THE INFLUENCE OF MOLYBDENUM, CALCIUM AND AGAR ON NITROGEN FIXATION BY *AZOTOBACTER INDICUM.*

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(Plate xxiii; two Text-figures.)

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Introduction.

Azotobacter indicum Starkey and De (1939) was isolated from rice soils in India and was found to grow and fix nitrogen over a range of reaction from pH about 3 to about 9 (Starkey, 1939). It thus forms a remarkable contrast to other species of *Azotobacter* in which nitrogen fixation ceases at pH 6 and less (e.g., Burk *et al.*, 1934). It is not known whether *Az. indicum* also occupies a special position with regard to molybdenum (or vanadium), which is essential or at least stimulating for nitrogen fixation by other *Azotobacter* species (e.g., Horner *et al.*, 1942), and calcium, which appears to be a generally essential element (Krzemieniewska, 1910; Burk and Lineweaver, 1931; Horner and Burk, 1934; Burk and Burris, 1941). Some experiments in this direction have been made with a strain of *Az. indicum* kindly supplied to me by Dr. R. L. Starkey, New Jersey Agricultural Experiment Station, New Brunswick, N.J., U.S.A.

Methods.

The basal medium had the following composition, unless otherwise stated: carbon compound (mostly sucrose), 20·0 gm.; K_2HPO_4, 0·25 gm.; KH_2PO_4, 0·25 gm.; $MgSO_4$, 0·2 gm.; NaCl, 0·2 gm.; $FeSO_4$, 0·02 gm.; distilled water, 1,000 ml. Calcium, usually as chloride, and molybdenum, as sodium molybdate, were added in varying concentration. The salts were of ordinary analytical purity. Duplicate, or sometimes triplicate, cultures were grown in 25 ml. medium in 250-c.c. Erlenmeyer flasks or round flasks of Pyrex glass; the former gave more rapid growth, probably owing to the larger surface area of the liquid (cf. Wilson and Burris, 1947), but upon the whole the growth in favourable media was very vigorous and accompanied by abundant mucus formation, which after a few weeks gave the cultures the appearance of a flour-paste. Incubation took place at 30–32°C., in some cases 35–36°C.; the temperature seemed to make little difference within these limits. Nitrogen was determined by the Kjeldahl method, with selenium as a catalyst and n/28 sulphuric acid and sodium hydroxide for the titration. The whole culture was analysed, except in a few instances, when an aliquot was taken for glucose determination by the method of Lane and Eynon. The nitrogen content of cultures analysed immediately after inoculation was subtracted from that of the incubated cultures to give the net gain of nitrogen, which in the subsequent tables is always expressed as mgm. per 25 ml. medium. Control cultures of *Pseudomonas pyocyanea*, *Rhizobium meliloti* and *Aspergillus niger*, incubated together with the cultures of *Azotobacter*, showed after two to four weeks mere traces of growth and no significant gains of nitrogen (less than 0·1 mgm.); assimilation of combined nitrogen from the incubator-atmosphere may thus be practically disregarded. The inoculum of *Az. indicum* consisted of a drop of cell suspension from young agar slope cultures, or of liquid cultures in Mo- or Ca-free solution when these elements were to be tested.

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Experimental Results.

Preliminary Tests. Very little nitrogen (0·1 to 0·7 mgm. per flask in 14 or 15 days) was fixed in glucose or sucrose medium to which no molybdenum was added. The fixation rose to 4·0–6·5 mgm. in medium with 5 parts per million of sodium molybdate, but the same quantity of vanadium sulphate or ammonium vanadate had no effect. Nitrogen fixation was equally strong in the presence and absence of 0·025% calcium chloride; 0·2% potassium oxalate had no inhibitory effect on *Az. indicum*, although it completely suppressed nitrogen fixation by *Az. chroococcum*. In subsequent experiments, where molybdenum was not the factor tested, 5 p.p.m. sodium molybdate (= 2·4 p.p.m. Mo) was added; calcium was only added where actually stated.

Various organic growth factors, such as pure biotin, potato extract, lucerne-root extract and soil extract, did not significantly increase nitrogen fixation, but 0·1% agar had a conspicuous effect on the fixation, which in ten days amounted to 9·5–10·0 mgm. in medium with agar, against 1·5–2·3 mgm. in control medium (cf. Rippel, 1936).

Qualitative tests with various sources of carbon (organic acids as sodium salts) showed no growth, or trace only, with butyl alcohol, butyric acid, valeric acid, citric acid, xylose, maltose, lactose, and starch. Scant growth was produced with ethyl alcohol, acetic acid, and lactic acid, but fair to good growth with glycerol, mannitol, sorbitol, succinic acid, malic acid, arabinose, glucose, levulose, galactose, sucrose, raffinose, and inulin.

