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QUANTIZATION OF A FLUX RATIO IN CHAROPHYTES?

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SUMMARY

1. The ratio of the Cl^- influx into the vacuole to the Cl^- influx into the whole cell has been measured for a large number of internodal cells of *Chara corallina*. The distribution of the values of this ratio shows no tendency to cluster about preferred values in integer ratios with each other.

2. The claim of MACROBBIE[1], that values of this ratio for *Nitella translucens* do cluster in this way, is shown to be unsupported by her data.

3. It is concluded that there is no evidence for the phenomenon of "quantization"[1] of this flux ratio.

INTRODUCTION

Recently MACROBBIE[1,2] has discussed the kinetics of the uptake of Cl^- by cells of Nitella and Tolypella. Ion uptake in plant cells has generally been described in terms of the three-compartment model[3,4] (in which outside, cytoplasm and vacuole are the compartments), though recently LARKUM[5] and LARKUM AND PRING[6] have considered four-compartment models in which the chloroplasts form the fourth compartment. MACROBBIE[1,2] has concluded from a number of features of the kinetics, particularly at short times of uptake, that both three- and four-compartment models of the cell could be rejected, and has argued instead that salt uptake can be described in terms of a model including pinocytosis or vesicular transport. Since this concept could be of fundamental importance in the study of ion uptake, we are examining some of the kinetic features she has described.

Here, we discuss the phenomenon of "quantization"[1]. When charophyte cells are allowed to take up radioactive Cl^- for a short time (about 10 min), the distribution of this Cl^- between cytoplasm and vacuole may be determined[1,2]. MACROBBIE[1] contended that the proportion of Cl^- reaching the vacuole, $(M_{ov}/M_T)^*$, tended to take on, in any single experiment, discrete values related in the ratios $1:2:3:4\cdots\cdots$ Thus in one experiment on 22 cells the results for M_{ov}/M_T were interpreted as clustering round the following series of values of $n\alpha$: 0.051, 0.102, 0.153, $\cdots\cdot$, 0.306; while

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine *N'*-2'-ethane sulphonc acid.

* For ease of cross-reference we will use the symbols of MACROBBIE[1] throughout this paper, *i.e.* M_{ov}, influx to vauole from outside solution; M_T, influx into cell from outside solution; α, a constant determined for each experiment; n, an integer constant assigned to a group of values of M_{ov}/M_T. The constants α and n occur in the equation of MACROBBIE $M_{ov} = n\alpha M_T$.

in another experiment on 18 similar cells they were said to cluster round the values: 0.035, 0.070, 0.105, ····, 0.175. The combined distribution of $M_{ov}/\alpha M_T$ in a number of experiments is said to support this claim.

If this clustering effects exists, it should appear in the distribution of values of M_{ov}/M_T for a sufficiently large number of strictly comparable cells. There should be no need to assume that there is a different value of α for each group of cells, if all cells are similar and are similarly treated. This should remove an unsatifactory feature of the work of MACROBBIE[1]. If a single value of influx time is used, it will not be necessary to assume (as MACROBBIE[1] seems to have done) that values of M_{ov}/M_T are independent of time at short times. The evidence on this point is far from satisfactory (MACROBBIE[2], Fig. 4).

In the present paper we report the results of two large experiments on *Chara corallina*, in each of which the distribution of M_{ov}/M_T was studied. The method of treating the data used by MACROBBIE is also critically examined.

MATERIALS AND METHODS

Chara corallina, collected from Ross, Tasmania, is held in culture at Flinders University, South Australia. It is grown in mud and nutrient solution, and frequently replanted. The same species is similarly grown at the University of Sydney, N.S.W., but the starting material was collected at Gledswood, near Sydney. (The Tasmanian material was originally named *C. australis* var. *australis*, the N.S.W. material *C. australis* var. *nobilis*.)

Internodal cells, 3.5–8.0 cm in length, were harvested, prepared as usual for flux experiments and stored some days in Flinders pond water[7]. The cells were pretreated in light in the following solution (Nuffield artificial pond water) for 2 h prior to influx measurement: 2.0 mM *N*-2-hydroxyethylpiperazine *N'*-2'-ethane sulphonic acid[8] (Calbiochem) (HEPES) and 0.7 mM NaOH, 1.0 mM NaCl, 0.1 mM KCl, 0.05 mM $CaSO_4$ (pH 7.0).

The influx solution was Nuffield artificial pond water labelled with $^{36}Cl^-$ (Radiochemical Centre, Amersham) at a high specific activity (7 mC/g Cl^-). This helped to reduce the counting errors for small samples.

