# THE CELL SAP OF HYDRODICTYON*

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It is obvious that biological findings are enhanced by comparative studies made on a variety of materials; this has certainly proved to be the case in plants with large multinucleate cells, so much utilized in the last 15 years for problems of permeability, salt accumulation, and bioelectric phenomena. Those most employed have fallen in three orders: Charales, or Charophyta (*Nitella*); Siphonocladiales (*Valonia*); and Siphonales (*Halicystis*). Their comparative study has often revealed common principles, as well as startling differences, which in some cases at least, have been reconciled by experimental treatment. While there are other, somewhat less satisfactory genera in each of these orders (respectively: *Chara*; *Batophora* and *Ernodesmis*; *Bryopsis*), a member of a still different order is even more likely to yield new results, or extend the validity of general principles. The present paper deals with a member of a group almost wholly new for such studies, namely the Chlorococcales; these are generally microscopic algae, but one genus, *Hydrodictyon*, has multinucleate cells reaching considerable size. In *H. reticulatum*, the common water-net, found in ponds the world over, the cells are large enough to be seen by the naked eye, and have been used (under the microscope) for studies of the penetration of alcohol by plasmoptysis or bursting.[1] They are not, however, large enough for individual handling, sap extraction, and bioelectric contacts. Three other species have been described, with larger cells: *H. africanum*, Yamanouchi,[2] with net cells becoming separated into spheres a centimeter in diameter; *H. indicum*, Iyengar;[3] and *H. patenaeforme*, Pocock.[4] The latter, occurring in the same ponds as *H. africanum*, has only recently been described, from South Africa, but it appears to be the

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[1] Holdheide, W., *Planta*, 1931, **15**, 244.

[2] Yamanouchi, S., *Bot. Gaz.*, 1913, **55**, 74.

[3] Iyengar, M. O. P., *J. Indian Bot. Soc.*, 1925, **4**, 315.

[4] Pocock, M. A., *Tr. Roy. Soc. South Africa*, 1937, **24**, 263.

species reported upon here. Smith[5] indicated its occurrence in California several years before Pocock's description, but did not give it a name. It turns out to be identical in almost every respect, however, with *H. patenaeforme*, and will be so designated, lacking sufficient distinction to make it a new species.

Sap analysis of the same species from Africa would be of interest, in comparison with the present form. There is some evidence that *H. africanum* may occur in California, and it may be possible later to report upon it.

### *Material and Sap Extraction*

The Californian *H. patenaeforme* occurs nearly every year in the same spot on the Stanford campus, in a low-lying pasture which is flooded from January to April or May. The young nets appear usually in March as very delicate flat plates or saucers (hence the name), and grow rapidly for 2 months or more, often becoming contorted and twisted into ropes or balls, but sometimes remaining extended in great flat nets up to 20 cm. in diameter, with interstices 2 or 3 cm. wide between the cells. The latter usually join at their ends in threes, although cells at the margin of the net may extend outward alone or in chains. The individual cells become 2 or 3 cm. long, and 1 (rarely 2) mm. in diameter, before assuming the golden, opaque appearance which precedes isogamete formation and collapse of the cell (usually on illumination).

The net is purely and simply aquatic, having no rhizoids or holdfasts like *Nitella*, *Valonia*, and *Halicystis*, which might conceivably influence the absorption of elements from the soil or substrate. In *Hydrodictyon* absorption must be from the surrounding water by the actual cells themselves.

Its very rapid growth might be another advantage for experimental absorption studies, since it grows from an almost invisible fragile net to very large size in about 2 months—an increase of easily 1000 per cent in volume. The season can be extended somewhat by bringing the nets into cooler, shaded pools in the botanical garden, but they rarely survive beyond June in any case.

The cells are readily separated from each other by a slight bend at their junctions, and they then live several weeks when so isolated from the net. This makes them convenient objects for sap extraction free from contaminating material, and for bioelectric contacts at two points on the surface. The constitution of the cell sap is itself of interest, as extending the rather meagre list of organisms from which sap can be obtained in pure state; it also should be known for bioelectric studies, in order to analyze the rôle played by various ions in the maintenance of the normal potential, resistance, and capacity from vacuole to exterior.