Experiments with Molybdenum. The source of carbon in these tests was commercial sucrose (ordinary white table sugar), which previous experiments with *Az. chroococcum* had shown to be very poor in available molybdenum and/or vanadium, and which was further purified by filtration through animal charcoal (cf. Horner *et al.*, 1942). In the first experiment a strongly nitrogen-fixing strain of *Az. chroococcum* was grown in medium with 0·1% calcium carbonate for comparison with *Az. indicum*.

The figures in Table 1 show that both species fix only a very small, and *Az. indicum* hardly a significant, amount of nitrogen in the molybdenum-free medium. The stimulating effect of molybednum on *Az. indicum* begins at a concentration of 0·1 to 1·0γ per litre, and increases with some irregularity up to 1 mgm. per litre. Vanadium is entirely without effect. It is further noteworthy that the omission of calcium carbonate from the medium causes almost complete cessation of the nitrogen fixation by *Az. chroococcum*, while *Az. indicum* grows well in the calcium-free medium with sufficient molybdenum concentration. Its growth, however, is much slower than that of *Az. chroococcum*.

Higher concentrations of molybdenum, and another form of vanadium, were tested in a second experiment, recorded in Table 2. The gains of nitrogen are somewhat higher and more consistent than in Table 1, and the optimum seems to be reached at a molybdenum concentration of 0·1 p.p.m. Vanadium is ineffective also as sulphate. These results show clearly that the nitrogen-fixing enzyme system of *Az. indicum* resembles that of other *Azotobacter* species in so far as molybdenum is stimulatory and apparently essential for its activity, but it differs by not responding to vanadium; in this respect *Az. indicum* resembles several strains of nitrogen-fixing clostridia (Jensen and Spencer, 1947).

Experiments with Calcium. Media with pure sucrose (Gurr, "Bacteriological") and glucose (Mallinckrodt) were first tested, with increasing doses of calcium chloride. An attempt was made to purify the sucrose medium further by the procedure of Waring and Werkman (1942): repeated addition of 8-hydroxyquinoline and extraction with chloroform at pH 10. (According to Prodinger (1940), calcium is completely precipitated by 8-hydroxyquinoline at pH 9·2 and above.) This, however, proved unsuccessful; less than 0·1 mgm. Ca was extracted from a solution of 20 gm. commercial sucrose which by direct analysis was found to contain approximately 0·5 mgm. Ca.

As shown in Table 3, vigorous growth and nitrogen fixation took place, but calcium has no stimulating effect; neither is there any sign of inhibition by calcium carbonate

TABLE 1.
Effect of Molybdenum on Nitrogen Fixation by Azotobacter indicum *and* Azotobacter chroococcum.

Az. indicum.		*Az. chroococcum.*	
Medium.	Gain of N, mgm. per Culture.	Medium (+0·1% $CaCO_3$).	Gain of N, mgm. per Culture.
Control (−Mo)	0·09–0·11	Control (−Mo)	0·19–0·23
0·0001 p.p.m. Mo	0·20–0·20		
0·001 ,, ,,	0·38–0·59	0·001 p.p.m. Mo	0·34–0·44
0·002 ,, ,,	0·73–0·75		
0·005 ,, ,,	0·83–1·04		
0·01 ,, ,,	1·56–2·32		
0·05 ,, ,,	1·74–2·42		
0·1 ,, ,,	1·35–1·53		
1·0 ,, ,,	2·25–3·93	1·0 p.p.m. Mo	7·81–8·03
0·2 ,, V	0·08–0·10	(1·0 ,, ,, −$CaCO_3$)	0·14–0·24
2·0 ,, ,,	0·07–0·11		

25 ml. sucrose medium in 250 c.c. round flasks. Initial N-content, 0·08 mgm. Incubation 18 days 35° C. (*Az. chroococcum* 8 days). Vanadium as NH_4VO_3.

TABLE 2.
Effect of Molybdenum on Nitrogen Firation by Azotobacter indicum.

Medium.	Gain of N, mgm. per Culture.
Control (−Mo)	0·13–0·14
0·1 p.p.m. Mo	3·81–4·10
1·0 ,, ,,	3·65–4·28
5·0 ,, ,,	3·60–3·69
10·0 ,, ,,	4·14–4·48
1·0 ,, V (as $VOSO_4$)	0·16–0·19

25 ml. sucrose medium in 250 c.c. round flasks. Initial N-content, 0·07 mgm. Incubation 21 days 30–32° C.

in the glucose medium, as reported by Starkey (1939). It thus appears that calcium is neither essential nor stimulatory, or else the impurities of the medium must have been sufficient.

The calcium content of the sugars was determined by ashing 30 gm. sugar in a silica basin and taking up the minute residue in a small volume of hot dilute hydrochloric acid from which the calcium was precipitated with ammonium oxalate at pH 5. The precipitate was collected and washed by centrifugation, and calcium was determined by titration with n/20 potassium permanganate, using a microburette with 0·01 ml. divisions. The glucose and the sucrose were found to contain 0·20 and 0·23 mgm. Ca, respectively, in 30 gm. The sugars thus impart contents of 0·13 and 0·16 p.p.m. calcium to the medium when added in amounts of 20 gm. per litre. These quantities together with the possible impurities of the other constituents thus seem fully to cover the calcium requirements (if any) of *Az. indicum*.

