SOME NEW ASPECTS OF THE MECHANISM OF NITROGEN FIXATION IN THE SOIL.

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(*Read at Symposium, August 29-30, 1936.*)

During recent years a large volume of work has been directed towards the elucidation of the mechanism of non-symbiotic nitrogen fixation in the soil. These studies have been mostly carried out with pure strains of the genus *Azotobacter*. This is partly due to the historical association of those organisms and partly to their ability to fix fairly large amounts of nitrogen in the artificial media that are generally employed. On the other hand, very little information is available regarding the precise manner in which these organisms function in the soil ; as to whether they stand competition with other organisms, and the extent to which the combined activities of all the organisms contribute towards nitrogen fixation in the soil.

Much valuable work has already been carried out on the nitrogen transformations attendant on fixation. Only meagre data are available, however, regarding the changes undergone by the carbonaceous materials which are utilised in the process, the nature of their degradation products and the manner in which they are transformed into the ultimate products of fixation. The relation of these products to the growth of the organisms and their ability to fix nitrogen are also awaiting solution.

It is well known that, under ordinary field conditions, there is comparatively very little fixation of atmospheric nitrogen ; indeed, the various attempts that have been made during the past few decades to enrich the soil by artificial inoculation of *Azotobacter* and allied organisms have not proved very successful (Lipman, 1908 ; Bottomley, 1914 ; Emerson, 1918 ; Brown and Hart, 1925). On the other hand, Wilsdon and Barkat Ali (1922) and a few other workers have reported considerable increase in the nitrogen contents of certain arid tracts which could be accounted for only by fixation from the atmosphere. Moreover, a number of workers have reported periodical increase in the nitrogen contents of soils (Sahasrabuddhe and co-workers, 1928, 1931, 1936 ; Annet *et al.*, 1928, and others) which cannot be entirely accounted for by non-biological transformations. These observations would suggest that, although, under normal conditions, the extent of fixation may be almost negligible, there are yet certain special conditions under which quite large quantities may be fixed. A fuller understanding of the foregoing and other observations will be possible only if the various factors influencing the efficiency of fixation in the soil can be satisfactorily determined.

With a view to throwing some light on these and allied problems, the present enquiry was undertaken.

Experimental

Previous studies on carbon transformations during fixation of nitrogen by *Azotobacter*, particularly by Stoklasa (1908), would suggest that organisms belonging to that genus derive their nutrition chiefly from carbohydrates. Working with *Azotobacter chroococcum*, Ranganathan and Norris (1927) observed that decomposition of sugar was comparatively slow ; that the fixation of nitrogen was complete before the entire quantity of sugar was used up. Regarding these observations, it is difficult to determine whether it was due to the slow action of *Azotobacter* or to the smallness of the inoculum. Moreover, as the sugar was present all through in the medium, it is not possible to ascertain whether any of the products of fermentation was utilised in the fixation of nitrogen.

The following experiments were therefore carried out with an active strain of *Azotobacter chroococcum*, Beij., isolated from the local soil. Heavy growths of the organism were obtained on Ashby's mannite agar medium using the cellophane technique of Bhaskaran *et al.* (1936). The growth (which was free from the solid medium) was shaken up with sterile water and the resulting suspension used for inoculation. The liquid medium used for the study was similar to Ashby's original composition with the difference that glucose was substituted for mannite. At stated intervals, the distribution of carbon and nitrogen in the medium was determined according to Bhaskaran and Subrahmanyan (1936).

Table I.

Distribution of Carbon.

Time in days	Organic carbon (in mg.) in 25 c.c. of medium				Lost as gas (mg. of carbon)
	Total	Present in slime and bact. cells	Present as sugar	Present in other soluble forms	
0	92·9	0·50	92·0	0·4	*nil*
3	88·7	9·8	70·1	8·8	4·2
6	76·0	10·5	64·9	....	16·9
10	48·0	12·0	28·7	7·3	44·9
14	30·0	15·7	*nil*	14·3	62·9
18	31·6	15·6	,,	16·0	....
22	22·1	16·1	,,	6·0	70·8
26	17·8	16·2	,,	1·6	75·1

TABLE II.

