The Biochemistry of the Nitrifying Organisms

3. COMPOSITION OF *NITROSOMONAS*

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In recent years a number of investigations have been carried out on the composition of proteins, polysaccharides and whole cells of micro-organisms. Mazur & Clarke (1942) examined protein hydrolysates of a number of algae. Camien, Salle & Dunn (1945) investigated *Escherichia coli* and four strains of *Lactobacilli* and reviewed earlier work on this subject. Figures for amino-acids for various bacteria and yeasts are given by Stokes & Gunness (1946) and Freeland & Gale (1947). Recently, Fowden (1952) published the results of an analysis of the bulk proteins of *Chlorella vulgaris*. Bell & Northcote (1950) found that the cell-wall polysaccharides of baker's yeast consisted only of glucose. Work (1950) isolated a hitherto unknown amino-acid, $\alpha\epsilon$ -diaminopimelic acid, from *Corynebacterium diphtheriae*.

No information, however, is available on the composition of chemosynthetic organisms. These organisms are of special interest as they derive their energy entirely from simple inorganic reactions and use carbon dioxide or inorganic carbonates as their only source of carbon.

This paper gives the results of an investigation on the composition of *Nitrosomonas*, the chemosynthetic organism oxidizing ammonium ions to nitrite ions.

MATERIALS AND METHODS

Nitrosomonas was grown as described by Lees (1952) in 400 ml. medium.

Preparation of organisms for analysis. When the amount of nitrite formed in a culture vessel had risen to about 300 μg . $\text{NO}_2\text{-N/ml.}$, corresponding to about 10 mg. organic carbon in the whole culture (Hofman & Lees, 1952), the culture was centrifuged and the insoluble inorganic salts with the adhering organisms were collected. After several washings with water the residues were shaken with about 150 ml. 5% acetic acid to dissolve the inorganic salts. The suspension was filtered through a very coarse sintered-glass filter to remove any large particles remaining and the filtrate was again centrifuged and the residue washed several times with small quantities of water.

Amino-acid analysis. The organisms were hydrolysed for 18 hr. with 6N-HCl at 110° in a sealed tube under an atmo-

sphere of N_2 . Excess HCl was removed by evaporation and the residue taken up in 1 ml. of water.

Nitrogen determinations. Total N was estimated in the hydrolysate by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943); α -amino N was determined by the method of Pope & Stevens (1939); free $\text{NH}_3\text{-N}$ estimations were carried out in the usual way on a Markham micro-distillation apparatus (Markham, 1942).

Amino-acid estimations. These were carried out essentially according to the method described by Fowden (1951). It was found, however, that under the conditions of our experiments more constant results could be obtained when control chromatograms of amino-acid solutions of known composition were run simultaneously with the mixtures of amino-acids to be analysed. These control solutions were of approximately the same composition as the unknown. A preliminary estimate of the composition of the unknown was obtained by following exactly the procedure as described by Fowden (1951). The amount of each amino-acid in the *Nitrosomonas* hydrolysate was then determined colorimetrically on a Unicam diffraction grating spectrophotometer at a wavelength of 570 $\text{m}\mu$. using the corresponding amino-acid from the control chromatograms as a standard. Proline was estimated at 440 $\text{m}\mu$.

Most of the amino-acids present in the hydrolysates could be separated on two-dimensional chromatograms (Consden, Gordon & Martin, 1944) using phenol-water 4:1 (v/v) in the presence of 0.1% ammonia as the first, and butanol-acetic acid-water (Partridge, 1948) as the second, solvent.

Leucine, isoleucine, methionine and phenylalanine did not appear as separate spots under these conditions. They were separated by using water-saturated *tert*-amyl alcohol (Work, 1949) on a one-dimensional chromatogram and developing for 4 days in the presence of diethylamine vapour. In agreement with Fowden (1952) it was found that phenylalanine did not occupy the same relative position in this solvent as that described by Work (1949), but appeared between methionine and isoleucine.

Sugar analysis. For the qualitative analysis of the sugar components about 12 mg. of organisms were hydrolysed with 2 ml. $\text{N-H}_2\text{SO}_4$ for 3 hr. at 100°. The insoluble cell fragments were filtered off and the H_2SO_4 removed as BaSO_4 or by treating the hydrolysate with an anion-exchange resin (Amberlite IR-4B) in a column of 0.7×10 cm. The resin was washed first with N-HCl for 2 hr., then with N- NH_3 solution for 2 hr. and finally with distilled water until the reaction of the effluent was neutral. The filtered hydrolysate (about 4 ml.) was added to the column and washed down with 5 ml. of water. The first 2 ml. of effluent were discarded. The next 5 ml. (about pH 6.0) were collected,

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Table 1. *Amino-acid composition of hydrolysates of whole cells of Nitrosomonas in comparison with Corynebacterium diphtheriae, Chlorella vulgaris and brewer's yeast*

Amino-acid	Amino-acid N (as % total N)			
	<i>Nitrosomonas</i>	<i>C. diphth.</i> (Work, 1949)	<i>Chlorella</i> (Fowden, 1952)	Yeast (Lindan & Work, 1951)
Aspartic acid	5	4	6.4	8
Glutamic acid	8	8	7.8	10
Serine	2	2	3.3	4
Glycine	8	5	6.2	8
Threonine	4	1	2.9	7
Alanine	8	8	7.7	10
Tyrosine	1	2	2.8	2
Leucine	3	4	6.1	6
Isoleucine	4	4	3.5	4
Valine	7	7	5.5	8
Histidine	3	6	3.3	8
Arginine	8	20	15.8	10
Lysine	8	5	10.2	9
Phenylalanine	4	3	2.8	1.6
Methionine	2	3	1.4	0.5
Proline	4	5	7.2	3
Cystine	—	—	0.2	0.9
Tryptophan	—	—	2.1	0
Ammonia N	8	—	6.1	—
Total N	87	87	101.3	100

—, Indicates that no estimation has been made.

concentrated *in vacuo*, and the residue, taken up in water (0.2 ml.), was used for analysis.

