NITROGEN-FIXATION BY 'AZOTOBACTER CHROOCOCCUM.'

By S. Ranganathan and Roland V. Norris.

A better knowledge of *Azotobacter* metabolism is highly desirable in view of the important role this organism plays in maintaining soil fertility by fixing nitrogen from the atmosphere. Though much time has been devoted to its investigation our knowledge of the subject is far from being satisfactory. The papers hitherto published have dealt mainly with the participation of *Azotobacter* in the nitrogen cycle, and little attention seems to have been paid to its carbon metabolism. The latter is the more important of the two, as it is through the oxidation of carbohydrates and similar organic compounds that the organism derives energy to carry on its endothermic nitrogen-fixation.

In 1908 Stoklasa (*Zentr. Bakt.*, II, 1908, 484, 620) working upon dextrose fermentation by *Azotobacter*, detected the following compounds:—Carbon dioxide, formic acid, acetic acid, lactic acid and ethyl alcohol. In the same year Krzemieniewski (*Bull. Intr. Ac. Sci. Cracovia*, 1908, 929) and recently Bonazzi (*J. Bact.*, 1921, **6**, 331) were unable to detect any volatile acid or lactic acid in the same dextrose medium fermented by *Azotobacter*. The first object of the present investigation was, therefore, to throw further light on this point and ascertain what intermediate compounds are really produced.

EXPERIMENTAL.

ISOLATION OF THE ORGANISM.

Ashby's mannite medium (*J. Agric. Sci.*, 1907–8, **2**, 35) was prepared, sterilised and inoculated with about one gram of ordinary garden soil per 100 c.c. of medium. The culture medium was sterilised for a comparatively long time in the steam steriliser in preference to the usual sterilisation in an autoclave for 15 minutes at 130°. In the latter case the medium turns brownish and partial decomposition of the sugars often takes place. The pH of the medium was always maintained between 7·0 and 7·4. About 0·5 gm. of sterile calcium carbonate was added per 100 c.c. of medium.

In about three days there was a heavy growth in the inoculated flask as was shown by frothing and the formation of a scum on the surface of the liquid. A loopful was taken from the surface and

inoculated into another flask containing the same medium. This operation of sub-culturing was continued till a microscopical examination revealed that most of the contaminating organisms had been eliminated. The culture was then plated out on a mineral salts-mannite-agar medium. Sub-cultures were made several times on the same medium till nothing but milky white colonies of *Azotobacter* were to be found. Such selective sub-cultures yielded finally a pure culture of *Azotobacter chroococcum*. After about ten days' growth on the plates the colonies were brownish-black owing to the pigment formation so characteristic of this organism.

During the above isolation considerable difficulty was experienced in separating a fungus which accompanied *Azotobacter*. Attempts to grow this fungus independently of *Azotobacter* failed.

Preservation of the Culture.

The organism was initially preserved in Ashby's mannite-agar-medium, sub-cultures on plates being made every ten or twelve days. In the early stages of the investigation the organism grew quite luxuriantly in this medium and good colonies were obtained in 48 hours; but after about 3 months the growth of the organism and the activity decreased so rapidly that it was not possible to obtain a good growth on the plates even after the expiry of ten days. Experiments to stimulate growth by adding a minute quantity of ammonium nitrate were of no avail. The improved silica-jelly method (Legg, *Biochem. J.*, 1919, **13**, 107) was also tried, but ultimately a soil extract-agar medium was found to be more useful and quite good results were achieved by its use.

This was prepared by autoclaving at 25 lbs. pressure for 30 minutes 500 gms. of garden soil with 1 litre of distilled water. The extract was then filtered through a film of kieselguhr. The filtered extract was quite clean but coloured strongly yellow due to iron in the soil. Washed agar (2 per cent.) was used for solidification. The medium prepared in this way was neutral to litmus and, with its use, bright colonies of the organism could be obtained in less than 24 hours. The organism was sub-cultured once in 10 or 12 days on this medium.

Carbohydrates Metabolism.

Dextrose-fermentation and nitrogen-fixation.—In the following experiments, unless stated to the contrary, sugar was estimated by the Bertrand method after removing protein and other impurities by means of dialysed iron (Shaffer, *J. Biol. Chem.*, 1914, **19**, 285). Nitrogen was estimated colorimetrically after kjeldahling the solution.

Two 2-litre flasks each containing 1000 c.c. of sterile dextrose-ashby medium were inoculated with 10 c.c. each of a fresh culture of *Azotobacter*. The flasks were neutralised at room temperature and 5·0 gms. of sterile calcium carbonate added to each. Samples (50 c.c.) were taken daily from each by means of a sterile pipette and analysed for sugar and total nitrogen. In all cases a microscopical examination of the sample was made as a test for its purity. The results obtained are recorded in the following table :—

TABLE I.