Distribution of Nitrogen.

Time in days	Nitrogen (in mg.) in 25 c.c. of medium			C-N ratio of slime and bact. cells
	Total	Present in slime and bact. cells	Present in the supernatant	
0	0·14	0·04	0·10	14·25
3	0·43	0·33	0·10	29·30
6	0·95	0·82	0·13	12·80
10	1·07	1·03	0·04	11·75
14	1·20	1·09	0·11	14·30
18	1·24	1·12	0·12	13·90
22	1·28	1·24	0·06	12·8
26	1·22	1·14	0·08	14·2

There was steady fall in the total organic carbon content of the medium. The sugar was used up in the course of 14 days, but considering the heaviness of the inoculum, the progress should be regarded as being comparatively slow. The decomposition proceeded very much more rapidly in presence of the mixed flora of the soil, the entire quantity of sugar being used up in the course of 4 days (*vide infra*). The major part of the sugar was converted into gaseous products, but a small part was also taken up by the organisms. The growth of the organisms came more or less to a stop after the sugar was used up. The residual organic matter is accounted for by certain water-soluble forms, chiefly organic acids (Ranganathan and Norris, *loc. cit.*). These forms increased up to a point but were then used up by the organisms. They did not contribute to the dry weight of the organisms but were converted into gaseous forms, chiefly carbon dioxide.

A comparison of the above with the distribution of nitrogen showed that the major part of the fixation took place before the sugar was entirely used up. The subsequent fixation was almost negligible, so that it may be concluded that the residual organic matter was not utilised in the fixation. The fixed nitrogen was mostly in the slime and in the bacterial cells, but a small portion was always left in the supernatant. The C-N ratio of the growth showed wide fluctuations in the early stages but attained a more or less steady value after the 14th day.

The foregoing observations would show clearly that fixation by *Azotobacter* is closely linked up with the availability of the carbohydrate. The residual organic matter was not utilised to any appreciable extent in the fixation.

The above results are supported by observations on the growth of the organisms and storage of organic carbon and nitrogen in the cells and slime.

TABLE III.

Growth in relation to fixation of carbon and nitrogen in cells and slime.

Time in days	Bacterial count in millions per c.c.	Dry matter of cells and slime (in mg.)[1]	Organic carbon of cells and slime (in mg.)	Organic nitrogen of cells and slime
0	61·0	2·4	0·5	0·04
3	90·1	25·3	9·8	0·33
6	151·3	36·0	10·5	0·82
10	....	38·0	12·0	1·03
14	190·5	36·6	15·7	1·09
18	192·5	38·6	15·6	1·12
22	181·0	36·5	16·1	1·12
26	....	39·7	16·2	1·14

A comparison with the previous tables would show that there was no increase in bacterial numbers after the disappearance of sugars : nor was there any appreciable increase in the dry matter, organic carbon or organic nitrogen content of the cells.

An entirely different set of results was obtained when the soil itself (representing the mixed flora) was used for inoculation into a similar medium.

TABLE IV.

Distribution of carbon.

Time in days	Organic carbon (in mg.) in 50 c.c. of medium.				Lost as gas (as mg. of carbon)
	Total	Present as sugar	Present in sediment[2]	Present in supernatant	
0	173·7	170·4	....	....	....
2	111·7	83·6	13·6	94·8	62·0
4	55·0	*nil*	24·2	27·5	118·0
8	53·4	....	39·8	10·3	120·3
12	52·5	....	39·9	9·3	121·2
16	50·5	....	39·4	7·8	123·2

[1] Correction has been made for the weight of unused calcium carbonate which separated with the organisms.

[2] The soil used for inoculation contained 3·3 mg. of carbon, but correction has been applied for this.

TABLE V.

Production and Distribution of organic acids.

