THE ACCUMULATION OF ELECTROLYTES

XI. Accumulation of Nitrate by Valonia and Halicystis

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(Accepted for publication, January 6, 1938)

In the course of preliminary experiments on the penetration of nitrates into *Valonia macrophysa*, Kütz., in Bermuda in the winter of 1935–36, it was found that living cells invariably contained significant concentrations of nitrate ions.

Previously Wodehouse[1] had shown that *Valonia* sap gave a test for nitrate by means of the nitron reagent, although the surrounding sea water did not. An analysis made in this laboratory on preserved sap[2] yielded the low value of 0.002 M, and it was conjectured that it might be higher in fresh sap. In 1934, Ullrich,[3] working in Naples, found that freshly collected cells had no nitrate detectable by the phenol-disulfonic method but that after 5 days in the circulating sea water of the station the nitrate concentration rose to the neighborhood of 0.015 M, which was also the average concentration of nitrate in the circulating sea water at the time of the experiments. He did not find accumulation of nitrate in any of his experiments.

On the basis of earlier work we had assumed that the nitrate concentration of the sap could be neglected. The object of the present paper is to show that in *Valonia macrophysa* in Bermuda nitrate is accumulated to a large extent, as Wodehouse has already shown qualitatively.

Analyses made on *Halicystis Osterhoutii*, Blinks and Blinks in 1936–37 showed nitrate inside greater than outside by 500 times or more.

The work on *Valonia* falls into two parts, 13 analyses made in the

[1] Wodehouse, R. P., *J. Biol. Chem.*, 1917, **29**, 453.

[2] Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, **5**, 225.

[3] Ullrich, H., *Planta*, 1934, **23**, 146.

winter of 1936–37 on the sap of cells which had been exposed to the circulating salt water system of the Bermuda Biological Station for varying periods from a few hours up to 6 weeks, and 10 analyses made in the winter of 1937–38 on sap extracted in the field immediately after collection.

Analyses

The presence of nitrate in the sap and in the sea water was demonstrated qualitatively by the method of Atkins,[4] using diphenylbenzidine, and in the case of sea water this method was used for the quantitative analysis. Because of the large amount of nitrate present it was not suitable for the sap and a method based on the use of Devarda's alloy, which reduces nitrate and nitrite to ammonia, was substituted.

The analysis was carried out as follows. 0.1 ml. of centrifugalized sap was transferred to the distillation flask of a micro-ammonia steam distillation apparatus similar to that described by Teorell.[5] 5 mg. of Devarda's[6] alloy was then added and the flask was connected to the apparatus. Then 2 ml. of 30 per cent sodium hydroxide was admitted to the flask and reduction started by applying a small flame for a few moments. The escaping hydrogen was made to pass through the condenser, through 10 ml. of 0.05 N sulfuric acid, and finally into the air through a trap consisting of glass pearls wet with 0.05 N sulfuric acid. In this way loss of ammonia during the reduction stage was avoided. After 10 minutes reduction, the ammonia was distilled in the steam current into the sulfuric acid and its quantity was determined by Nessler's reagent, using the Zeiss-Pulfrich step-photometer as described in a previous paper.[7]

A determination of ammonia was made on each sap sample by steam distillation without the addition of the reducing alloy, and the result of this was subtracted from the total nitrogen found by the first procedure, to give nitrate + nitrite nitrogen.

Several samples of sap also were tested for nitrite, using the dimethyl aniline hydrochloride test. These invariably gave less than 0.0001 M nitrite nitrogen which was about the lower limit of sensitivity as we used the method. Nitrite nitrogen may therefore be neglected.

The diphenylamine test for nitrate is, as Ekkert[8] has pointed out, also given

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[4] Atkins, W. R. G., *J. Marine Biol. Assn. United Kingdom*, Plymouth, 1932, **18**, 167.

[5] Teorell, T., *Biochem. Z.*, Berlin, 1932, **248**, 246.

[6] Devarda, A., *Chem. Zentr.*, 1897, **2**, 64.

