STUDIES ON THE MECHANISM OF BIOLOGICAL NITROGEN FIXATION.

Part I. Economy of Carbon during Fixation of Nitrogen by the Mixed Flora of the Soil.

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In view of its profound scientific interest and very great practical importance, biological fixation of atmospheric nitrogen has attracted considerable amount of attention during recent years. In addition to numerous studies on soil or seed inoculation, special attention has also been paid to the physiology of the organisms concerned and the various factors influencing fixation. Much interest has centred round the mechanism of fixation, especially on the nitrogen side and the energy changes attendant thereon. Valuable work has also been done on the related carbon transformations, especially by Stoklasa (1908), Bonazzi (1921) and Ranganathan and Norris (1927), but further information is needed on certain important aspects of the problem. Thus, it is not definitely known as to how the different forms of organic matter are utilised in the fixation; at what particular stage of the decomposition the fixation begins; what portion of the degraded organic matter is taken up by the organisms and as to how far the growth of the organisms is related to the fixation. With a view to throwing some light on these and related aspects of the problem, the present series of investigations were undertaken.

Experimental.

Technique.—In the study of carbon transformations, it would be desirable to separate living organisms from other carbonaceous substances present in the medium. Unfortunately, this is not possible because the presence of calcium carbonate is essential for the fixation. The carbonate always comes down with the organisms and any method of separating them will be only partially successful. The analytical procedure was therefore modified to include carbonates and may be described as follows:—At stated intervals, samples of suspension containing the mixed flora of the soil were taken out and analysed for their carbonate, organic carbon and total nitrogen

contents (Subrahmanyan, Narayanayya and Bhagvat, 1934; A.O.A.C., 1930). Parallel samples were centrifuged and the suspended matter, together with the insolubles, separated from the clear supernatant. They were then analysed for their organic carbon and nitrogen contents. In a further sample, the residual sugar, if any, was estimated (Bhaskaran *et al.*, 1934). From these results, the distribution of carbon was calculated as follows :—The difference between the total carbon originally taken and that left behind at

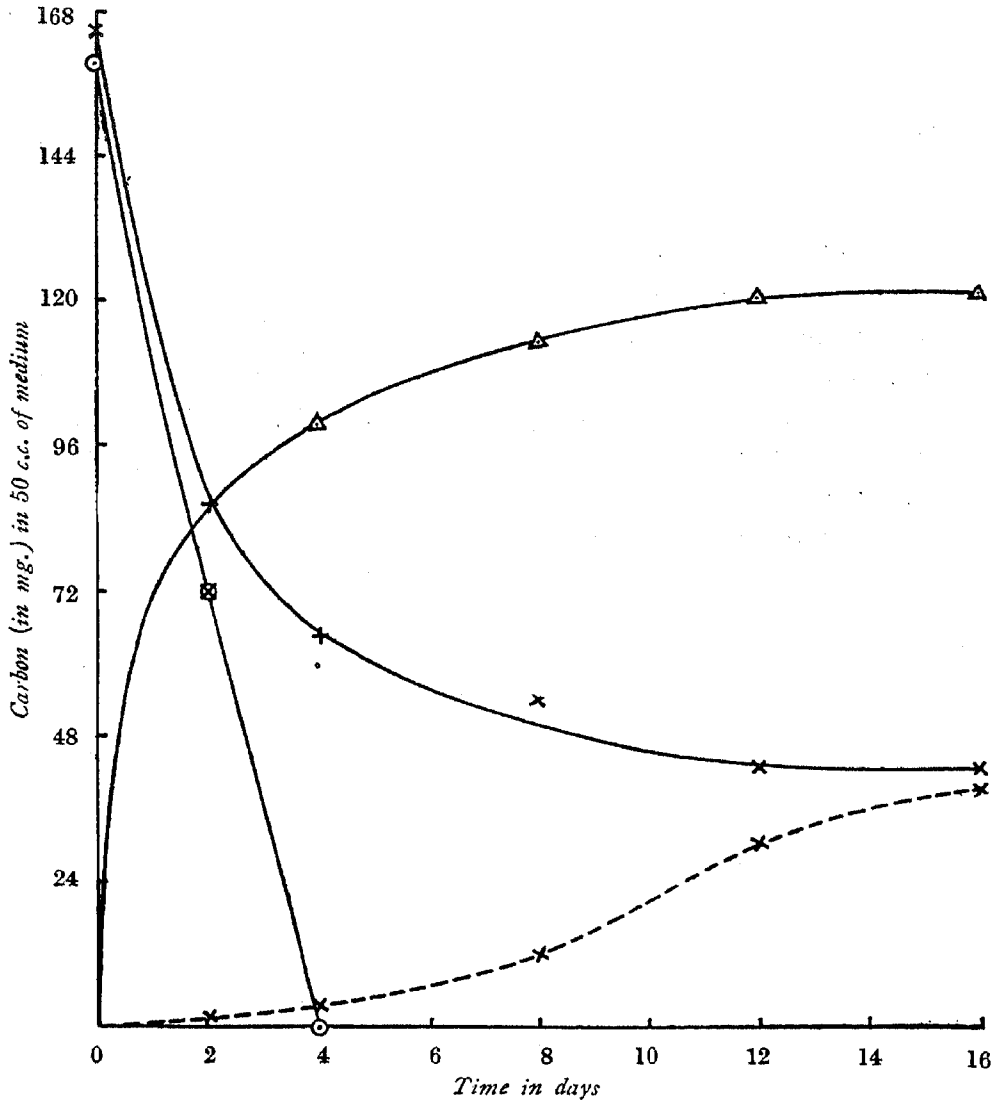

FIG. 1. Distribution of Carbon

Carbon (Total) present in the system
,, present in the sediment
,, lost as gas
,, present as sugar