	Experiment I			Experiment II		
Duration of growth	Amount of sugar in 100 c.c. Mgs.	N fixed per 100 c.c. Mgs.	Per cent. of sugar consumption	Amount of sugar in 100 c.c. Mgs.	N fixed per 100 c.c. Mgs.	Per cent. of sugar consumption
0	827·5	0·48	...	815·0	0·48	...
1 day	802·5	1·68	3·02	785·0	1·76	3·68
2 days	715·0	4·8	13·59	637·5	5·2	21·78
3 ,,	557·5	5·2	32·63	510·0	5·6	37·42
4 ,,	462·5	6·0	44·11	381·5	6·4	53·19
5 ,,	356·5	6·4	56·92	321·0	6·4	60·61
6 ,,	310·5	6·8	62·48	228·0	6·8	72·02
7 ,,	194·5	7·6	76·50	130·0	7·6	84·05
8 ,,	46·0	8·8	94·44	27·0	8·8	96·70
9 ,,	nil	9·6	100·00	nil	9·6	100·00

From the above it can be seen that the organism isolated was a very active one, capable of fermenting the sugar completely within eight days. It will moreover be noticed that more than 50 per cent. of the nitrogen fixed from the atmosphere was assimilated within the first three days.

Relation between concentration of sugar and nitrogen-fixation.—According to Stoklasa there is a definite relationship between the carbon-content of a soil and the nitrogen assimilated. In order to ascertain whether the same holds good in pure culture solutions of *Azotobacter* the following experiments were made. To six 125 c.c. conical flasks, each containing 50 c. c. of Ashby's medium (the salts being taken in double the original concentration), were added 0·25 gm.,

0·5 gm., 1·0, 2·0, 3·0 and 4·0 gms. of dextrose respectively. The volume in each flask was made up to 100 c. c. and the flasks sterilised in the steam steriliser for three hours. They were then each inoculated with 1 c. c. of a fresh culture of *Azotobacter* and 0·5 gm. of sterile calcium carbonate added to each the next day. The flasks were incubated at 36° and shaken at frequent intervals, the solutions being analysed for total nitrogen after the expiry of three weeks. The results are given below:—

TABLE II.

Influence of Sugar-concentration on Nitrogen-fixation.

No.	Amount of sugar per 100 c.c.	Total N fixed	N fixed per gm. dextrose
1	0·25 gm.	2·4 mgs.	9·6 mgs.
2	0·50 ,,	3·2 ,,	6·4 ,,
3	1·0 ,,	4·0 ,,	4·0 ,,
4	2·0 ,,	4·4 ,,	2·2 ,,
5	3·0 ,,	4·4 ,,	1·13 ,,
6	4·0 ,,	4·2 ,,	1·05 ,,

It is thus obvious that there is no regular increase in nitrogen-fixation with increasing amounts of sugar in the medium. On the other hand the amount of nitrogen fixed per gram of dextrose decreases rapidly with increase in the sugar-concentration. In other words the fixation takes place much more efficiently in dilute solutions of sugar.

Relation between Phosphorus and Nitrogen-fixation.

J. E. Greaves (*Soil Sci.*, 1918, **6**, 163) maintains that phosphates greatly accelerate nitrogen-fixation and economise the carbohydrate-consumption. Stoklasa calculated the relationship between phosphorus utilised and nitrogen fixed in soils and found this, in the case of *Azotobacter*, to be 5·0 to 5·7 gms. of free nitrogen per gram of phosphorus used. Experiments were therefore conducted to ascertain whether phosphates really effect any economy in sugar-consumption in pure cultures. The general outline of the experiment was the same as that previously described in the case of sugar and nitrogen-fixation. Dextrose-ashby medium was used with the addition of varying amounts of acid potassium phosphate neutralised by sodium hydroxide.

Together with the total nitrogen estimations the residual sugar was also determined, the analyses being made after the expiry of three weeks. The results are given in the table below :—

TABLE III.

Influence of Phosphate on Nitrogen-fixation.

No.	Amount of phosphate, Gm.	Total N fixed, Mgs.	Amount of unfermented sugar, Mgs.	Sugar fermented, Mgs.	N fixed per gram of dextrose, Mgs.
1	Control (Inoculated and sterilised)	...	827·5	...	...
2	0·008	4·8	340·6	486·9	9·86
3	0·02	5·0	242·1	585·4	8·54
4	0·04	5·4	220·0	607·5	8·89
5	0·08	5·6	220·0	607·5	9·22
6	0·10	6·0	192·2	635·3	9·44
7	0·20	6·0	181·3	646·2	9·28

Though there is apparently increasing fixation of nitrogen with the increase in concentration of phosphate in the medium, the nitrogen fixed per 1000 mgs. of dextrose fermented remains practically constant. There is thus no real sugar-economy. The main function of the phosphate appears to be to increase the amount of sugar fermented as shewn by the figures in column 5 of Table III.