A more purified medium was made up in the following way. Concentrated solutions (1/20 of the final volume) were prepared in two portions, the phosphate being kept separate from the rest of the constituents, sufficient potassium oxalate was added to give a final concentration of 0·1%, the solutions were left overnight, the slight precipitates were removed by centrifugation, and the medium was finally made up to

volume with triple-distilled water as used for blood transfusion. The phosphate was supplied as sodium salts in order to avoid ionic imbalance. Calcium was added as 0·1% calcium chloride, which suffices to precipitate the oxalate and leave a small surplus of calcium in the solution.

The results, which are given in Table 5, show good growth in the calcium-free medium and no beneficial effect of the calcium; in fact, one culture in glucose medium shows an inexplicable reduction in nitrogen fixation.

TABLE 3.
Nitrogen Fixation by Azotobacter indicum *in Medium with varying Calcium Content.*

Sucrose Medium.	Gain of N, mgm. per Culture.	Glucose Medium.	Gain of N, mgm. per Culture.
Control (–Ca)	3·39–3·42	Control (–Ca)	6·51–7·09
20 p.p.m. Ca	2·46–2·78	10 p.p.m. Ca	5·63–6·11
100 ,, ,,	3·51–4·15	100 ,, ,,	6·01–6·29
250 ,, ,,	3·88–3·03	250 ,, ,,	5·27–5·76
500 ,, ,,	2·55–2·85	250 ,, ,, (as $CaCO_3$)	6·82–lost

Sucrose medium: 25 ml. in 250 c.c. round flasks. Initial N-content, 0·06 mgm. Incubation 20 d. 30–32° C. Glucose-medium: 25 ml. in 250 c.c. Erlenmeyer flasks. Initial N-content, 0·06 mgm. Incubation 19 d. 30–32° C.

TABLE 4.
Nitrogen Fixation by Azotobacter indicum *in Oxalate-purified Medium.*

	Glucose Medium.		Sucrose Medium.	
	Control (–Ca).	+0·1% $CaCl_2$.	Control (–Ca).	+0·1% $CaCl_2$.
Gain of N, mgm. per culture—				
a	7·70	7·72	9·22	8·52
b	7·88	(3·92)	9·47	7·33
c	7·29	7·49	9·60	9·46
Mean ..	7·6	6·4 (7·6)	9·4	8·6

25 ml. medium in 250 c.c. Erlenmeyer flasks. Initial N-content, 0·07 mgm. (glucose), 0·10 mgm. (sucrose). Incubation 18 days. 30° C.

To eliminate the possible effect of the small amount of calcium remaining in solution as oxalate, an experiment was performed in medium made up with triple-distilled water and extra pure glycerol, distilled *in vacuo* from A.R. grade glycerol, which could be regarded as practically free from calcium. Since glycerol is only a moderately good source of carbon, 3% was given instead of the customary 2%, and the cultures were incubated for four weeks. The results in Table 5 show vigorous nitrogen fixation in the calcium-free medium and, except for a remarkably high gain in one of the cultures with 100 p.p.m. Ca, no stimulating effect of calcium chloride. Calcium carbonate, at least in the higher dose, shows in this case a definite inhibitory effect.

A final experiment was designed to provide for the possibility of small amounts of calcium coming into solution from the Pyrex glass, which was stated by the manufacturers to contain only 0·4% CaO. The cultures were grown in silica evaporation dishes of 9 cm. diameter, covered with Petri dish lids. The medium contained 2% vacuum-distilled glycerol; one portion had the customary composition and was given

two concentrations of calcium chloride; another portion was made up with sodium phosphate and potassium oxalate as above (no visible precipitate appeared). The result of this test is seen in Table 6. The growth in the oxalate-treated medium was slow and irregular, but good in the plain medium, and in both cases the addition of calcium shows no effect.

It seems justified to conclude that calcium is neither essential nor stimulatory for nitrogen fixation by *Az. indicum*, or else quite infinitesimal amounts must be sufficient for maximum yield. In this respect as well as with regard to reaction *Az. indicum* thus differs strikingly from *Az. chroococcum*, which for optimum nitrogen fixation requires a supply of 0·3–0·4 mgm. calcium per gm. glucose (Krzemieniewska, 1910).