[7] Jacques, A. G., *J. Gen. Physiol.*, 1937–38, **21**, 665.

[8] Ekkert, L., *Pharm. Zentralhalle*, 1925, **66**, 649 (quoted from *Chem. Abstr.*, 1926, **20**, 158).

by iodates, chlorates, perchlorates, bromates, chromates, and bichromates, tungstates, molybdates, vanadates, ferricyanates, and peroxides. Atkins points out that the same substances must also give the diphenylbenzidine test. He adds to the list arsenate and ferric iron. Of these substances, the heavy metal acids are most unlikely to be present in the sap in detectable amounts. Ferric iron has been found to be absent by testing with thiocyanate. Iodate and bromate have been excluded by adding KI to the sap in the presence of dilute sulfuric acid and chloroform. If either iodate or bromate had been present in appreciable amounts the iodine produced would have colored the chloroform. This test is better for iodate than for bromate, but the absence of an important amount of the latter is also proved. Chlorate and bromate were tested for by the sea water method of Korenman,[9] and found to be absent. Perchlorate is scarcely likely to be present in any important amount and this is true also for peroxides. In any case the absence of these latter was proved by a test with "luminol," or 3-amino phthalhydrazide (not "luminal"), by a method suggested to the writer by O. Baudisch.[10]

It seems clear therefore that the strong diphenylbenzidine test we obtained is valid evidence of a considerable amount of nitrate in the sap.

The analyses, however, were made by reducing the nitrate and determining ammonia. It is therefore necessary to consider how the results may be affected by other sources of ammonia. Brandt[11] used aluminum amalgam in a solution made alkaline by magnesium oxide to reduce nitrate and nitrite, but his method has been criticized by Harvey[12] for the very small amounts of nitrate in the sea water on the ground that the treatment may decompose organic nitrogen compounds, such as amino acids and proteins. His criticisms were applied to the case of sea water. They can scarcely apply to our analyses of the sap where the nitrate content is at least several thousand times greater. The sap contains too little protein, even if it could be all decomposed by reduction, so as to give its nitrogen in the form of ammonia, to affect our results seriously and the absence of all but a doubtful faint trace of amino acid has been established by testing the sap according to the method of Folin.[13] We may say therefore with considerable confidence that our method gives reasonably accurate values for nitrate in the sap.

This applies chiefly to *Valonia*. *Halicystis* sap has not been studied so much,

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[9] Korenman, I. M., *Mikrochemie*, 1936, **20**, 225.

[10] According to this method, a dilute alkaline solution of 3-amino phthalhydrazide is treated with a few drops of a dilute hemin solution, and to this is added a trace of the test solution. In the presence of peroxides the mixture luminesces strongly. *Valonia* sap failed to excite the luminescence, but a drop of a very dilute H_2O_2 solution did so.

[11] Brandt, K., *Nova acta Abh. d. k. Leop.-Carol. Deutsch. Akad. Naturf.*, Halle, 1915, Vol. C, No. 4.

[12] Harvey, H. W., *Rapp. conseil perm. internat. expl. mer*, 1929, **53**, 72.

[13] Folin, O., *J. Biol. Chem.*, 1922, **51**, 377.

and moreover the sap used here contained much larger, and more variable, amounts of ammonia, as determined by distillation from alkaline solution and Nesslerization. This is in distinct contrast with the previous analyses of *Halicystis* sap[14] which contained no appreciable amount of ammonia. Blinks[15] has suggested that the difference in ammonia content of *Halicystis* cells probably depends on age and condition. Old cells, such as were used in the present case, which have gone through several reproductive periods, invariably contain considerable quantities of dark deposits in the sap and it is presumed that the decomposition of this material furnishes ammonia, since cells with the dark material always have ammonia in them. Before analysis the sap was centrifugalized, but there remains the possi-