Chromatography. For one-dimensional paper partition chromatograms, 20–40 μ l. of hydrolysate were used and the following solvents: butanol-acetic acid-water (Partridge, 1948), butanol-pyridine-water, 3:1:1, and butanol-ethanol-water, 4:1:1:0.9 (Hough, Jones & Wadman, 1950). Two-dimensional chromatograms were obtained using phenol-water, 4:1, as the first and the butanol-acetic acid-water mixture as the second developing agent. *p*-Anisidine hydrochloride (3%, w/v, in butanol) was used as a spray reagent (Hough *et al.* 1950).

RESULTS AND DISCUSSION

Table 1 shows the average values obtained from six determinations. Under the conditions used in these experiments it was not found possible to reduce the probable error of the individual values below about 10%. For this reason all the values are given to the nearest whole figure.

It can be seen from the table that the distribution of the amino-acids is similar to that obtained for *Corynebacterium diphtheriae* (Work, 1949) and for *Chlorella vulgaris* (Fowden, 1952) and for brewer's yeast (Lindan & Work, 1951). The content of total basic amino-acids and of tyrosine is lower than in the other three organisms.

The presence of tryptophan has been confirmed by paper chromatography of a barium hydroxide hydrolysate using *p*-dimethylaminobenzaldehyde for spraying. Cystine was present in small amounts only. No attempts have been made to estimate either tryptophan or cystine. $\alpha\epsilon$ -Diaminopimelic

acid, which was found to be part of a great number of micro-organisms (Work & Dewey, 1952) but does not seem to occur in material of non-bacterial origin, was not detected in our hydrolysates.

Attempts were made to extract and estimate free amino-acids from the cells. For this purpose the organisms were extracted repeatedly with 95% ethanol, prior to treatment with acetic acid. The pooled extracts after they had been reduced to a very small volume did not contain any detectable amount of amino-acids.

Table 2. *Sugar composition of hydrolysates of Nitrosomonas*

(Detected on paper chromatograms after spraying with 3% *p*-anisidine-HCl in *n*-butanol.)

	Intensity of spot	
	In visible light	In ultraviolet light
Galactose	+++	+++
Ribose	+++	+++
Rhamnose	++	++
Xylose	+	?
Unknown	+	+++

+++ , strong; ++ , moderate; + , weak, ? , doubtful.

In Table 2 results of a paper-chromatographic analysis of the sugar components are presented. One-dimensional chromatograms were run with the mixture of the unknown and with known sugars on the same sheet. On two-dimensional chromatograms the unknown mixture and known sugars as markers were run together. When the spots of the

unknown and the marker appeared as one single spot it was assumed that the unknown sugar was identical with the marker. On all chromatograms run with four different solvent mixtures there appeared an unidentified spot which reacted with *p*-anisidine hydrochloride to give a pale yellow colour and a fairly strong bluish white fluorescence in the ultraviolet light. Its R_f value of 0.05–0.11 in all four solvents tested was different from that of a series of known hexoses and pentoses.

It is of interest to find that the sugars, galactose, rhamnose and xylose, which were present in the hydrolysates of *Nitrosomonas* were also found by Hough, Jones & Wadman (1952) in hydrolysates of carbohydrates extracted from the freshwater alga *Nostoc*. These carbohydrates contain in addition glucose, which could not be detected in our hydrolysates, uronic acids, and an unidentified component. From the information available it is not possible to obtain any indication as to whether the unidentified

component in the *Nitrosomonas* and *Nostoc* hydrolysates could be the same.

SUMMARY

1. Hydrolysates of whole cells of *Nitrosomonas* have been investigated for amino-acids and sugars.

2. The results of a quantitative study of the amino-acid composition were compared with figures for *Corynebacterium diphtheriae* and *Chlorella vulgaris*.

3. Four sugars were detected on paper chromatograms. Their chromatographic properties were identical with those of galactose, ribose, rhamnose and xylose. In addition an unidentified spot was found.

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A Radiochemical Method for the Micro-estimation of α -Amino-Acids Separated on Paper Partition Chromatograms

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Consden, Gordon & Martin (1944) successfully separated mixtures of amino-acids on paper partition chromatograms and thereby provided a sensitive method for the qualitative analysis of protein hydrolysates. Although the amounts of amino-acids thus separated were only of the order of micrograms, the elegance of the technique stimulated many workers to attempt their quantitative estimation. Bull, Hahn & Baptist (1949) used a standard method for the colouring of amino-acid spots on paper chromatograms with ninhydrin, and estimated their amino-acid contents by scanning

them with a photometer. The differences in the size of amino-acid spots on chromatograms formed the basis of the method used by Fisher, Parsons & Morrison (1948), who showed that the area of a spot was linearly related to the logarithm of its amino-acid content. Between pH 8 and 12 cupric ion will react with α -amino-acids to give complexes of the type $(H_2N.CHR.COO)_2Cu$. This reaction was employed by Martin & Mittelmann (1948) and Woiwod (1949), who used copper phosphate suspensions at pH 9 to form the soluble copper complexes of amino-acids; the former authors estimated