Carbon Balance.

In order to determine the carbon balance, the organism was grown on dextrose-ashby medium in the apparatus shown in Fig. I. The flask A (Duro-glass wide-mouthed conical flask), its three-holed rubber stopper through which pass tubes B and C, the separating funnel P and the tube with the glass tap E, were sterilised in the steam steriliser for two hours on three successive days, the glass tubes and the rubber corks being in the first place treated with alcohol. Dextrose-ashby medium (100 cc.) was run into the conical flask by means of a pipette and the flask again sterilised for one hour. It was then inoculated with a loop of fresh *Azotobacter* from a plate culture. Taking all precautions to maintain sterility the flask was connected to the respiration apparatus shown in the diagram. The culture flask itself was kept in an electrically heated box maintained at 29–31°.

Dry air free from carbon dioxide was bubbled through the culture medium by means of an aspirator, the air entering through B and passing out by C. The absorption tube F was filled with pumice

soaked in sulphuric acid to absorb water from A. A second absorption tube G filled with calcium chloride was connected with F to catch any water which might diffuse backwards from the potash bulb H. The end of H was in turn connected to another absorption tube I containing both soda-lime and calcium chloride. Tube I was connected to a guard-tube J containing soda-lime and calcium chloride and finally terminating in the guard-tower containing calcium chloride and soda-lime to prevent back diffusion of water-vapour and carbon dioxide from the atmosphere.

The culture flask was aerated in this manner by means of sterile air free from carbon dioxide for 21 days at the rate of about ten bubbles per minute. In about three days the medium, which was initially clear, developed turbidity and the characteristic film. These are both indications of good growth. After the expiry of the three weeks about three c.c. of syrupy phosphoric acid was dropped into the incubation flask through the separating funnel P and a stream of air was again passed through to remove any carbon dioxide held in the medium in chemical combination. G, H and I were then weighed, the increase in weight representing the amount of carbon dioxide evolved during the fermentation of the dextrose. The contents of the flask A were then filtered through a Gooch crucible lined with a thick layer of previously ignited asbestos to remove the growth, and the filtrate made up to 250 c.c. Aliquot portions were taken for sugar estimation by Bertrand's method. The carbon of the bacterial bodies and also of the filtrate was then estimated separately according to the wet combustion method of Rogers and Rogers (*Amer. J. Sci. and Arts*, Series 2, 1848, **5**, 352) as modified by Gortner (*Soil Sci.*, 1916, **2**, 395), the apparatus used being shown in Fig. II. Before the method was adopted for this purpose, it was tested with pure substances such as uric acid and glycine.

TABLE IV.

Carbon balance results.

No.	———	Expt. I Gms.	Expt. II Gms.
1	Amount of original sugar in culture flask ...	1·05	1·160
2	Amount of sugar after fermentation ...	0·76	0·8275
3	Glucose utilised	0·29	0·3325
4	CO_2 equivalent of 3	*0·4263*	*0·4877*
5	CO_2 evolved during experiments	0·3059	0·3682
6	CO_2 evolved during the combustion of the bacterial residue	0·0587	0·0543
7	CO_2 evolved from the combustion of the filtrate (after making allowance for the residual sugar)	0·0385	0·0428
8	Total CO_2 thus accounted for	*0·4031*	*0·4653*

Fig. II.

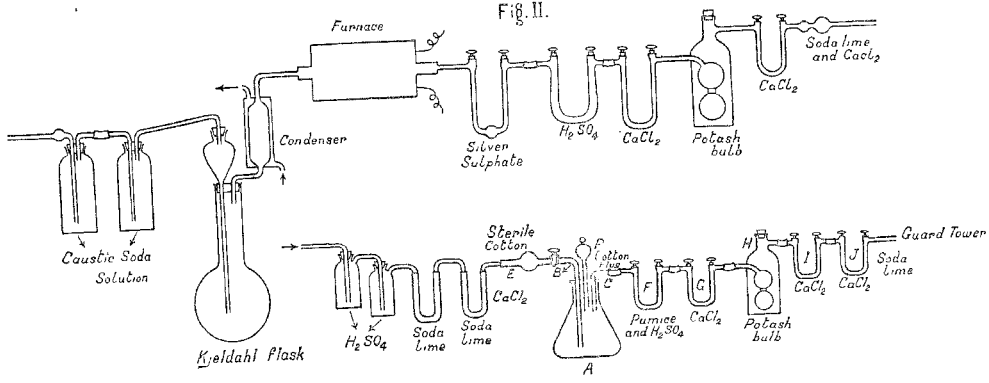
Furnace
Soda lime and $CaCl_2$
$CaCl_2$
Condenser
Silver Sulphate
H_2SO_4
$CaCl_2$
Potash bulb
Caustic Soda Solution
Sterile Cotton
Guard Tower
Soda lime
$CaCl_2$
H_2SO_4
Soda lime
Soda lime
Kjeldahl Flask
Pumice and H_2SO_4
$CaCl_2$
Potash bulb
$CaCl_2$
$CaCl_2$
A

Fig. I.