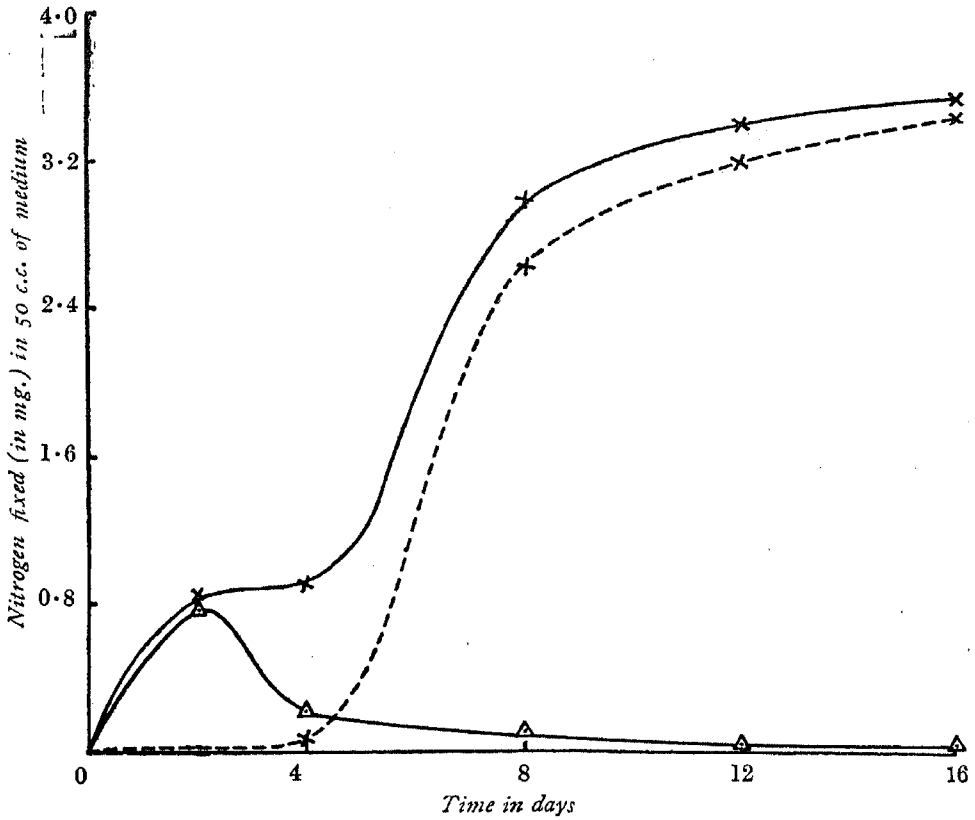

FIG. 2. Distribution of Nitrogen

Total nitrogen in the system
Nitrogen present in sediment
„ „ „ supernatant

each stage gave an estimate of the quantity lost as gas. The difference between total carbon in the sediment and the carbonate present in it yielded the figure for organic carbon in the organism. The difference between the total organic carbon of the supernatant and that actually present as sugar represented the soluble products formed by fermentation. The separate estimations of nitrogen helped to differentiate between that present in the organism and that occurring in soluble form in the supernatant.

In nitrogen fixation studies, mannitol is generally used as the source of carbon, but as there is no accurate method for estimating that hexitol, glucose was used in its place. Otherwise, the medium was similar to that originally proposed by Ashby (1907). Since, under natural conditions, the mixed microflora—rather than any single organism—are concerned with the various transformations, soil itself (0·1 g. of air dried material for 50 c.c. of medium) was used for inoculation. The incubations were at 30° and the results have been presented in Figs. 1 and 2.

It may be seen from the above that all the sugar was used up in the course of the first four days. The corresponding loss of nitrogen in gaseous form was also considerable. The subsequent changes, though slow, were highly significant from the point of view of fixation. The organic matter in solution was steadily converted into insoluble forms representing mostly the tissue material of the organism. There was also marked fixation of nitrogen, especially between the fourth and the eighth days. The major part of the fixed nitrogen was present in soluble form but a small part was also present throughout in soluble condition.