TABLE 5.
Nitrogen Fixation by Azotobacter indicum *in Pure Glycerol Medium.*

Medium.	Gain of N, mgm. per Culture.
Control (-Ca)	3·91–4·05
10 p.p.m. Ca as $CaCl_2$	3·77–3·85
100 ,, ,, ,, ,,	3·90–5·60
250 ,, ,, ,, ,,	3·38–3·76
160 ,, ,, ,, $CaCO_3$	2·87–3·03
500 ,, ,, ,, ,,	1·82–2·57

25 ml. 3% glycerol medium in 250 c.c. Erlenmeyer flasks. Initial N-content, 0·05 mgm. Incubation, 28 days 30–32° C.

TABLE 6.
Nitrogen Fixation by Azotobacter indicum *in Glycerol Medium with and without Oxalate.*

Medium.	Gain of N, mgm. per Culture.
Plain, −Ca	4·28–4·72
,, 10 p.p.m. Ca	4·22–4·37
,, 100 ,, ,,	2·82–4·10
Oxalate-purified, −Ca	1·23–2·04–2·59
,, ,, +0·1% $CaCl_2$	0·52–2·87–2·88

25 ml. glycerol medium in silica basins. Initial N-content, 0·08 mgm. Incubation, 21 d. 30–33° C.

Experiments with Agar. The effect of agar, observed in the preliminary tests, was studied in more detail in the customary medium with sucrose and 0·1% fibrous agar. Two other colloidal substances, soluble starch and pectin (commercial, from citrus fruits), were also tried, and a test with calcium carbonate was included. The results are seen in Table 7. Corrections were made for the small amounts of nitrogen present as impurities in starch (0·07%), agar (0·14%) and pectin (0·38%).

The accelerating effect of agar on the growth is very striking, especially after ten days. Starch has a similar but lesser effect, while pectin is inactive. Calcium carbonate seems to have no significant influence in this case.

Another experiment with starch (Table 8) confirms the small stimulating effect of starch in comparison with agar, and shows that the starch itself does not serve as material for nitrogen fixation.

The fact that *Az. indicum* is not stimulated by various organic substances, as observed in the preliminary tests, renders it unlikely that the effect of agar was due

TABLE 7.

Effect of Colloidal Substances and Calcium Carbonate on Nitrogen Fixation by Azotobacter indicum.

Medium.	Gain of N, mgm. per Culture, after		
	5 Days.	10 Days.	20 Days.
Control	0·61–0·62	2·08–2·29	4·82*–6·80
0·1% agar	1·18–1·74	7·23–8·08	10·56–10·81
0·2% starch		4·45–4·68	
0·5% pectin		2·04–2·07	
1·0% $CaCO_3$			5·71– 6·25

25 ml. sucrose medium in 250 c.c. Erlenmeyer flasks. Initial N-content of control medium, 0·05 mgm. Incubation at 30–32° C.

* Some ammonia was lost during the distillation.

TABLE 8.

Comparative Influence of Starch and Agar on Nitrogen Fixation by Azotobacter indicum.

Medium.	Gain of N, mgm. per Culture, after	
	8 Days.	18 Days.
Control	0·89–1·29	3·70–3·98
0·2% starch	0·90–1·08	4·24–5·30
0·4% „	1·19–1·23	4·91–5·09
0·1% agar	1·53–3·02	6·30–7·22
0·4% starch (–sucrose)		0·05–0·05

25 ml. sucrose medium in 250 c.c. round flasks. Initial N-content of control medium, 0·06 mgm. Incubation at 30–32° C.

to any growth compound or other organic impurity. However, an experiment was made with varying concentration of agar purified by maceration in water for five days at 30°C., extraction with dilute acetic acid, and washing with distilled water. The results in Table 9 show that the accelerating effect increases with the agar concentration, particularly from 0·1 to 0·2%. The purification has indeed lessened the effectiveness of the agar in comparison with the untreated material, but this may well be due to a notable loss of gel strength in the purified agar (cf. Rippel, 1936), and not necessarily to removal of impurities.

A final experiment was made in 2·5% glucose medium with 0·1% untreated agar; glucose consumption as well as nitrogen fixation was estimated. The bacterial substance was precipitated, together with the agar, by addition first of a 10% solution of aluminium chloride and then of sufficient normal sodium hydroxide to precipitate the aluminium as hydroxide, which carried down most of the slimy growth and the agar, where present. The precipitate was centrifuged down, the sediment was washed with distilled water, the centrifugate was made up to a definite volume, of which an aliquot was taken for glucose determination, and separate nitrogen determinations were made in the rest of the centrifugate and in the sediment. From 7 to 20% of the total nitrogen was found in the centrifugate, but this does not represent strictly extra-cellular nitrogen, since complete precipitation of the bacterial substance was difficult to obtain. Table 10 gives the results.

The economy of nitrogen fixation, 15 to 18 mgm. per gm. consumed sugar, is much the same as in other species of *Azotobacter*. The effect of the agar seems to

TABLE 9.
Influence of Varying Agar Concentration on Nitrogen Fixation by Azotobacter indicum.