Time in days	Organic acids (as mg. of c. in 50 c.c. of medium)					Total
	Non-volatile	Volatile				
	Lactic	Acetic	Propionic	Butyric	Total	
2	6·1	2·7	0·6	3·8	7·1	13·2
4	12·8	3·1	0·4	3·5	7·0	19·8
8	*nil*	....	....	....	5·8	5·8
12	*nil*	....	....	....	4·8	4·8

TABLE VI.

Distribution of Nitrogen.

Time in days	Nitrogen fixed (as mg.) in 50 c.c. of medium			C-N ratio of mucilage
	Total nitrogen	Nitrogen in sediment	Nitrogen in supernatant	
2	0·78	0·39	0·39	43·9
4	1·26	0·39	0·87	62·1
8	2·42	1·93	0·49	20·6
12	3·07	2·90	0·17	13·8
16	3·15	2·95	0·20	13·4

It may be seen that the sugar was decomposed at a very rapid rate, the entire quantity being used up within 4 days. Only less than a third of the organic carbon was left behind in the medium, the rest being lost as gas. The organic carbon in the supernatant was present mostly as acids (chiefly lactic acid), but this was used up between the 4th and 8th days for conversion into microbial tissue.

The study of the nitrogen transformations also showed that the fixation took place mostly between the 4th and 12th days, that is, after the sugar was entirely used up. A small quantity of nitrogen always remained in solution, but this bore no relation to the rate of fixation. The C-N ratio tended steadily to narrow down to a constant value.

These observations were supported by direct experiments with the soluble organic matter left after the disappearance of sugar. On being sterilised and then inoculated with the mixed flora, the soluble residues fixed nearly two-thirds as much nitrogen as the original sugar itself.

TABLE VII.

Fixation of Nitrogen by the water soluble residue left after decomposition of sugar.

Time in days	Nitrogen fixed (in mg.) by 50 c.c. of:	
	Filtrate sterilised and freshly inoculated	Original sugar medium
4	0·25	2·10
8	1·62	2·62

A further observation of interest was that the supernatant liquid (consisting chiefly of the mixed calcium salts of organic acids) was used up in the subsequent fixation. The solid sediment consisting of the living cells together with the insoluble products formed in the medium did not fix any appreciable quantity of nitrogen when left as such.

TABLE VIII.

Relative efficiencies of supernatant and sediment in fixing nitrogen.

Time in days	Nitrogen fixed (in mg.) in 50 c.c. of medium		
	Supernatant	Sediment	Unsterilised (control)
4	1·25	0·39	2·10
8	1·62	0·44	2·62
12	1·67	0·44	2·77

It would be seen from the foregoing, that, in presence of the mixed flora, the carbohydrate was extremely unstable and was rapidly destroyed. The products of decomposition, though small in quantity, were economically utilised in the fixation. These observations would suggest that if, by some means, the carbohydrates can first be converted into the mixed acid products before inoculation with the soil flora, then it may be possible to obtain greater return of fixed nitrogen for the carbon utilised than would otherwise be the case.

It has already been shown (Bhaskaran, 1936) that if the carbohydrate is first fermented under conditions of restricted air supply, then the major part of the organic carbon is converted into acid products. These can be neutralised with lime and utilised as their mixed calcium salts.

With a view to standardising the conditions for the application of the mixed calcium salts, some experiments were carried out substituting them for sugar in Ashby's medium. It was found that when they were used in fairly large quantities (on the same carbon basis as the original sugar), only a very small portion was used up. The corresponding fixation was also small, but the ratio of carbon utilised to nitrogen fixed was quite satisfactory, being of the order of 17 : 1. This would compare very favourably with the result obtained when sugar was used as the source of carbon. In the latter case, the ratio of carbon utilised to nitrogen fixed was of the order of 60 to 1 (Bhaskaran and Subrahmanyan, *loc. cit.*).

TABLE IX.

Effect of applying large quantities of mixed calcium salts.

Time in days	Total organic carbon (in mg.)	Total nitrogen (in mg.)
0	132	*nil*
4	120	0·42
8	115	0·95
12	106	1·50

The dilute residue left after fermentation of sugar was utilised to a greater extent than the concentrated product. There was also greater fixation of nitrogen, but the ratio of carbon utilised to nitrogen fixed was more or less the same as in the previous experiment. Combination of the mixed calcium

TABLE X.