Microscopic examination of the supernatant obtained after centrifuging, especially between the fourth and the eighth days, showed that it was not completely free from living cells. Moreover, it was not clear whether the acids, which were the chief products of fermentation, would account for all the organic matter in the supernatant. A fresh series of experiments was, therefore, carried out, first adding a flocculent to precipitate the organism and then studying the distribution of carbon and nitrogen. A number of flocculents, Kieselguhr, calcium sulphate, freshly precipitated barium sulphate, copper hydroxide, dialysed iron and alumina cream, (preparation C of Willstätter and Kraut, 1923) were tried, but the last was found to be most effective in precipitating the organisms, especially in the early stages, when it is so difficult to separate out the young cells. In addition to the above, the quantities of organic acids present at different stages were determined. Lactic acid was estimated according to Subrahmanyan (1929), the total volatile fatty acids by the method of Birkinshaw and Raistrick (1931) and the distribution according to Virtanen and Pulkki (1928). The results have been presented in Tables I to III.

TABLE I.

Distribution of carbon (suspension treated with alumina cream and then centrifuged).

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*	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

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

TABLE II.

Production and distribution of organic acids.

Time in days	Organic acids (as mg. of C) in 50 c.c. of medium					
	Non-volatile	Volatile				Total
	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 III.

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

The above observations show that a part of the carbon in the sediment was derived from the sugar itself while the rest originated from the immediate products of decomposition. The major part of the carbon in the supernatant was accounted by the organic acids. The latter diminished in quantity after the 8th day and were presumably taken up by the organisms.

Working with *Azotobacter chroococcum*, Ranganathan and Norris (*loc. cit.*) observed that the decomposition of sugar was comparatively slow; that the fixation of nitrogen was complete before the entire quantity was used

up. They record, however, that there was a sudden decrease in volatile acids between the 9th and the 13th days. Regarding the first observation, it is difficult to determine whether it was due to the slow action of *Azotobacter* or the smallness of the inoculum. As the sugar was present all through in the medium, it is not possible to ascertain whether any of the products of fermentation was used up in the fixation. The disappearance of a portion of the volatile acids is, nevertheless, significant and lends indirect support to our observation.

The C-N ratio of the sediment narrowed rapidly between the 4th and the 8th days and attained a steady value by the 16th day.

Starting with the products left on the 4th day, it would appear that the return of nitrogen for the carbon utilised was favourable. When reckoned, however, on the basis of the original sugar, it would be found to be very small (1 : 55).

Fixation of Nitrogen by the residue after decomposition of sugar :— The fermented suspension (4th day) was filtered through Kieselguhr and the clear filtrate subjected to discontinuous steam sterilization. It was then inoculated with the soil and the extent of fixation at the end of 4 and 8 days compared with that in unsterilised control. (The quantity of inoculum was increased to 1 g. so as to correspond to the growth during the first four days. The necessary correction for added nitrogen was made when calculating the quantities actually fixed.)

In another series, the supernatant was separated by centrifuging (4000 r.p.m. ; 15 min.) and inoculated with soil. To the sediment sterile water was added to make up the volume and the suspension incubated as before.

The quantities of nitrogen fixed in the two sets of experiments have been presented in Tables IV and V.

TABLE IV.

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

TABLE V.

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

The above observations afford direct proof to show that the residue after decomposition of sugar can fix nitrogen. The supernatant is the more potent fraction, while the sediment (containing the bulk of the organisms), when left by itself, does not fix any appreciable quantity.

Discussion.

The results of the present enquiry are of much practical interest. In the first place, they show that in presence of the mixed flora—which represent the natural conditions of the soil—the major part (about 70 per cent.) of the added sugar is wasted. The return of nitrogen for the carbon spent is very small. On the other hand, the residual organic matter, consisting of the acids and other products of fermentation, is more efficiently utilised not only for the growth of the organisms but also for the fixation of nitrogen.

It is well known that nitrogen fixers can use organic acids (or their salts) as sources of energy. Since in presence of the soil, sugars are so easily decomposed, it would appear to be desirable to convert them into more stable products before application to the field.

Our researches have already shown that, under conditions of restricted air supply, gas production is greatly reduced. The major part of the sugar is converted into organic acids, chiefly volatile fatty acids (Bhaskaran, private communication). If these could be subsequently applied to the field in the form of their mixed calcium salts, then there would be greater fixation of nitrogen than would otherwise be possible.

In recent years, much interest has centred round the possibility of utilising cane molasses as fertiliser. The mechanism of the fertilising action is still not fully understood, but the recent observations of Dhar and Mukherji (1934, 1935) would suggest that it is at least partly due to the fixation of atmospheric nitrogen. On the other hand, it has been shown by a number of previous workers that molasses rapidly decomposes in the soil so that the

return of nitrogen for the carbon utilised would be very small. The procedure described above would appear to provide a convenient means of applying molasses to land and, at the same time, obtaining greater fixation of nitrogen.

Further researches on the foregoing and allied aspects of the problem are in progress and will be described in subsequent communications.

Summary.

During decomposition of glucose by the mixed flora of the soil, a considerable part (over two-thirds) of the sugar is converted into gaseous forms in the course of the first four days. The residue is present in water-soluble forms, chiefly as organic acids. The latter are largely utilised for the fixation of nitrogen.

The water-soluble products left after the decomposition of sugar, if inoculated with the mixed flora of the soil, can fix nearly two-thirds as much nitrogen as the original sugar itself. The solid sediment (including the major part of the organism), when left by itself, does not fix any appreciable quantity of nitrogen.

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