Medium.	Gain of N, mgm. per Culture.
Control (–agar)	3·33–3·34
0·05% agar, purified	4·84–5·14
0·1% „ „	5·43–5·51
0·2 „ „	7·95–8·18
0·4 „ „	8·89–9·22
0·1% „ untreated	8·30–8·32

25 ml. sucrose-medium in 250 c.c. Erlenmeyer flasks. Initial N-content of control medium, 0·09 mgm. Incubation, 12 days 30–32° C.

TABLE 10.
Nitrogen Fixation and Glucose Consumption by Azotobacter indicum.

Medium	Control (–Agar).		+0·1% Agar.	
Incubation days	8	16	8	16
Gain of N, mgm. per culture—				
a	1·39	6·52	5·88	9·51
b	1·49	6·77	6·17	9·66
Consumption of glucose, mgm. per culture				
a	92	416	332	588
b	96	415	347	588
Mgm. N fixed per gm. glucose consumed				
a	15·1	15·8	17·7	16·2
b	15·5	16·3	17·8	16·4

25 ml. medium in 250 c.c. Erlenmeyer flasks. Initial N-content, 0·12 mgm. Incubation at 30–32° C.

consist in a simple growth acceleration, in agreement with the findings of Rippel (1936). The economy of fixation in the presence of agar is only slightly higher after eight days, and after sixteen days the difference has disappeared. It is noteworthy that in another case (Table 7) the economy appears even higher, reaching at least 21 mgm. nitrogen per gm. sucrose. The growth acceleration is probably, as in other species of *Azotobacter*, due to the physical effect of the agar, which supports growth near the surface of the medium and thus provides better access of oxygen and nitrogen than in the agar-free solution, where *Az. indicum* grows exclusively as a slimy bottom-deposit.

Growth with Combined Nitrogen. The growth rates in media with free and combined nitrogen were compared in order to see if the effect of molybdenum is a specific catalysis of the process of nitrogen fixation, as in other *Azotobacter* species.

Az. indicum was first grown in glucose medium with and without 5 p.p.m. sodium molybdate, with free nitrogen as well as with 400 p.p.m. nitrogen in the form of sodium nitrate and ammonium sulphate, this quantity corresponding approximately to the highest observed yield of fixed nitrogen. Duplicate cultures were grown in 15 ml. medium in 100-c.c. Erlenmeyer flasks and tested after 5, 10 and 14 days. The growth rate was estimated by turbidity measurements with a Hilger "Spekker" electrophotometer, and by determination of residual glucose.

The results are summarized in Text-figure 1. The growth with free nitrogen is notably stimulated by molybdenum, as in the previous experiments where nitrogen

was determined. With nitrate the molybdenum appears to have, if anything, a retarding effect, and with ammonium sulphate its effect is hardly significant; the very poor growth in the last series was probably due to rapidly increasing acidity of the medium: after 10 days the initial pH of 6·6–6·7 had fallen to 3·0–3·1, and this did not change significantly after 14 days (pH 2·9–3·1).

Another experiment was performed in medium with combined nitrogen only, but with two concentrations of molybdenum: 0·05 and 5·0 p.p.m. Mo. Calcium carbonate (0·2%) was sterilized separately and added to the medium with ammonium sulphate in order to prevent acidification, and was dissolved before the turbidity measurements