Nitrogen fixation by the residue left after anaerobic decomposition of sugar.

Time in days	Total organic carbon (in mg.)	Total nitrogen (in mg.)
0	93	*nil*
4	82	0·70
8	58	1·54
12	55	2·06

salts together with the sugar did not lead to more economic fixation of nitrogen except when the quantity of the former was comparatively small.

TABLE XI.

Effect of combining sugar with the mixed calcium salts.—Changes in carbon.

Time in days	Organic carbon (in mg.)			
	Sugar solution alone (control)	Sugar solution +20 mg. equivalent of mixed calcium salts	Sugar solution +40 mg. equivalent of mixed calcium salts	Sugar solution +80 mg. equivalent of mixed calcium salts
0	100·2	120·2	140·2	180·2
4	97·0	118·5	137·7	170·0
8	48·8	84·0	98·8	103·0
12	47·0	66·8	75·5	86·4

TABLE XII.

Effect of combining sugar with the mixed calcium salts.—Changes in nitrogen.

Time in days	Total nitrogen (in mg.)			
	Sugar solution alone (control)	Sugar solution +20 mg. equivalent of mixed calcium salts	Sugar solution +40 mg. equivalent of mixed calcium salts	Sugar solution +80 mg. equivalent of mixed calcium salts
0	*nil*	*nil*	*nil*	*nil*
4	0·84	0·93	0·97	0·93
8	2·60	3·01	3·11	3·10
12	3·50	4·21	4·46	4·70

The concentrate added to the fermented medium after the sugar was entirely used up did not fix more nitrogen than that used as such, the ratio of nitrogen fixed to carbon utilised (1 : 20·6) being similar to that previously obtained.

TABLE XIII.

Effect of adding the acid concentrate after the sugar was used up.

Time in days	Organic carbon as mg. in 100 c.c. medium		Nitrogen fixed (in mg.) by the mixed calcium salts
	Sugar alone (control)	Sugar +20 mg. equivalent of mixed calcium salts	Experimental *minus* Control
0	28·4	48·7	*nil*
4	....	38·1	0·70
8	28·0	34·8	0·80
12	24·0	31·6	0·83

Experiments with two different types of soils (one a local specimen and the other an alkali soil from Sindh) showed that when the concentrate was applied at 20 mg. to every 10 g. of soil, the efficiency of fixation was of the same order as those previously reported.

TABLE XIV.

Fixation of nitrogen by the mixed calcium salts in different soils.

Time in days	Organic carbon (in mg.)		Nitrogen fixed (in mg.)	Ratio of nitrogen fixed to carbon utilised
	Soil (10 g.)+20 mg. equivalent of mixed calcium salts	Carbon utilised	Experimental *minus* Control	
		Bangalore Soil		
0	70·5	*nil*	*nil*	....
4	65·1	5·4	0·32	1 : 16·9
12	61·6	9·9	0·88	1 : 11·3
17	58·2	12·3	0·93	1 : 13·2
		Kalar Soil		
0	69·0	*nil*	*nil*	....
4	66·3	3·6	0·50	1 : 7·2
8	62·3	7·6	0·66	1 : 11·5
12	57·1	12·8	....	....
17	54·7	15·2	0·91	1 : 16·7

In both the cases, the untreated soil did not show any appreciable variation in organic carbon, so the corresponding figures have not been recorded.

During the first 4 days, the rate of fixation in the soil was very high, the ratio of nitrogen fixed to carbon utilised being as narrow as 1 : 7 in one of the soils. Although there was some slackening in the later stages, the final results should still be regarded as satisfactory.

The quantities of the mixed calcium salts added in the previous experiments would correspond, approximately, to 2 tons of organic carbon per acre. The nitrogen fixed by these would amount to about 2 cwts., i.e., about 13·5 cwts. of protein or about 2·5 tons of a good seed cake.

