668 mg. of brucine and that of caffeine and yeast water was 0.282 mg.

The rate of growth was very similar to that of Genge nodule bacteria and no growth was possible in chinine while the large organisms were found in caffeine, strychnine and morphine hydrochloride.

From the results obtained in the foregoing experiments, the change of nitrogen contents will be shown in Table 7.

Table 7.
Influence of Chemicals on the Change of Nitrogen Contents
in Nodule Bacterial Culture.

Chemicals.	'Without'-Initial.			'With'-Initial.			'With'-'Without'.					
	Number of strains.			Number of strains.			Number of strains.					
	+	-	±	+	-	±	+	-	±			
Control.	0	4	1	(mg.) -0.1974	0	5	0	(mg.) -0.2538	0	2	3	(mg.) -0.0564
Guanidine.	1	4	0	-0.1974	1	4	0	-0.3384	1	4	0	-0.1330
Pyridine.	1	3	1	-0.2678	0	5	0	-0.3666	1	3	1	-0.0988
Caffeine.	1	4	0	-0.2114	3	2	0	-0.0356	2	3	0	+0.1618
Strychnine.	1	3	1	-0.1890	4	1	0	+0.3890	5	0	0	+0.5780
Strychnine nitrate. . . .	3	2	0	+0.0706	3	1	1	+0.1550	3	2	0	+0.0844
Brucine.	1	4	0	-0.378	1	4	0	-0.1832	2	2	1	+0.0464
Chinoline.	2	1	2	+0.1972	4	0	1	+0.296	2	2	1	+0.0988
Chinine sulfate.	1	4	0	-0.1974	2	3	0	-0.1550	2	1	2	+0.0424
Chinine hydrochloride. .	3	1	0	-0.1974	2	3	0	-0.0080	0	3	2	-0.0846
Morphine hydrochloride.	1	2	2	-0.1128	2	3	0	-0.0704	1	3	1	-0.0704
Sodium succinate. . . .	1	4	0	-0.1692	0	5	0	-0.2396	1	3	1	-0.1552
Anthraquinone.	1	4	0	-0.2538	1	3	1	-0.2256	2	1	2	-0.0846
Emodine.	1	3	1	-0.0846	0	3	2	-0.1692	3	2	0	+0.0562
Yeast water.	4	1	0	+0.0988	3	2	0	-0.0614	1	4	0	-0.1614

As shown above, in a majority of cases, nitrogen decreased in a series of uninoculated over the initial, and the increase took place in strychnine nitrate, chinine hydrochloride and yeast water so far as the cultures are concerned while the nitrogen increase was noted only in strychnine nitrate, chinoline and yeast water and in all the other cases, the nitrogen decreased.

Among the inoculated over the initial, the increase of nitrogen was found in caffeine, strychnine, strychnine nitrate, chinoline and yeast water so far as the

cultures are concerned and the others all decreased. The nitrogen increased in strychnine, strychnine nitrate and chinoline.

In the inoculated over the uninoculated, the nitrogen increased in strychnine, strychnine nitrate and emodine as the number of cultures are concerned, and that of decrease was noted in guanidine, pyridine, caffeine, chinine hydrochloride, morphine hydrochloride, sodium succinate and yeast water, and in a majority of the controls, no appreciable change took place. As the number of cultures is concerned, the cases where the increase took place were about the same with those decreased while the nitrogen alone is considered the increase took place in seven more cases than the decrease among which strychnine was very marked.

So far as the growth is concerned, it is very similar to that of the preceding report¹⁾ and the good growth took place in guanidine, morphine hydrochloride, anthraquinone and pyridine, and the poor growth was obtained in chinine compounds and chinoline. With a few exception, the large cells were found in caffeine and strychnine, and no marked difference was observed from those on the solid medium.

Discussions.

In connection with the question of the nitrogen fixation by the nodule bacteria, it has been a problem as to the method of determination of nitrogen in the control. The following two methods have been generally employed: 1.) the nitrogen in the culture medium is determined right after the inoculation and the amount of nitrogen found then is taken as the control, and after a definite interval, the determination of nitrogen is made to find the difference; 2.) right after the inoculation, a disinfectant is added to destroy the inoculant and incubated along with the normally inoculated culture, and the nitrogen is determined in both at the different intervals to ascertain the difference. According to LÖHNIS⁷⁾, it was reported that the nitrogen in some kind of culture medium for the nodule bacteria decreases during the incubation but when the same medium was inoculated with the nodule bacteria, the original nitrogen was assimilated by the bacteria so that the loss of nitrogen is prevented, and often the difference of nitrogen contents in these two cases is considered by mistake as the amount of nitrogen fixed by the nodule bacteria. In this experiment, the difference of nitrogen contents between the initial and the inoculated series was taken as the index, and as the results, in all the cases except two or three, the nitrogen decreased as reported by LÖHNIS. Thus in both cases where the difference between the inoculated and the initial, and between the inoculated and the uninoculated, the error caused by the incubation could not be eliminated. In the former, the amount of nitrogen fixed is noted extremely small while the latter calculation give too large figure. For these reasons, the fixation of nitrogen by the nodule bacteria has been subjected to various discussions by the different investigators. Further it is questioned as to the nitrogen might have been brought in with the inoculum but