It will be seen that in experiment I, 0·4031 gms. of carbon dioxide was accounted for out of 0·4263 gms., whereas in the second experiment, out of 0·4877 gms. of carbon dioxide 0·4653 gms. has been traced. The results indicate that rather more than 70 per cent. of the carbon due to sugar utilised appeared as carbon dioxide and about 12 per cent. was assimilated by the bacterial cells, leaving about 18 per cent. constituting the carbon of the fermentation products other than carbon dioxide. Stoklasa (*Zent. Bakt. Par.*, 1908, **21**, ii, 620) working in the same direction and starting with 15·89 gms. of dextrose recovered 7·9 as carbon dioxide, 0·3 as ethyl alcohol, 0·2 as formic acid, 0·7 as acetic acid and 0·2 as lactic acid. Only once, working under anaerobic conditions, he obtained also a small quantity of butyric acid and hydrogen. All these amounted to only 9·379 gms. and he was not able to account for the remaining 6·5110 gms. of sugar. In view of the results of the present experiments, it is obvious that Stoklasa must have missed some other by-products of sugar fermentation.

Detection and estimation of by-products in the sugar fermentation by Azotobacter.

Detection.—Two litres of dextrose-ashby medium was sterilised and inoculated with a fresh culture of *Azotobacter*. Sterile calcium carbonate was added and the growth continued for over a month so that most of the sugars might be fermented. After fermentation the liquid was tested for the common fermentation products such as alcohol, aldehyde, acetone, acetylmethylcarbinol and formic, acetic, butyric, lactic acids, etc.

The reaction of the liquid after fermentation was neutral to phenolphthalein and methyl red. The supernatant liquid was decanted from the solid sediment and 500 c.c. of this was distilled till 100 c.c. of distillate collected. The distillate was redistilled and the second distillate, containing only volatile non-acidic products, tested for the usual fermentation products.

Volatile non-acidic portion.

Substance tested for	Test	Result
Ethyl alcohol	Iodoform test	Perceptible smell of iodoform
	Benzoyl chloride test ...	Characteristic smell of ethyl benzoate
Aldehyde	Amm. solution of $AgNO_3$	Precipitate of Ag
,	$K_2Cr_2O_7$ and HNO_3 ...	No coloration at first, but intense blue coloration after standing for some time
Acetone	Sodium nitroprusside test ...	Negative
Acetylmethylcarbinol ...	Peptone test	Negative

Volatile acidic portion.—Some of the residue from the previous distillation was acidified with tartaric acid and then distilled, the product giving the following reactions.

Substance tested for	Test	Result
Formic acid ...	Ammn. solution of $AgNO_3$ ...	Precipitate of Ag
	$K_2Cr_2O_7$ and HNO_3 ...	Blue coloration after standing for some time
Acetic acid	Ethyl acetate	Fruity smell of ethyl acetate

Non-volatile portion.—The residue remaining from the first distillation was concentrated to less than half its volume on a water bath and tested as follows :—

Substance tested for	Test	Result
Lactic acid ...	Formation of zinc lactate ...	Characteristic crystals of zinc lactate obtained
Tartaric acid	Resorcinol and H_2SO_4 ...	Fine rose colour at first changing to violet-red either on standing or on slight warming

It is thus seen that in the fermentation of dextrose by *Azotobacter* the following by-products were obtained : Carbon dioxide, ethyl alcohol, aldehyde and formic, acetic, lactic and tartaric acids. The presence of tartaric acid has not been recorded by previous investigators.

Estimation of by-products.—Two litres of dextrose-ashby medium was sterilised in a 3-litre flask and inoculated with 10 c.c. of a fresh culture of *Azotobacter*. Exactly 50 c.c. was removed by means of a sterilised pipette and analysed for initial sugar by Bertrand's method. About 10 gms. of sterile calcium carbonate was added to the flask and the latter incubated at 36° for one month. The culture solution after removal of proteins by dialysed iron was then analysed for unfermented sugar and for the various decomposition products identified above, with the following result :—

1. *Sugars fermented.*—

Original amount of sugar in 1950 c.c of medium .... = 17·35 gms.
Sugar not fermented .... = 6·3 ,,
Sugar actually fermented .... = 11·05 ,,

2. *Total acids.*—These were estimated by Harden's method (*J. C. S.*, 1901, **79**, 613) and expressed as grams of calcium held in solution by the acids. Fifty c. c. of the fermented culture was boiled with excess of pure calcium carbonate, the solution filtered and the calcium in the filtrate determined volumetrically.