The recent work of Burk and Horner (1936) would suggest that the fixed nitrogen is easily assimilated by plants. The foregoing observations would show that the preparation of the mixed calcium salts from a cheap carbohydrate waste like molasses and its subsequent application to land would be an economical way of indirectly supplying a useful quantity of readily available nitrogen to the soil.

Discussion.

Perhaps the most striking observation arising from the present enquiry is that the fixation of nitrogen by the mixed flora of the soil (which is the nearest approach to soil conditions) follows a different course from that of *Azotobacter* alone. The latter is comparatively slow in decomposing sugar. The fixation proceeds only as long as the sugar lasts: the residual organic matter is not utilised to any appreciable extent in the fixation. On the other hand, the mixed flora of the soil, though fewer in number, are extremely rapid in decomposing the sugar. Only a small quantity of nitrogen is fixed in presence of the sugar while the major part (amounting to over two-thirds of the total nitrogen) is fixed in the later stages. It has also been demonstrated that the products of decomposition of sugar, consisting chiefly of organic acids, are utilised in the subsequent fixation. These observations would suggest that, although *Azotobacter* may be potent by itself, it does not play any important part in presence of the other organisms of the soil. The latter decompose the sugar at a very rapid rate, so that, even if the *Azotobacter* is active in the soil, it will receive only a limited amount of organic nutrient and will, in consequence, fix only a small amount of nitrogen. Since *Azotobacter* does not utilise the residual organic matter to any appreciable extent, it has to be inferred that the fixation observed after the disappearance of sugar is presumably due to the agency of the other organisms of the soil.

It is well known that many of the soil organisms (other than those of the genus *Azotobacter*) are capable of fixing nitrogen. It is probable that some of them are active in the soil and are primarily responsible for the fixation consequent on the application of sugar. The nature of these active organisms and the manner in which they function are still obscure. Further work is needed to throw light on this aspect of the problem.

A further observation, which is also of considerable importance, is that the residue left after the decomposition of sugar is highly potent in fixing nitrogen in the soil. The return of nitrogen to carbon utilized is highly favourable (being under 1 : 20) and thus brings it to a high degree of efficiency which can stand comparison with those of the chemical and electrochemical methods now in vogue. The nitrogen thus fixed will be in organic combination and, being also readily available, will be very much more valuable than the inorganic forms that are now being manufactured.

The success of the new method of fixation would naturally depend on the availability of cheap sources of organic carbon and on the efficiency of conversion of the available forms into organic acids. Among the raw materials, molasses is one of the most abundant and is, at present, cheaply available. The conditions for the fermentation of molasses have already been standardised, rate of air supply being the most important factor. Under favourable conditions, 80–90 per cent of the total organic carbon can thus be converted into acids. The other sources of organic carbon would include the various

types of plant residues which can also be fermented, under similar conditions, to yield organic acids. Further work is needed, however, to standardise the conditions for the concentration of the acid products and their supply in a dry, non-hygroscopic form to the consumers. Work in this direction is in progress.

The nature of the components that are utilised in the fixation by the mixed flora of the soil is still obscure. It would be of considerable interest to determine the rates at which the different organic acids are being used up. The available evidence would suggest that lactic acid is being most readily utilised, but direct experiments with that acid, both in the free condition and as the calcium salt, would be needed before any conclusion can be drawn. There are also other components, organic as well as inorganic, which may play an important part in the fixation. Further studies on the mechanism of the related changes will not only throw more light on the problem but may also suggest new means of increasing the efficiency of fixation.

It has already been shown that the fixed nitrogen is readily available. Further work is, nevertheless, needed to determine whether it is available, with the same facility, to all types of crops and under different soil and climatic conditions. Vegetation experiments with the different types of crops are already in progress and it is hoped that, before long, it will be possible to throw some further light on this aspect of the problem.

Summary.

Decomposition of sugars by even heavy inocula of *Azotobacter* proceeds comparatively slowly. After the sugar is entirely used up, there is very little growth of the organism : nor is there appreciable fixation of nitrogen. The residual organic matter is decomposed by the organism, but is not utilized in the fixation. These observations are supported by plate counts and the study of the distribution of organic carbon and nitrogen.