it is very small in our case. As it was reported previously²⁾, 20 cc. of four days old culture contained less than 10 mg. of nitrogen and in a majority of cases 5—8 mg. of nitrogen was found, and for inoculation, 20 cc. were diluted to 100 cc. and only 1 cc. was taken so that the nitrogen which was added by the inoculum was more or less than 0.1 mg. Again the bacteria cells contain about 10 per cent of nitrogen so that the bacterial cells alone carry only 0.01 mg. of nitrogen which is insignificant. Considering these factors, it seems to be reasonable to take the difference of nitrogen contents between the inoculated and the initial as the index of nitrogen fixation by the nodule bacteria in such an investigation as this.

As to the method of determination of nitrogen, HOPKINS⁸⁾ recommended DAVISSON-PARSON method⁹⁾ since GUNNING method does not give the satisfactory results in the presence of nitrate nitrogen but KJELDAHL method was used in this investigation.

Further concerning the fixation of nitrogen by the leguminous seeds and their relation to alkaloids, VITA¹⁰⁾, HARITANTIS¹¹⁾, GIRTSCHANOFF¹²⁾, and others had presented their different views which have interesting connection with the results obtained in this investigation in regards to the influence of strychnine, strychnine nitrate and caffeine on the nodule bacteria, although BARTHEL¹³⁾ failed to obtain the increase of nitrogen by adding caffeine to the nodule bacterial culture.

The growth as well as the size of cells of the nodule bacteria obtained in this investigation varied somewhat from those observed in the preceding investigation²⁾ but the general tendency was very similar. It was not recognized in this investigation that the liquid culture favors the formation of bacteroids better than the solid medium.

Summary.

In this investigation, the influence of some stimulating chemicals with special reference to the alkaloids on the nitrogen fixation by the nodule bacteria was investigated by using three strains of Genge, one of each bean and clover nodule bacteria, and the yeast-mannit liquid culture medium was used as the basic medium. Also the chemical analyses of the bacterial cells themselves were made since their composition has an important relation to the nitrogen fixation. The results obtained are summarized as follows:

- 1.) The cells of nodule bacteria contained about 5—10 per cent of nitrogen, 45—50 per cent of carbon, 10 per cent of hydrogen and 2—4 per cent of ash.
- 2.) The fixation of nitrogen by the nodule bacteria as a whole was not clearly shown since the variation of nitrogen was within the experimental error except strychnine which showed the increase of nitrogen somewhat clearly.
- 3.) With a few exception, the nitrogen content in the culture medium decreased during the incubation.
- 4.) The growth, morphology and the size of cells were about the same in both cases of solid or liquid medium.

5.) No definite relation between the morphology of cells and the fixation of nitrogen was noted in general but in case of strychnine the cells were large where the fixation of some nitrogen took place.

The authors wish to acknowledge with thanks, the financial support rendered by the Japanese Society for Promotion of Scientific Investigation (Nippon Gakuzyutu Sinko Kwai) to carry out a part of investigation.

Literature.

- 1.) ITANO, A. and A. MATSUURA, In manuscript.
 - 2.) Ibid.
 - 3.) HOPKINS, E. W., PETERSON, W. H., and FRED, E. B., *Jour. Biol. Chem.*, 85: 21, 1929.
 - 4.) VIRTANEN, A. I., und von HAUSEN, S., *Ztschr. Pfl. Diing. Bodenk., A.*, 21: 57, 1931.
 - 5.) OMELIANSKY, W. I., und SIEBER, N. O., *Ztschr. Phys. Chem.*, 88: 445, 1913.
 - 6.) RUCHANAN, R. E. and FULMER, E. I., *Physiology and Biochemistry of Bacteria*. 1930.
 - 7.) LÖHNIS, M. P., *Zentbl. Bakt.*, 2 Abt., 80: 342, 1930.
 - 8.) HOPKINS, E. W., *Soil Science*, 28: 433, 1929.
 - 9.) DAVISSON, B. S., and PARSONS, J. T., *Jour. Ind. Eng. Chem.*, 11: 306, 1919.
 - 10.) VITE, N., *Biochem. Ztschr.*, 252: 278, 1932.
 - 11.) HARITANTIS, B. J., *Ztschr. Pfl. Diing. Bodenk., A.*, 34: 257, 1934.
 - 12.) GIRTSCHANOFF, K., *Zentbl. Bakt.*, 2 Abt., 92: 349, 1935.
 - 13.) BARTHEL, C., *Meddel. 308 Centralanst. Försöksv. Jordbruksomradet Bakt. Avd. 43.*
(Abstract.)
-