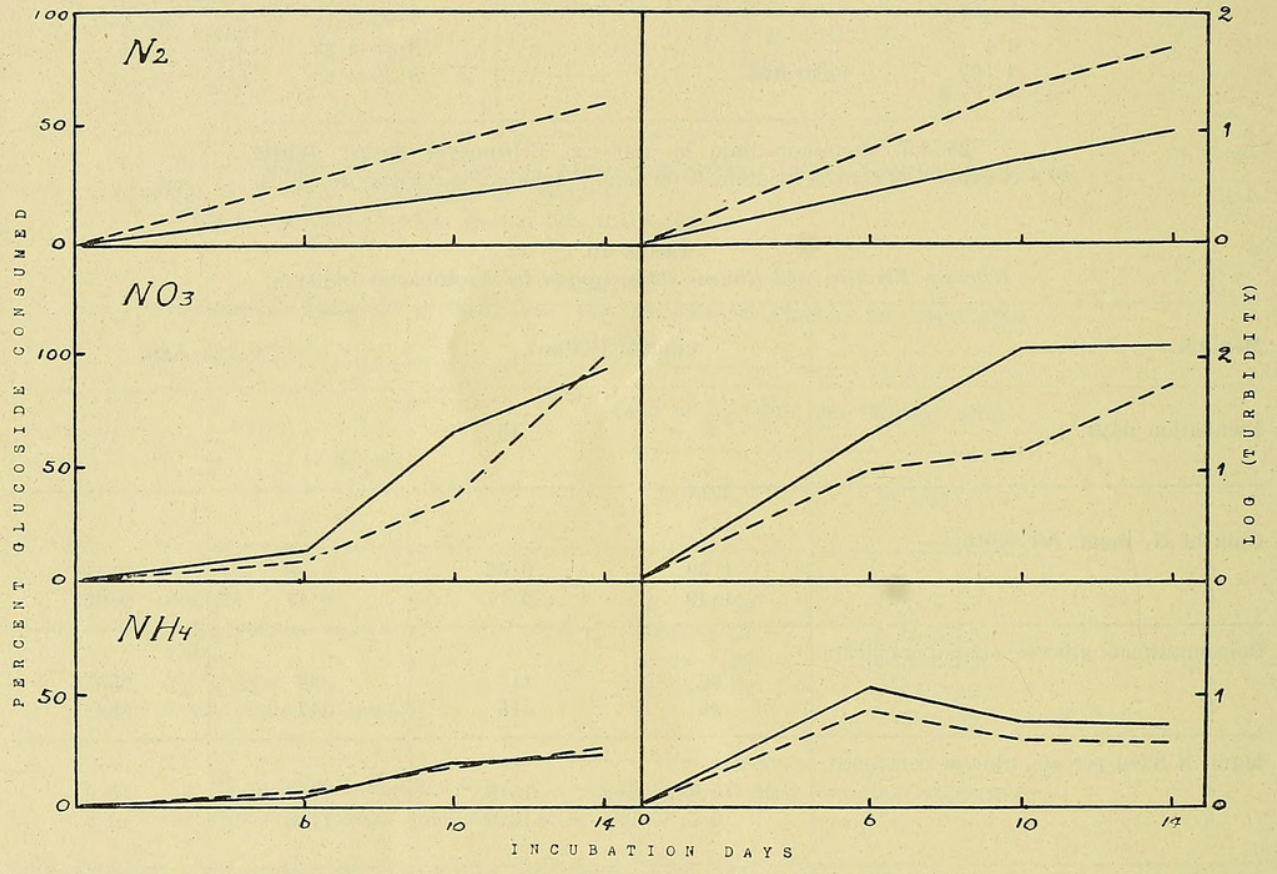

Fig. 1.—Influence of molybdenum on growth of *Az. indicum* with free nitrogen, sodium nitrate, and ammonium sulphate. Left: sugar consumption. Right: turbidity readings. Continuous lines: –Mo; broken lines, 2·4 p.p.m. Mo.

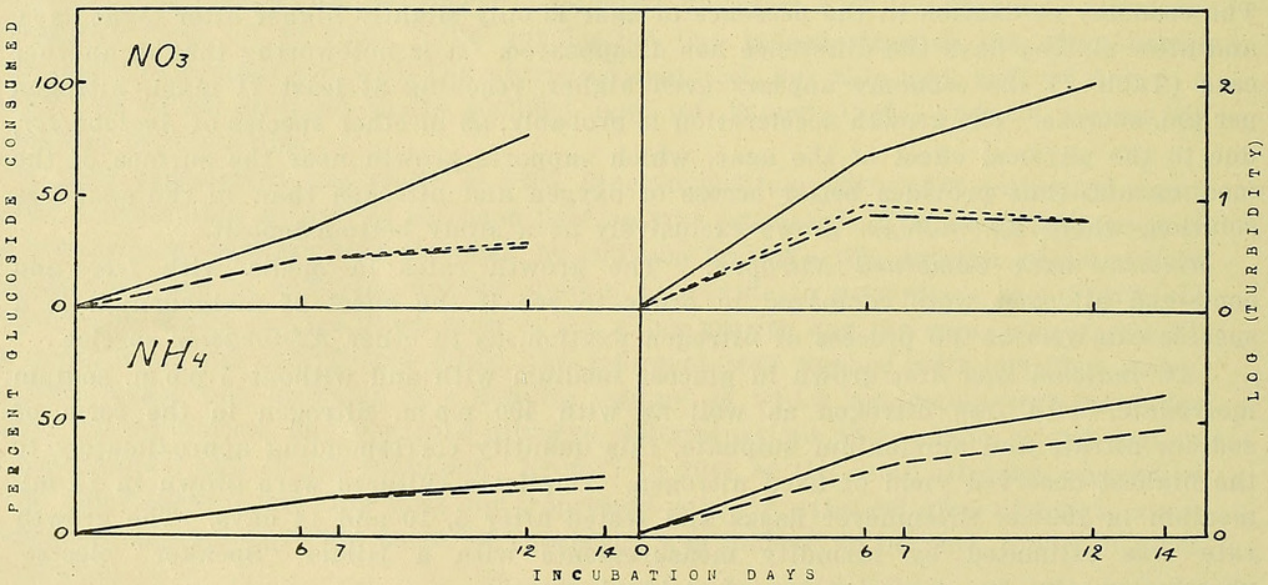

Fig. 2.—Influence of molybdenum on growth of *Az. indicum* with sodium nitrate and ammonium lactate. Left: sugar consumption. Right: turbidity readings. Continuous lines: –Mo; broken lines: 0·05 p.p.m. Mo; dotted lines: 5·0 p.p.m. Mo.