The following methods of obtaining sap were employed:

(*a*) For the most careful extraction, the cells were separated and washed in distilled water, then, one at a time, gently drained and dried on filter paper, and placed on a clean glass slide. There the end of the cell was gently pricked with a fine glass needle.

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[5] Smith, G. M., Fresh water algae of the United States, New York, McGraw, Hill, 1933, 487.

Some sap spurted out upon the slide as the cell collapsed; this, and the remainder in the cell was immediately picked up by capillarity or micrometer screw suction into a mounted micropipette, without pressure upon the cells, so that little or no protoplasm or chloroplasts were included in the clear sap. Each cell yielded about 0.01 cc. of sap in this manner, and about 0.5 cc. could be collected in an hour. In all, several cubic centimeters were thus obtained, for samples used to determine the more abundant constituents (K, Cl), as well as for H ion, $NH_3$, and sulfate tests without the chance of contamination by protoplasm. Several different microtitrations of the chloride were performed on different samples, in order to gain an idea of the variations in Cl at different times, and with the advancing season. It was found to be fairly constant between 0.05 and 0.06 M, older cells generally showing a slightly higher concentration. This pure sap also gave, surprisingly enough, large tests for sulfate, comparable to that in sea water, as borne out by larger scale analyses mentioned below. A charring test was negligible, as was ammonia, by Nessler reagent. Nitrate and phosphate were negligible. There was not enough of sample *a* to analyze for Na, Ca, Mg, or $SO_4$.

(*b*) To obtain larger samples for analysis of these less abundant elements, but still with a minimum of contamination by outside solution or by protoplasm, groups of smaller cells or complete nets were thoroughly washed in distilled water, drained on filter paper, and then exposed in large crystallizing dishes to a current of warm air until quite dry and flattened to the glass. They were then soaked in several successive small volumes of distilled water to leach out the soluble salts without crushing the cells or releasing any colloidal constituents of the protoplasm, this still being held back by the intact cellulose wall. The volume of this wash water was made up to equal approximately that of the original undried cells, since the cell volume is easily 95 per cent sap. When this was done, the chloride and potassium content, as well as K:Cl ratio, were found to be not very different from that in the directly extracted sap, indicating that the very thin protoplasmic film had not yielded up, or absorbed, appreciable amounts of these elements. This makes it seem likely that the other mineral constituents, chiefly sodium, calcium, and sulfate, are also much the same in such a leached solution as in the pure cell sap; danger of contamination appears the more remote since when protoplasm is definitely included, as in the less pure samples below, these elements are also much the same. On the other hand, magnesium appears in such protoplasmic inclusions, as would be expected from the presence of chlorophyll.

(*c*) Another method of killing the cells, and releasing the sap constituents, was to bring the washed and drained cells to a nearly boiling temperature on a water bath, pouring off the copiously released sap, and leaching again with distilled water to the proper volume, as above. This sap, even from uncrushed cells, was darker in color and tended toward prompter putrefaction than samples *a* and *b*, indicating much greater contamination with protoplasm, organic matter now being released across the cell wall. The inorganic analysis is not very different, however, from that of *a* and *b*.

(*d*) For still cruder extracts, masses of cells, after washing and draining, were crushed with a glass rod, and the copiously extruded sap filtered free of plastids and large protoplasmic particles. Here considerable contamination was inevitable, but the analysis of the ash gave values rather similar to *a*, *b*, and *c*, except for the presence of magnesium and phosphate, obviously derived from the protoplasm.

(*e*) Finally, the residue from this crushing and filtering was saved, and after ashing, was analyzed to give the mineral constituents of the protoplasm and cell wall. These

are not notably different from the sap, possibly due to the still strong admixture of the latter with the residue. Strong tests were also obtained here for iron, silica, and phosphate, as might be expected.