Total amount of dissolved calcium in 1950 c. c. medium .... = 1·4524 gms.

3. *Volatile non-acidic portions.*—Alcohol was estimated by distilling 800 c. c. of the fermented culture till one-third remained in the flask. On distilling the distillate and collecting 100 c. c. in a standard measuring flask the density was found to be 0·996 at 27°. Hence the percentage of alcohol by volume, applying the corrections for temperature, is 1·50 and the percentage by weight is 1·19; the amount of alcohol in the entire medium is 2·975 gms.

4. *Volatile acids.*—The method employed was Duclaux's distillation and titration process. One hundred c.c. of the fermented medium was acidified with tartaric acid, diluted to 200 c.c. and distilled till 100 c.c. of the distillate were collected. The volume of the distillate was made up to 110 c.c. and redistilled till 10 c.c. remained in the flask. Fractions (10 c.c.) were collected and titrated by standard lime water. The titration value for each 10 c.c. fraction is given below.

TABLE V.

Titration of volatile acid fractions.

Fraction No.	Lime water required	Total amount of distillate	Total lime water required	Lime water utilised in c.c. Total as 100
1	1·15 c. c.	10 c. c.	1·15 c. c.	6 63 c. c.
2	1·25 ,,	20 ,,	2·40 ,,	13·84 ,,
3	1·45 ,,	30 ,,	3·85 ,,	23 20 ,,
4	1·60 ,,	40 ,,	5·45 ,,	31·42 ,,
5	1·70 ,,	50 ,,	7·15 ,,	41·22 ,,
6	1·75 ,,	60 ,,	8·90 ,,	51·31 ,,
7	1·85 ,,	70 ,,	10·75 ,,	61·98 ,,
8	1·95 ,,	80 ,,	12·7 ,,	73·21 ,,
9	2·15 ,,	90 ,,	14·85 ,,	85 63 ,,
10	2·5 ,,	100 ,,	17·35 ,,	100·00 ,,

On plotting the results obtained, and also the figures for pure formic acid and acetic acid, the curve indicates the presence of traces of formic mixed with a much larger proportion of acetic acid.

Taking all the volatile acids to be acetic acid, this was estimated as follows: A fermented medium (100 c.c.) previously acidified with tartaric acid was distilled and the distillate titrated against standard decinormal sodium hydroxide. The acetic acid content of the medium was 0·72 gm.

5. *Non-volatile acids*: *Lactic acid.*—The acidified fermented medium (400 c.c.) was steam-distilled and the residue extracted several times with ether. The ethereal extracts were mixed, filtered and the ether evaporated. The lactic acid remaining in the syrupy residue was determined by boiling the latter with excess of pure calcium carbonate and estimating the dissolved calcium volumetrically. The total amount of lactic acid in the entire medium, as estimated by the above method, was 0·675 gm.

Tartaric acid.—About 2 gms. of potassium bromide were added to 100 c.c. of the fermented liquid. This was evaporated into a syrupy consistency on a water bath and allowed to crystallise slowly. When crystallisation was complete, the crystals were washed several times with 40 per cent. alcohol previously saturated with potassium bitartrate. The liquid was decanted to a filter, care being taken not to remove the crystals. The crystals were then dissolved in water and the boiling solution titrated to standard alkali. One c.c. of N/10 sodium hydroxide is equivalent to 0·0188 gm. of potassium bitartrate. Adopting this method the amount of tartaric acid in the medium was found to be 1·47 gms.

6. *Acetaldehyde.*—The estimation was unfortunately spoiled, but the amount was very small.

The 11 gms. of sugar fermented, therefore, yielded in addition to the carbon dioxide evolved, 2·98 gms. alcohol, 0·72 gm. acetic acid, 0·675 gm. lactic acid, 1·47 gms. tartaric acid and traces of aldehyde and formic acid.

It was observed from the results of a few preliminary experiments that the sugar fermentation is not uniform, and evidence was obtained that there is a definite change in the nature of the fermentation after a few days, no doubt owing to a secondary fermentation being set up, the volatile acids suddenly diminishing. With the object of obtaining greater insight into the nature of this fermentation, the following experiments were carried out. Four 3-litre flasks, containing

2 litres each of sterile dextrose-ashby medium, were inoculated with 50 c.c. of an active culture of *Azotobacter*. The initial sugar contents were analysed by taking samples immediately after inoculation. In flask No. 1 the initial nitrogen content and the volatile acids also were estimated. Sterile calcium carbonate in sufficient quantity was added to each of the flasks 24 hours after inoculation.