by addition of a small amount of hydrochloric acid. The inhibitory effect of molybdenum on growth with nitrate is in this case quite unmistakable, as shown in Text-figure 2. (It may here be mentioned that the sodium nitrate itself contained less than 0·1 p.p.m. Mo.) The growth with ammonium sulphate was extremely weak; the turbidity was barely visible, and only some 7 to 10% of the sugar was used up. No explanation for this remarkable inhibitory effect of the calcium carbonate can be offered. Another test was made, with nitrogen as ammonium lactate, and with 2·4 p.p.m. Mo, 20 ml. medium in 250-c.c. Erlenmeyer flasks, glucose and turbidity determinations after seven and fourteen days. The result is included in Text-figure 2, which shows that the moderately good growth with ammonium lactate is also somewhat retarded by molybdenum, although the effect on glucose consumption is not significant.

The ability of *Az. indicum* to utilize various forms of combined nitrogen was finally tested in molybdenum-free sucrose medium. Sodium nitrite, ammonium lactate, hydroxylamine (as hydrochloride), urea, glycine, alanine, asparagine, aspartic acid, glutamic acid and leucine were used in concentrations corresponding to 400 p.p.m. of nitrogen. The solutions of hydroxylamine-HCl, aspartic acid and glutamic acid were adjusted to original reaction (pH 6·6–6·7), and the solutions of hydroxylamine and urea were sterilized by Seitz-filtration instead of by autoclaving. Cultures with free nitrogen, in medium with and without 5 p.p.m. sodium molybdate, were included for comparison.

Table 11 gives the results, which show that aspartic acid is by far the best source of nitrogen, and the only one equal to elementary nitrogen (with molybdenum). Good growth is also produced with glutamic acid, leucine, and ammonium lactate, but asparagine is an inferior source of nitrogen, and urea gives little better growth than free nitrogen in the absence of molybdenum (cf. Starkey, 1939). The remaining four compounds are definitely inhibitory. This is hardly surprising in view of the relatively high concentration of reducing compounds like hydroxylamine and nitrite, but it is

TABLE 11.
Growth of Azotobacter indicum *with Different Sources of Nitrogen.*

Source of N.	log (Turbidity).	Source of N.	log (Turbidity).
N_2	0·245–0·248	Asparagine	0·333–0·382
$NaNO_2$	0·048–0·057	Aspartic acid	1·32 –(contamin.)
NH_4-lactate	0·566–0·642	Glutamic acid	0·800–0·865
Hydroxylamine	0·012–0·015	*l*-Leucine	0·600–0·620
Urea	0·278–0·292	N_2 Mo (2·4 p.p.m.)	1·28 –1·40
Glycine	0·023–(lost)	Sterile medium	0·020
Alanine	0·022–0·022		

20 ml. Mo-free sucrose medium in 250 c.c. round flasks. Incubation 12 days at 32–35° C.

TABLE 12.
Influence of Glycine and Alanine on Growth of Azotobacter indicum.

Source of Nitrogen.	log (Turbidity).
Free nitrogen	1·61 –1·73
Glycine, 80 p.p.m. N	0·822–1·17
,, 400 ,, ,, ...	0·036–0·038
Alanine, 80 ,, ,, ...	1·37 –1·57
,, 400 ,, ,, ...	0·038–0·042
Sterile medium	0·022

25 ml. sucrose-medium, with 5 p.p.m. Na_2MoO_4, in 250 c.c. Erlenmeyer flasks. Incubation 14 days 31–33° C.

more remarkable that glycine and alanine are not merely non-assimilable, but cause actual inhibition; however, a similar action of single amino-acids has been observed in other instances (Anderson, 1946).

Another experiment, recorded in Table 12, shows that the inhibitory action of glycine and alanine is also displayed in the presence of molybdenum; the lower concentration of glycine (80 p.p.m. N) also causes a significant partial inhibition.

Hydrogenase Production. It may finally be mentioned that a hydrogenase was found in cell material grown with free nitrogen, but not in cells grown with ammonium lactate or glutamic acid. The hydrogenase formed in sucrose and glycerol media was very active towards methylene blue as a hydrogen acceptor, but not towards nitrate, nitrite, or hydroxylamine. With respect to hydrogenase formation *Az. indicum* thus resembles other species of *Azotobacter* (Phelps and Wilson, 1941; Lee and Wilson, 1943).