*Pond Water.*—For comparison with the sap, and to indicate the ratio in which the several elements were accumulated in the sap above their external concentration, a large water sample was collected from the shallow pond in which the cells were growing. Since it was very dilute in most of the elements, 5 liters were carefully evaporated down to 500 cc., and the residue, including precipitates, analyzed by the methods employed for the sap. The findings were then divided by 10 to give the original values. The ratios to sap are given in Table II.

## *Analytical Methods*

Owing to the small quantities of sap available, it was necessary to determine as many cations as possible on a single sample. This frequent biological need may justify a

TABLE I

*Recoveries from a Known Solution (0.1 Per Cent Glucose Solution Containing Na, K, Ca, and Mg in the Amounts Noted)*

| | Added | Recovered |
|---|---|---|
| | *mg.* | *mg.* |
| Na | 1.10 | 0.54; 0.57 (using Cd amalgam) |
| | | 1.10; 1.13 (using granular zinc) |
| K | 3.90 | 3.20; 3.90; 3.80; 3.76; 3.86 |
| Ca | 1.00 | 0.96; 1.04; 1.02; 1.02; 0.99 |
| Mg | 0.90 | 0.87; 0.88; 0.90; 0.98; 0.94 |

detailed description of methods. A study of the literature[6–9] indicated that it would be possible to adapt some of the recent semi-micro methods to a quantitative procedure for the determination of the desired elements. After the various methods had been fitted together into a procedure such that no reagents containing interfering elements were added, it was tested on known quantities of a 0.1 per cent glucose solution containing all of the elements to be determined. Table I shows the results of these recovery experiments.

It will be noted that the first two recoveries on sodium were poor. Holmes and Kirk[6] in their method for the determination of sodium recommend the reduction of the uranium in the sodium zinc uranyl acetate precipitate by cadmium amalgam. We found that this reagent gave low results (first pair of recoveries), but when granular zinc was substituted for the Cd amalgam a complete reduction was obtained and good recoveries resulted (second pair). Recently Linder and Kirk[10] have published a method

[6] Holmes, B., and Kirk, P. L., *J. Biol. Chem.*, 1936, **116**, 377.

[7] Cruess-Callaghan, G., *Biochem. J.*, London, 1935, **29**, 1081.

[8] Murer, H. K., *Ind. and Eng. Chem., Analytical Edition*, 1937, **9**, 27.

[9] Wilcox, L. V., *Ind. and Eng. Chem., Analytical Edition*, 1937, **9**, 136.

[10] Linder, R., and Kirk, P. L., *Mikrochemie*, 1938, **23**, 269.

for sodium in which is discussed the relative efficiencies of various reducing agents for the uranium, with the conclusion that either granular zinc or cadmium spirals are best suited for the method.

The special reagents employed were the following:

*A*. For calcium:

1. Saturated $(NH_4)_2C_2O_4$
2. $KMnO_4$ 0.05 N

*B*. For magnesium:

1. 1 gm. of 8-hydroxyquinoline dissolved in 89 ml. of absolute alcohol. Add 10 ml. concentrated $NH_4OH$ and 1 ml. concentrated HCl. 2 ml. good for 1 mg. Mg.
2. 2.7 gm. $KBrO_3$ and 25 gm. KBr dissolved in 500 ml. $H_2O$.
3. 6 gm. $Na_2S_2O_3 \cdot 5\ H_2O$ dissolved in 1 liter $H_2O$. Standardize against $KIO_3$.

*C*. For sodium:

1. 10 gm. uranyl acetate $\cdot 2\ H_2O$ and 6 gm. (30 per cent) acetic acid. Make up to 65 gm. with $H_2O$.
2. 30 gm. $Zn(Ac)_2 \cdot 3\ H_2O$ and 3 gm. (30 per cent) acetic acid. Make up to 65 gm. with $H_2O$.
3. Warm Nos. 1 and 2 until complete solution is obtained, and then mix together. Allow to stand for 24 hours and filter.
4. 0.02 N ceric sulfate.
5. Phenanthroline ferrous sulfate indicator.
6. Glacial acetic acid saturated with sodium zinc uranyl acetate.