Flask No. 1 was allowed to ferment completely; while thus fermenting, samples (60 c.c.) were taken at intervals of 24 hours and analysed for sugars, volatile acids produced and nitrogen fixed. No. 2 was allowed to ferment undisturbed until there was a sudden drop in the production of volatile acids in No. 1, when a complete analysis of the various by-products was made. No. 3 was likewise subjected to a complete analysis, but only when the fermentation was complete, this being determined by the absence of sugar in No. 1. Flask No. 4 differed from the rest in containing 1·0 gm. of sodium bisulphite per litre, the object being to side-track the intermediate by-products at the aldehyde stage. A complete analysis of No. 4 was made along with No. 3.

When the experiments were begun it was observed that there was appreciable growth in the first three flasks within 48 hours while there was not even a trace in No. 4. It was only after nine days from beginning the experiment that any growth was discernible in the bisulphite flask. Evidently the addition of bisulphite retarded the growth of *Azotobacter* for some time, though fermentation subsequently proceeded actively. The results of the various experiments are given below.

FLASK No. 1.

Duration of fermentation	Residual sugars, Gms.	Sugar fermented, Gms.	N fixed, Mgs.	Total volatile acids (c. c. N/20 NaOH neutralised), c. c.
1st day	18·24	...	6·0	20·0
2nd ,,	18·04	0·20	28·0	24·0
3rd ,,	17·92	0·32	40·0	44·0
4th ,,	17·92	0·52	48·0	44·0
5th ,,	17·40	0·84	68·0	48·0
6th ,,	15·68	2·56	96·0	68·0
7th ,,	14·46	3·78	112·0	104·0
8th ,,	12·72	5·52	144·0	128·0
9th ,,	10·68	7·56	160·0	140·0
10th ,,	8·06	10·18	164·0	*104·0*
11th ,,	5·40	12·84	172·0	108·0
13th ,,	2·78	15·46	184·0	148·0
14th ,,	1·48	16·76	192·0	144·0
15th ,,	1·15	17·09	192·0	152·0
16th ,,	0·72	17·52	192·0	164·0
17th ,,	...	18·24	192·0	160·0
18th ,,	...	18·24	192·0	160·0

(The figures in all cases have been calculated to the original volume of the medium i.e., 2000 c.c.).

FLASKS Nos. 2, 3 and 4.

	No. 2 Gms.	No. 3 Gms.	No. 4 Gms.
Original sugar	18·24	18·24	18·24
Residual sugar at the time of analysis	3·88	1·54	0·09
Sugar actually fermented	14·36	16·70	18·15
Total acids expressed in Ca equivalent	0·862	0·445	2·788
Alcohol	trace	trace	trace
Aldehydes expressed as acetaldehyde	0·0035	*Nil.*	0·0722
Total volatile acids expressed in c. c. N/20 NaOH required for neutralisation.	356	195	1170
Lactic acid ... ... Gms. ...	0·396	0·751	0·603
Tartaric acid ... ... ,, ...	1·824	1·352	1·36
Nitrogen fixed per 100 c. c. ... ... Mgs. ...	7·2	7·2	2·4

Methods used.—The sugars were determined by Bertrand's method. Total acids were determined by boiling an aliquot with excess of calcium carbonate, filtering and estimating dissolved calcium volumetrically. The alcohol estimation was made by density measurements. Aldehydes were estimated by Ripper's method (*Monatsh.*, **21**, 1079) as modified by M. Thomas (*Biochem. J.*, 1925, **19**, 945). Tartaric acid was estimated by Perentzy's method (*J. C. S.*, 1907, *Abstracts* ii, 991) and the filtrate from four determinations was used for the lactic acid estimation by extracting this with ether for six hours in a continuous ether extractor. The ether extract was later boiled with excess of calcium carbonate, filtered and the dissolved calcium estimated and the amount of lactic acid calculated.

It will be noted that No. 2 which was analysed at the intermediate stage, i.e., when the figures in No. 1 suggested that the secondary fermentation was beginning, contains far more volatile acids than No. 3 in which the fermentation was allowed to proceed to completion, though the sugar consumed in the latter case was naturally more than in No. 2. The tartaric acid is also increased in No. 2 while the lactic acid is diminished as compared with No. 3.

The influence of bisulphite (flask 4) on the other hand is shewn by an increase in the amount of aldehyde, as might be expected, and

a very large increase in the volatile acids while the amount of nitrogen fixed was exceedingly small. Further work to elucidate the mechanism of these chemical changes is contemplated.

Mechanism of Nitrogen-fixation.

A 2-litre flask containing 1200 c. c. of sterile dextrose-ashby medium was inoculated with a fresh culture of *Azotobacter*. Sterile calcium carbonate in the proportion of 0·5 gm. per 100 c.c. medium, was added and the culture flask incubated at laboratory temperature. Samples (100 c. c.) were removed by means of a sterile pipette every alternate day and examined for total, ammoniacal, nitrite and nitric, monoamino and diamino-nitrogen. The two forms of amino-nitrogen were determined by the method outlined by Barker and Cohoe (*J. Biol. Chem.*, 1905–6, **1**, 234). The results are given below.