TABLE I

Analyses of Sap of Valonia for Nitrate

Description of locality	Concentration nitrate + nitrite* in sap	Concentration nitrate + nitrite in sea water	Ratio $\frac{(NO_3)_{sap}}{(NO_3)_{s.w.}}$
	M	M	
Shallow lagoon with circulation for about 12 hours a day	0.0022	5×10^{-6}	440
Shallow tidal pool sheltered from the sun	0.0134	7×10^{-7}	19143
Deep pond with slow circulation	0.0242	9×10^{-6}	2690
Deep pond with slow circulation	0.0221	5×10^{-6}	4420
Tidal inlet	0.0187	1×10^{-5}	1870
Tidal shallow cove	0.0377	1×10^{-5}	3770
Open shore	0.0300	1×10^{-5}	3000
Artificial ponds at head of tidal inlet	0.0172	1×10^{-5}	1720
Tidal pool	0.0113	9×10^{-6}	1256
Tidal pool	0.0115	9×10^{-6}	1277
Station circulating salt water system (3 days)	0.0111	5×10^{-6}	2220
Average	0.0181	7.5×10^{-6}	2387
Average deviation from the mean	0.0077		

* Nitrite was shown to be negligibly small in both sap and sea water.

bility that soluble nitrogenous substances, arising from the decomposition of the insoluble organic material, may have given rise to ammonia on reduction. However, it seems probable that there is some nitrate in normal *Halicystis* sap, although the amount is smaller than in sap of *Valonia*.

In the first stage of the work (season 1936–37) the average concentration of nitrate in *Valonia* sap was found to be 0.0143 M, and the

[14] Blinks, L. R., and Jacques, A. G., *J. Gen. Physiol.*, 1929–30, **13**, 733.

[15] Blinks, L. R., *J. Gen. Physiol.*, 1933–34, **17**, 109. Blinks, L. R., and Blinks, A. H., *Bull. Torrey Bot. Club*, 1931, **57**, 389.

average deviation from the mean 0.0023 M (13 samples). For *Halicystis* the average concentration of nitrate was 0.0043 M and the average deviation from the mean 0.0019 M (3 samples).

Each group of *Valonia* cells taken for analysis was different in its history but this seemed to make no great difference in the nitrate content. In all cases, however, the cells were exposed to the circulating salt water of the Biological Station. However, tests of the circulating sea water at various times gave only faint traces of nitrate. But in view of Ullrich's results which came to our attention after the completion of the first part of the work it seemed desirable to test sap samples expressed in the field and to compare the nitrate content with that of the sea water collected in the vicinity of the cells at the time the sap samples were expressed. The results of these analyses made in the winter of 1937–38 are given in Table I.

DISCUSSION OF RESULTS

As Table I shows the nitrate content of the sap was found to be in all cases much greater than that of the sea water surrounding the cells, the average accumulation in *Valonia* being more than 2000-fold. Moreover, it is not impossible that the nitrate values for the sea water may be too high, especially in view of the statement of Rakestraw[16] that "The vicinity of Bermuda is an area especially devoid of nitrite and other 'nutrients.' A number of surface samples were collected from the harbors and bays of the Bermuda Islands, and even from their fresh water streams and ponds, with no more than traces of nitrite, and minimal amounts of nitrates." As pointed out above the diphenylbenzidene test for nitrate is given by other oxidants and these may have been present in the sea water samples which were necessarily taken along the shore at low tide, when the cells are most easily collected. Under these conditions it would not be surprising if the sea water were modified considerably by metabolism.

The minimum amount of accumulation in *Valonia* is therefore more than 2000-fold.

This is of interest in view of a recent statement that it is doubtful whether *Valonia* can accumulate any other anion than chloride.[17]

[16] Rakestraw, N. W., *Biol. Bull.*, 1936, **71**, 133.

[17] Steward, F. C., and Martin, J. C., *Carnegie Institution of Washington, Pub. No. 475*, 1937, 87.