*D*. For potassium:

1. 0.02 N ceric sulfate
2. Sodium cobaltinitrite
3. 0.02 N ferrous ammonium sulfate.

## *Procedure*

10 gm. of the biological material are placed in a small silica or platinum evaporating dish. One half ml. of concentrated sulfuric acid is added and the material evaporated to dryness on a hot plate or water bath. The dish is then placed in a muffle furnace and ashed at 500–550°C. for about 3 hours or until a white ash is obtained. The ash is taken up with 1 ml. of concentrated HCl. A little water is then added and the dish heated for a few moments on a water bath. The solution is filtered to remove the silica. At this point the solution can be made up to a definite volume, and an aliquot representing 1/10 of the volume set aside for the potassium determination.

*Calcium.*—Add a few drops of bromine water to the remaining solution to oxidize any ferrous iron present. Boil to remove the bromine and make basic to methyl red with $NH_4OH$ to precipitate the iron and aluminum. The solution is then filtered and washed with hot water. Evaporate the filtrate to about 20 ml.; add 2 ml. of saturated $(NH_4)_2C_2O_4$ to precipitate the calcium; make basic to methyl red and allow to stand overnight. Filter out the $CaC_2O_4$ through a small, fine-pored, sintered (fritted) glass Gooch crucible and wash several times with dilute ammonia water (4 ml. $NH_4OH$ + 250 ml. $H_2O$). Save the filtrate for Mg determination. Wash off the bottom of the crucible and dissolve the precipitate with hot 1 N $H_2SO_4$, drawing it into a test tube placed inside the suction flask. Wash the contents of the tube into a beaker, heat to 90°C., and titrate with 0.05 N $KMnO_4$.[8]

*Magnesium.*—Evaporate the filtrate from the Ca determination to about 8 ml., and add 5 ml. of 8-hydroxyquinoline reagent. Heat to about 95°C. for a few moments and cool to room temperature. Filter the precipitate, using a Gooch crucible containing a fritted glass filtering disk and wash with 1 N $NH_4OH$. Transfer the filtrate to a silica evaporating dish and place on a water bath. Dissolve the magnesium precipitate with 4 ml. of 4 N HCl and suck into a 60 ml. test tube. Add 10 ml. of the bromate-bromide solution and immediately stopper the tube. Mix well and let stand for 5 minutes. Add a crystal of KI to reduce the excess bromine and titrate the released $I_2$ with 0.02 N $Na_2S_2O_3$. Magnesium = equivalents of bromine used/8.[7]

*Sodium.*—When the filtrate from the magnesium determination has evaporated to dryness it is placed in a muffle furnace and heated to 500°C. long enough to drive off the ammonium salts and the magnesium reagent. Cool and add 1 ml. of 1 N $H_2SO_4$ and transfer to a 50 ml. beaker. Evaporate to 1 ml. and add 40 ml. of cold, filtered zinc uranyl acetate reagent and let it stand for an hour. Filter through the previously mentioned glass Gooch crucible and wash with glacial acetic acid saturated with sodium zinc uranyl acetate. Dissolve the precipitate with 30 ml. of 2 N $H_2SO_4$ and suck into a test tube. Pour the solution into a 250 ml. flask and add 2 gm. of granular zinc. Heat to around 80°C. for a few moments to reduce the uranium from valence 6 to 3 and transfer to a beaker. Aerate the solution for a few moments, and oxidize the uranium to a valence of 6 with 0.01 N ceric sulfate.

*Potassium.*—This can be worked into the above series whenever there is time. Take the aliquot set aside for this determination and make up to 10 ml. Add 1 ml. of 1 N $HNO_3$. Weigh out roughly 1 gm. of sodium cobaltinitrite for each sample to be precipitated and dissolve in water to make a 20 per cent solution. (Do not make up an excess of the reagent as it does not keep well.) Add slowly, with stirring, 5 ml. of the reagent to the unknown potassium solution. Allow to stand for 2 hours at room temperature. Filter the solution through a Gooch crucible and wash with 0.01 N $HNO_3$ at least five times. Wash off the bottom of the crucible and place in a beaker containing an excess of 0.02 N ceric sulfate solution and 5 ml. of 1–1 $H_2SO_4$. Heat on a water bath until the precipitate is dissolved; allow to cool and titrate the excess ceric sulfate with 0.02 N ferrous ammonium sulfate. We have checked the factor 7.1084, given by Wilcox,[9] many times and found it to be correct.