TABLE VI.

Nitrogen distribution at different stages.

Sample No.	Ammoniacal N, Mgs.	Mono-amino N, Mgs.	Di-amino N, Mgs.	Total N, Mgs.	Miscellaneous N (by diffce.), Mgs.
1	1·14	1·14	*Nil.*	3·20	0·12
2	0·69	...	2·88	3·85	...
3	0·825	1·12	1·82	4 55	0·785
5	0·84	1·24	2·64	10·50	5·78
6	0·84	1·04	3·12	12·0	7·00
7	0·84	1·96	3·20	11·90	5·90
8	0·72	0·52	3·52	12·8	8·04
9	0·48	0·64	3·52	12·8	8·16
10	0·24	0·544	1·44	12·8	10·576

From the above it can be seen that, in a very young culture, the first product of atmospheric nitrogen-fixation appears to be ammonia; neither nitrites nor nitrates were found. As the culture grows older, the ammoniacal nitrogen gradually decreases and there is a corresponding increase in the complex nitrogen. Similarly the monoamino-nitrogen content gradually decreases with increasing age of the culture. The results in the last column show very clearly how the complex nitrogen content increases. It therefore seems likely that

the first product of nitrogen-fixation by *Azotobacter* is ammonia which is gradually worked up into more complex forms of nitrogen. This view is in agreement with those of several investigators, notably, Kostytschew and his co-workers (*Z. Physiol. Chem.*, 1926, **154**, 1).

Solubility of the nitrogen compounds formed.—One litre of sterile dextrose-ashby medium was inoculated with a fresh culture of *Azotobacter*. It was allowed to ferment for over three weeks; the contents were then divided into six portions and analysed as follows:—

Nos. 1 and 2.—Total nitrogen was estimated in the usual manner by kjeldahling, and amounted to 7·2 mgs. per 100 c.c.

No. 3.—On precipitation with lead acetate, the filtrate contained no nitrogen.

No. 4.—On pr ation with phosphotungstic acid, the filtrate after kjeldahling co d only a faint trace of nitrogen.

No. 5.—The fe d liquid was merely allowed to pass through a filter paper and t ogen estimated in the filtrate after kjeldahling. The amount o e nitrogen in 100 c.c. was 0·036 mgs.

No. 6.—Precipit f the proteins was effected by Merck's dialysed iron and sod phate. Nitrogen in the filtrate was nil and nitrogen in the pre e 7·2 mgs.

It is thus evident that in old cultures most of the nitrogen fixed by *Azotobacter* is either in an insoluble form or in a form which can be precipitated easily by dialysed iron or other protein-precipitants. This is the case, however, in old and well fermented cultures only; in fresh and young cultures there is an appreciable quantity of nitrogen not precipitated by any of the usual protein-precipitating agents; in other words there appears to be a gradual conversion of simple soluble nitrogen compounds into more complex insoluble substances.

Composition of the Azotobacter *Cell.*

The experiments already described having clearly indicated the synthesis by the organism of protein and other complex nitrogenous substances from the simple materials used as its food supply, there is obvious interest in attempting to seek information concerning the composition of the cell. Unfortunately before this could be carried out in a systematic manner the work had to be interrupted and therefore only a few preliminary results are at present available. As it is unlikely, however, that the work can be continued in the near future

it seems worth while to place these results on record, incomplete as they are.

Supplies of the organism were obtained (1) by growth on solid medium and (2) by growth in liquid culture.

Solid medium.—Soil extract-agar-medium was employed containing 2·5 per cent. agar. Large plates were made up containing 500 c.c. of the medium and a heavy inoculation made by the streak method with a fresh and vigorous culture of *Azotobacter*. Growth was allowed to continue for 45 days when the colonies were scraped off the surface, care being taken to remove as little of the agar as possible. The material thus obtained was transferred to a weighed Gooch crucible containing a thick wad of well ignited asbestos and the traces of agar removed by frequent washing in an autoclave, or by washing at the pump with boiling water. The substance was then dried, weighed and analysed.

Liquid culture.—A carboy was used as culture vessel, containing about 22 litres of glucose-ashby medium. This was sterilised by passing steam and, after cooling, incubated with 1·5 litres of an *Azotobacter* culture. About 100 gms. of sterile calcium carbonate was added to maintain a neutral reaction and the culture fluid was aerated daily for about three hours under sterile conditions. Growth was continued for 45 days. The solid matter was then allowed to settle and finally separated on the centrifuge and treated with dilute acetic acid to remove excess of calcium carbonate. The supernatant liquid obtained above was passed through a Sharple's super-centrifuge when a further small amount of solid material was obtained which was added to the solid obtained in the first operation. After washing, the mixed material was dried on a porous plate *in vacuo* and then analysed.