In considering the possible sources of energy responsible for the accumulations, there is apparently no nitrate with a gradient of chemical potential in the required direction. The manner in which the energy necessary for accumulation is applied is not clear, but a number of possibilities suggest themselves. (1) That nitrogen or nitrogenous compounds in the sea water, such as proteins, nitrites, or ammonia pass into the sap and are oxidized in the cell to nitrates.[18] (2) That the cell itself or bacteria found on the surface of the cell utilize nitrogen to form soluble nitrogenous compounds, and these pass into the cell and are there oxidized to nitrates,[19] and (3) that nitrate is formed by the cell itself or by bacteria at the external surface of the protoplasm. If bacteria manufacture the nitrate they supply the chemical energy and the nitrates pass into the cells by means of diffusional energy. In all other cases the cell itself supplies at least a part of the energy in the form of chemical energy.

It is clear that if the nitrate is manufactured inside the cell, the exit of nitrate must be very slow. The evidence in this respect is contradictory. Thus the results of Ullrich[3] described above suggest that nitrate passes freely in and out of the cell and comes rapidly into equilibrium in the sap and sea water. On the other hand we find that cells which have been kept as long as 3 months at 15°C. in dim light in sea water practically free from nitrate still contain appreciable amounts of nitrate. Moreover there is no evidence that the sea water in contact with them has gained nitrate. Under these circumstances we may assume either that the exit is very slow or that the cell gradually consumes the nitrate. This must be left to future investigation.

[18] Moore, Whitley, and Webster (Moore, B., Whitley, E., and Webster, T. A., *Proc. Roy. Soc. London, Series B*, 1921, **92**, 51) believe that *Enteromorpha compressa* is able to synthesize soluble nitrogenous compounds from nitrogen dissolved in sea water.

[19] At the present time the weight of the evidence seems to be against the direct fixation of nitrogen by green algae. It is now supposed that the fixation takes place through bacteria which, according to a number of investigators, are regularly found on the surface of certain algae. In the case of *Valonia macrophysa*, evidence of bacterial activity is found in the summer in Bermuda when cells are stored in still water. The cells and even the walls of the containing vessels become covered with thick layers of a jelly-like material which gives the cells a slippery feeling.

It should be pointed out, however, that (as shown in another paper[20]) when the nitrate content outside the cell is increased very greatly, its concentration inside also increases which indicates that nitrate can pass through the protoplasm.

Almost invariably in our work we have found the total cation concentration, ($K^+ + Na^+$) or in some cases ($K^+ + Na^+ + NH_4^+$), to be greater than the halide concentration.[21] On the basis of Van der Pyl's careful analysis Osterhout and Dorcas calculated the difference to be 1.9 per cent.[21] They suggested that the discrepancy would be accounted for if nitrate, bicarbonate, and organic anions were taken into account. From 25 analyses made at various times[22] on groups of cells under normal conditions the average concentration of ($K^+ + Na^+$) was 0.6231 M and for halide 0.6134 M, a difference of 0.0097 or 1.6 per cent. This discrepancy is more than covered by the concentration of nitrate in the sap for which an average[23] value of 0.0161 M has been obtained. The discrepancy is now 1.0 per cent in the other direction; *i.e.*, we have an excess of halide plus nitrate. This is perhaps due to small amounts of calcium, magnesium, and ammonia in the sap which are not taken into account.

Our results differ from those of Stewart and Martin[17] who state that in *Valonia macrophysa* of Tortugas the halide concentration is almost always greater than (K + Na) and that for the average of 29 analyses ($K^+ + Na^+$) = 0.620 M and halide = 0.624 M so that the excess halide = 0.64 per cent.

SUMMARY

The nitrate concentration in the sap of *Valonia macrophysa*, Kütz., is at least 2000 times that of the sea water, and in *Halicystis Osterhoutii*, Blinks and Blinks, at least 500 times that of the sea water.

[20] Jacques, A. G., *J. Gen. Physiol.*, 1937–38, **21**, 775.

[21] Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 301; 1931–32, **15**, 537; 1933–34, **17**, 727. Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924–25, **7**, 633.

[22] Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, **14**, 301; 1931–32, **15**, 537; 1933–34, **17**, 727. Also unpublished results.

[23] This is the average of all 24 analyses made in 1936–37 and 1937–38.