Phosphate was determined by the Zinzadze method.[11] A wet ashing with nitric and sulfuric acids was found satisfactory for decomposing the sample. Losses of phosphate as high as 30 per cent were observed when the material was ashed in a muffle furnace with sulfuric acid as an ash-aid.

Chloride was determined by the conventional $AgNO_3$ titration, using potassium chromate as an indicator.

Sulfate was determined gravimetrically, by precipitation with barium chloride.

## DISCUSSION

The results of the analyses are summarized in Table II.

It is clear that potassium strongly predominates, being fifteen to twenty times as concentrated as Na in the sap, although the ratio is markedly reversed in the pond water. Thus the actual accumulation ratio is tre-

[11] Zinzadze, C. H., *Ind. and Eng. Chem., Analytical Edition*, 1935, **7**, 227.

mendous, potassium being 4000 times as concentrated in the sap as in the pond water, at the time the analysis was made. It may be that some of this potassium was picked up earlier in the season, as released from other filamentous algae, which appear first in the pools, then die away before the *Hydrodictyon* begins to grow. However, it is at least retained against such a high gradient. That it exists as ionized salt in the sap is shown by the high electrical conductivity, as well as the effective osmotic pressure of the

TABLE II

*Composition of Various Samples of Cell Sap and Protoplasm from Hydrodictyon patenaeforme Pocock. Expressed in Moles Per Liter*

| Sample | K | Na | Ca | Mg | Cl | $SO_4$ | $HCO_3$, etc. |
|---|---|---|---|---|---|---|---|
| Pure sap, *a*, 1 | 0.08 | | | | 0.059 | | |
| Pure sap, *a*, 2 | 0.101 | | | | 0.063 | | |
| Dried and leached, *b* | 0.069 | 0.0047 | 0.0008 | — | 0.0523 | 0.0086 | 0.0043 |
| Heated, leached, *c* | 0.075 | 0.0039 | 0.0017 | — | 0.052 | | |
| Crushed, filtered, *d* | 0.062 | 0.0038 | 0.0012 | — | 0.048 | | |
| Crushed, filtered, *d*, 2 (1937) | 0.0722 | 0.0045 | 0.0036 | — | 0.0532 | (0.0056 phosphate) | |
| Crushed, (1937) *d*, 3 | 0.075 | 0.003 | 0.002 | 0.0015 | 0.06 | 0.0078 | 0.0067 |
| Cell residue from last, *e* | 0.0818 | 0.0025 | 0.0023 | 0.0025 | 0.058 | | |
| Average sap *S* (except *e*) | 0.0763 | 0.004 | 0.0018 | — | 0.0553 | 0.0082 | 0.0055 |
| Pond water *P* | 0.000019 | 0.0013 | 0.00108 | 0.0009 | 0.00108 | 0.00077 | |
| Accumulation ratio: (*S*/*P*) | 4000 | 3 | 1.65 | — | 51 | 10.7 | |

Specific conductivity (several samples, type *d*): 0.009 to 0.0096 (equivalent to 0.077 to 0.083 M KCl)

Tests on fresh *a* samples (purest sap):

pH, 5.5 to 6.0 (by glass electrode, Beckman capillary type)

$NH_3$ less than 0.001 M (Nessler test)

$SO_4$ roughly comparable to sea water (actually less by analysis)