On the other hand, the mixed flora of the soil (though comparatively small in number to begin with) decompose the sugar at a very rapid rate, the entire quantity being destroyed in under 4 days. During this period, there is some growth of the organisms, but very little fixation of nitrogen. Between the 4th and 12th days, the residual matter, consisting chiefly of organic acids, is used up not only for the growth of the organisms but also for the fixation. These observations would show that the mechanism of fixation of nitrogen by *Azotobacter* is different from that by the mixed flora of the soil.

Direct experiments with the residue left after the decomposition of sugar showed that they were utilised by the soil flora in a more efficient manner than the original sugar itself. When left by themselves, these organisms showed no ability to fix nitrogen.

It has been shown that the mixed calcium salts of organic acids left after anaerobic decomposition of carbohydrates can be utilised in the fixation of

nitrogen. These products are decomposed comparatively slowly, but the return of nitrogen fixed for carbon utilised (under 1 : 20) is more than three times that obtained when the carbohydrate is applied directly to the soil.

The efficiency of fixation by the mixed calcium salts under different conditions has been studied. It has been found that the maximum fixation takes place when the concentrate is applied in quantities corresponding to about 2 tons of organic carbon per acre. The nitrogen fixed by this will be 2 cwts. and would correspond to 13·5 cwts. of protein, which, in turn, would be equivalent to 2·5 tons of a good seed-cake. The presence of sugar or other fermentable matter does not appreciably interfere with the fixation by the calcium salts. When applied in equivalent quantities, nearly the same amounts of nitrogen are fixed in different soils.

The practical significance of the foregoing and other observations and their application in field practice are indicated.

References

1. Annet, H. E., Padmanabha Iyer, A. R. and Kayasth, R. N., *Mem. Dept. Agric. India*, Chem. Ser., Vol. **9**, p. 155, (1928).
2. Bhaskaran, T. R., *Proc. Ind. Acad. Sci.*, Vol. **3B**, p. 320, (1936).
3. Bhaskaran, T. R. and Subrahmanyan, V., *ibid.*, Vol. **3B**, p. 143, (1936).
4. Bhaskaran, T. R., Sreenivasaya, M. and Subrahmanyan, V., *Current Science*, Vol. **3**, p. 484, (1936).
5. Brown, P. E. and Hart, W. J., *J. Amer. Soc. Agron.*, Vol. **17**, p. 456, (1925).
6. Bottomley, W. B., *J. Roy. Soc. Arts.*, Vol. **62**, p. 372, (1914).
7. Burk, D. and Horner, C. K., *Soil Sci.*, Vol. **41**, p. 81, (1936).
8. Emerson, P., *Iowa Agric. Expt. Sta., Res. Bull.*, Vol. **45**, p. 27, (1918).
9. Lipman, J. G., *N.J. Col. Agri. Expt. Sta. Report*, No. 144, (1908).
10. Ranganathan, S. and Norris, R. V., *J. Ind. Inst. Sci.*, Vol. **10A**, p. 70, (1927).
11. Sahasrabuddhe, D. L., *Proc. Ind. Acad. Sci.*, Vol. **3B**, p. 310, (1936).
12. Sahasrabuddhe, D. L. and Daji, J. A., *Mem. Dept. Agric. India*, Chem. Ser., Vol. **8**, p. 53, (1927).
13. Sahasrabuddhe, D. L. and Kanitkar, N. V., *Ind. J. Agric. Sci.*, Vol. **2**, p. 455, (1932).
14. Sahasrabuddhe, D. L. and Ghatikar, B. H., *Ind. J. Agric. Sci.*, Vol. **1**, p. 631, (1931).
15. Stoklasa, J., *Zentrabl. Bakt.*, II, Vol. **21**, pp. 484, 620, (1908).
16. Wilsdon, B. H. and Barkat Ali, *Soil Sci.*, Vol. **14**, p. 127, (1922).