Observations on Calcium Requirement of Clostridium Butyricum. Attempts were made to grow *Cl. butyricum* in a calcium-free medium in order to ascertain whether calcium is essential for nitrogen fixation by this organism. The methods and medium were the same as stated in a previous communication (Jensen and Spencer, 1947), except that the calcium carbonate was replaced by a buffer mixture of 5·7 gm. Na_2HPO_4 and 0·3 gm. KH_2PO_4 per litre. The results were not altogether conclusive, because the medium rapidly became so acid (pH 3·9–4·5) that growth ceased and only comparatively small amounts of nitrogen were fixed; higher concentrations of phosphate buffer proved inhibitory. Moreover, the potato-extract concentrate contained a certain amount of calcium and imparted a Ca content of approximately 0·3 p.p.m. to the medium. This content, however, was so low that *Az. chroococcum* was capable of practically no nitrogen fixation, and addition of calcium chloride or purification of the medium with oxalate had no significant influence on the limited amount of nitrogen fixed by *Cl. butyricum*. The results suggest that calcium is not necessary for the anaerobic nitrogen-fixing bacteria, or at least their calcium requirements must be much smaller than that of *Az. chroococcum*.

General Conclusions.

Az. indicum obviously resembles the other species of *Azotobacter* in the pronounced activation of the nitrogen-fixing mechanism by small concentrations of molybdenum, and in the formation of a hydrogenase when nitrogen fixation takes place. Certain differences also exist, not only with regard to pH-tolerance, but also in so far as *Az. indicum* is not stimulated by vanadium and is inhibited by organic nitrogen compounds like glycine and alanine. The poor growth with asparagine (in comparison with aspartic acid) and with urea and the apparent toxicity of the two amino-acids might suggest that certain amino-compounds are able to block the nitrogen-fixing enzyme by virtue of a similarity in chemical structure to some key-compound of the fixation process. With regard to the utilization of combined nitrogen, *Az. indicum* resembles *Az. chroococcum* and *vinelandii* in its behaviour towards aspartic and glutamic acid (cf. Horner and Allison, 1944), of which particularly the latter appears to occupy a key position in nitrogen fixation (Wilson and Burris, 1947), but it differs in its ability to use leucine and its poor growth with urea and asparagine. The remarkable inhibitory effect of molybdenum on growth with nitrate, which also seems to have no parallel in other *Azotobacter* species, might conceivably be due to the molybdenum giving rise to some intermediate product of nitrate less readily assimilable than the intermediates formed in the absence of molybdenum. This is of course only put forth as pure hypothesis; it must be left for future experiments to decide whether all these phenomena indicate that the process of nitrogen fixation takes partly different routes in *Az. indicum* and in the other species of *Azotobacter*.

Unlike molybdenum, which is either essential or stimulatory to all definitely known types of biological nitrogen fixation (in *Azotobacter*, the clostridia, the blue-green algae, and legumes plus root nodule bacteria) calcium is according to the evidence of the present experiments not universally necessary for nitrogen-fixing

micro-organisms, a fact in agreement with the opinion of Burk and Burris (1941) that calcium, although essential for *Az. chroococcum* and *vinelandii*, is not needed specifically for the process of nitrogen fixation by these organisms.

Summary.

Azotobacter indicum Starkey and De was found to require molybdenum for nitrogen fixation. The effect appeared to begin at a molybdenum-concentration of 0·001 to 0·0001 parts per million, and to reach its optimum at 0·1 to 1·0 p.p.m. Molybdenum could not be replaced by vanadium.

A favourable effect of calcium on nitrogen fixation could not be detected. If this element is necessary at all, quite infinitesimal amounts seem sufficient for maximum fixation.

Addition of 0·1–0·4% agar to the liquid medium accelerated the nitrogen fixation strongly; starch had a similar but smaller effect. From 15 to 18 mgm. nitrogen were fixed per gm. glucose consumed, and occasional gains of more than 20 mgm. per gm. sucrose were observed.

Growth with nitrate was retarded by 0·05 to 5·0 p.p.m. molybdenum. Nitrate, aspartic acid and glutamic acid appeared to be the best sources of combined nitrogen, followed by *l*-leucine, ammonium lactate, asparagine, and urea, in the order mentioned. Nitrite, hydroxylamine, glycine and alanine had inhibitory effects. A hydrogenase was formed in nitrogen-free media, but not in media with combined nitrogen.

Tentative experiments suggested that *Clostridium butyricum* does not require calcium for nitrogen fixation.

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EXPLANATION OF PLATE XXIII.

1. Effect of molybdenum and vanadium on nitrogen fixation by *Az. indicum* (cf. Table 1) ; from left : –Mo ; 0·001 p.p.m. Mo ; 0·01 p.p.m. Mo ; 1·0 p.p.m. Mo ; 0·2 p.p.m. V. 2. Growth of *Az indicum* in oxalate-purified glucose medium (cf. Table 4). Left, –Ca ; right, 0·1% $CaCl_2$. 3. Effect of agar on growth of *Az. indicum* in glucose medium incubated 8 days (cf. Table 10). Left, 0·1% agar ; right, – agar. (The cultures were poured into wide test tubes and photographed immediately before analysis.) (S. Woodward Smith photos.)