Heat test: no charring, indicating little or no organic matter in sap

sap (by plasmolysis, mentioned below). The potassium is also consistently higher than the chloride, by about 0.02 M. This discrepancy is partly made up by the very high sulfate value, about 0.008 M or 0.016 N, nearly 30 per cent of the Cl in equivalents per liter. There is no question about this sulfate being high, since the purest sap, from a single cell without contamination from protoplasm gives a strong test with acid $BaCl_2$, roughly comparable to that in sea water. Even this does not quite balance anions against cations however, so a further search was made. Phosphate and nitrate being negligible in the pure sap, and organic acids probably missing

because of the absence of charring on heating, there remained the possibility of carbonate (bicarbonate at pH 5.5 to 6.0 in the fresh sap). On titrating with HCl to a point acid to methyl red, a figure of 0.0043 N was found for excess base in one case (dried and leached sample); and 0.0067 N in another (crushed sample). Assuming this to be bicarbonate, the cations nearly balance the anions, as shown in Table III.

That there are probably no other major constituents in the cell sap is shown by plasmolysis experiments. Intact cells of *Hydrodictyon* were placed in various dilutions of sea water (as representing a well balanced solution). The cells in sea water/8 and greater dilutions stayed turgid and lived well for several days. Those in sea water/4 showed prompt,

TABLE III

*Cell Saps, Expressed As Normality (Equivalents per Liter)*

| | *Hydrodictyon*, dried and leached, *b* | *Hydrodictyon*, crushed, *d* | *Nitella clavata*[12, 13] |
|---|---|---|---|
| K | 0.0690 | 0.075 | 0.0543 |
| Na | 0.0047 | 0.003 | 0.0100 |
| Ca | 0.0016 | 0.004 | 0.0204 |
| Mg | — | 0.003 | 0.0354 |
| Cations | 0.0753 | 0.085 | 0.1201 |
| Cl | 0.0532 | 0.06 | 0.0908 |
| $SO_4$ | 0.0172 | 0.0156 | 0.0166 |
| $HCO_3$ | 0.0043 | 0.0067 | 0.0036 ($H_2PO_4$) |
| Anions | 0.0747 | 0.0823 | 0.1110 |

continued plasmolysis, while those in sea water/6 were just perceptibly softened, indicating that sea water/7 was close to an isotonic solution. Since this corresponds to about an 0.08 N NaCl solution, which approximates the total normality of the analyzed salts in the sap, it is evident that these must account for practically all the osmotic pressure in the normal cell sap.

Finally, for comparison with the only other fresh water California plant which has had comparable cell sap analysis (and one of the few anywhere in the world), the figures of Hoagland and Davis for *Nitella clavata*[12, 13] are given in Table III.[14] While this *Nitella* grew in a more concentrated pond

[12] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, **5**, 629.

[13] Zscheile, F. P., Jr., *Protoplasma*, 1930, **11**, 481 (revision of Hoagland and Davis).

[14] For comparison of saps from several fresh water and brackish Charophyta of Finland, see Collander, R., *Protoplasma*, 1936, **25**, 201.

water than the *Hydrodictyon*, a general similarity is to be seen. However, *Hydrodictyon* has an even higher K:Na ratio (15 or 20:1, as against 5:1), and a very considerably lower actual Na, Ca, and Mg, than has *N. clavata*. On the other hand, it has about the same amount of sulfate. This may represent a characteristic of fresh water plants; if so they are in marked contrast to marine plants, which exclude sulfate. Permeability and electrical mobility studies of the sulfate ion in the two cases might be of interest therefore.

## SUMMARY

Analysis of the cell sap of *Hydrodictyon patenaeforme* Pocock, from California indicates the usual marked accumulation of potassium, which is 4000 times as concentrated as in the surrounding pond water. Small amounts of sodium and calcium were found. Chloride makes up about three-fourths of the anions, with a very high sulfate, and much lower bicarbonate concentration accounting for most of the remainder. Electrical conductivity and osmotic studies indicate that the analyzed elements are ionized, and account for most of the sap's osmotic pressure. pH is 5.5 to 6.0.

The analytical procedure was designed to determine as many of the cations as possible on one small sample.

*Hydrodictyon* is a large multinucleate cell belonging to an order (Chlorococcales) new to permeability and accumulation studies.