The culture fluid, freed as far as possible from *Azotobacter* cells, was now saturated with ammonium sulphate when a precipitate of protein material was obtained. This was separated by filtration and dialysed until free from ammonium sulphate.

The material available therefore consisted of (*a*) the *Azotobacter* cells and (*b*) a crude protein precipitate from the culture fluid. Both gave the xanthoproteic, Millon's and Hopkins' reactions.

The cell material contained 5·8 per cent. of nitrogen in the case of the agar culture and 4·6 per cent. in the liquid culture. Assuming this for the moment to be all protein these figures would correspond to about 36 and 29 per cent. of protein respectively in the two samples.

Hydrolysis indicated that it was rich in diamino-nitrogen, but a complete examination was precluded by insufficiency of material.

A further quantity of the cells (from liquid culture) was ground with quartz sand and extracted in a Soxhlet with ether, chloroform and alcohol successively, the residue being finally hydrolysed by boiling on the water bath with 15 per cent. hydrochloric acid for three hours. Ether extracted about 9 per cent. of the cells yielding a light brown mass melted at 72–75°. The fatty acids obtained on saponifying this material with 5 per cent. alcoholic potash melted at 56–60°. Chloroform extracted about 25 per cent. of the cell contents. The extract was insoluble in water and contained both nitrogen and phosphorus. Alcohol extracted only about 4 per cent. of the cell and the amount of material available was too small for examination.

The portion hydrolysed by hydrochloric acid was filtered and neutralised with sodium hydroxide when it was found to reduce Fehling solution, the sugars thus formed being possibly derived by the hydrolysis of reserve carbohydrate material or of nuclein. The above preliminary experiments indicate that fat and a phosphate-containing material are important constituents of the cell.

The Enzymes of the Cell.

In the course of experiments carried out in an investigation of the pigment produced by *Azotobacter* an examination was made of the enzymes present in the cell. For this purpose the cells were thoroughly ground with quartz sand and a little glycerol. The mass was centrifuged and the supernatant liquid used in the tests. The results are shortly summarised below.

Enzyme	Substance used as test	Result
Reducing enzymes	Methylene blue	?
Zymase	Glucose	Negative
Lipase	Olive oil emulsion	,,
Proteolytic enzyme	Gelatin	,,
Deaminase	Glycine	Positive
Carboxylase	Pyruvic acid	,,
Tyrosinase	Tyrosine	Negative
Oxidase	Tincture of guaiacum ...	Positive
Catalase	Hydrogen peroxide... ...	,,

Symbiosis between Azotobacter *and Fungi.*

It has been asserted that certain fungi, in association with *Azotobacter*, can fix atmospheric nitrogen, or at least increase the fixation of nitrogen by *Azotobacter*. Experiments to test this point were carried out with *Aspergillus*, two species of *Mucor* and with *Citromyces*.

Dextrose-ashby medium was used. In this medium the fungi alone grew very slightly and appeared to be incapable of nitrogen-fixation. In association with *Azotobacter* there was, however, a heavy growth, the fungi obviously using the nitrogen fixed by the *Azotobacter*. The amount of nitrogen fixed per 100 c.c. medium after three weeks growth was as follows.

1.	*Azotobacter* alone			....	6·4 mgs.
2.	,,	+	*Aspergillus*	....	7·0 ,,
3.	,,	+	*Mucor* (*a*)	....	6·2 ,,
4.	,,	+	,, (*b*)	....	6·4 ,,
5.	,,	+	*Citromyces*	....	8·8 ,

The presence of *aspergillus* or *citromyces* therefore does appear to have increased the nitrogen-fixation. Both these species break down dextrose very vigorously and it is possible that *Azotobacter* can utilise the intermediate products so formed more readily than dextrose itself

Summary and Conclusions.

1. Cultures of *Azotobacter* preserve their vitality and vigour much better in soil extract-agar-medium containing 1 per cent. mannite than in mineral salts-mannite-agar-medium.

2. Nitrogen-fixation increases in proportion to the amount of sugar fermented in the medium; more than 50 per cent. of the nitrogen fixed is assimilated within the first few days of the fermentation.

3. There is no regular increase in nitrogen-fixation with increasing concentrations of sugar in the medium.

4. Phosphates accelerate the process of nitrogen-fixation but do not economise sugar-consumption.

5. The carbon utilised by the organism could be accounted for within the limits of experimental error.

6. The following by-products were detected and estimated in the fermentation of dextrose by *Azotobacter*, carbon dioxide, ethyl alcohol, aldehyde and formic, acetic, lactic and tartaric acids.

7. It is likely that the first product of nitrogen-fixation is ammonia which is gradually worked up into more complex forms of nitrogen through mono- and diamino nitrogen compounds.

8. The cells appear to contain about 30 per cent. protein matter together with a considerable amount of fat and phosphatide material.

Department of Bio-Chemistry,
Indian Institute of Science,
Bangalore

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