After an influx period in Nuffield artificial pond water each cell was rinsed for 15 sec in Flinders pond water and blotted. When drying had reduced its turgor, its end was cut off, and a small drop of vacuolar sap ran out or was gently expressed. A measured volume of this sap, up to half the cell volume, was counted separately from the remainder of the cell. Cell walls and nodal cells were discarded from the remainder before counting. The sum of both counts gave the total radioactivity taken up, while the proportion in the sap could be found from the dimensions of the cell and the assumption that the vacuole occupied 94% of its volume. From these results the calculations gave M_T, M_{ov}, and M_{ov}/M_T.

Radioactivity was measured with a Nuclear–Chicago automatic gas-flow geiger counter. Most samples were counted to 3000 counts, but some to as small a total as 500, of which one-quarter was background. A quantitative estimate of the overall error is given below. No results were discarded due to low counting rate, (*cf.* MACROBBIE[1]) since this is not legitimate when the distribution of values is the aim of the experiment.

RESULTS

Expt. A was performed on 99 similar Chara cells from Flinders cultures, during the course of one day. The influx period was 10 min, and the cells were in light at 24°. The scatter diagram obtained by plotting M_{ov} against M_T for these cells is shown in Fig. 1. When the ratio M_{ov}/M_T is plotted against M_T, the scatter diagram of Fig. 2 results. The distribution of these values of M_{ov}/M_T is shown in Fig. 3a. This distribution is clearly not gaussian, and appears bimodal, but there is no appearance of "quantization", *i.e.* of peaks occurring regularly through the distribution.

There is some correlation between M_{ov}/M_T and M_T, as indicated by the results of Table I: the mean influx of cells from the lowest range of values of M_{ov}/M_T is half that of cells from the highest range. No correlation was observed between M_T

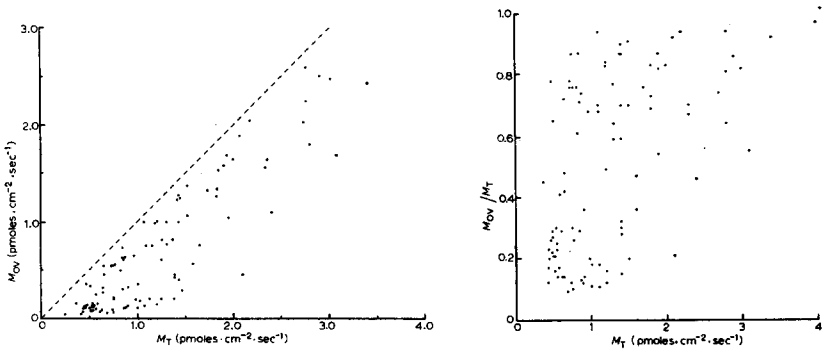

Fig. 1. The influx of Cl^- to the vacuole, M_{ov}, plotted against the influx to the whole cell, M_T, in *Chara corallina*, Expt. A. The influx period was 10 min. The dotted line shows the theoretical upper bound for the values, as the influx to the vacuole cannot exceed that to the whole cell.

Fig. 2. M_{ov}/M_T plotted against M_T, for *Chara corallina*, Expt. A.

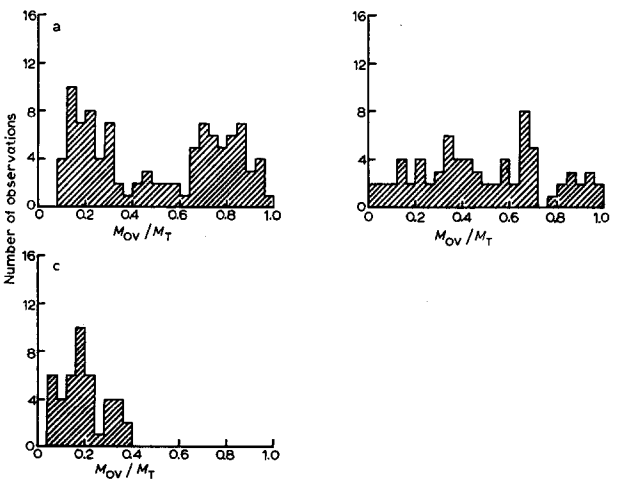

Fig. 3. Histogram showing the distribution of values of M_{ov}/M_T in (a) *Chara corallina*, Expt. A; (*b*) *Chara corallina*, Expt. B; and (c) *Nitella translucens*, Expt. NA3 of MacRobbie[1]. The interval in each histogram is 0.04.

and cell length, and nothing is known that would allow even a rough prediction of M_T or M_{ov} from the size or appearance of a cell.

A similar experiment (Expt. B) done on 74 cells of Chara from N.S.W. by W. J. CRAM, A. W. D. LARKUM AND M. G. PITMAN (personal communication) gave the distribution of M_{ov}/M_T shown in Fig. 3b. The influx period was 6 min. Again there is no sign of regularly spaced peaks, and again a wide scatter of individual values of M_{ov}/M_T.

TABLE I

CORRELATION BETWEEN M_{ov}/M_T AND M_T

Selected range of M_{ov}/M_T	0–0.35	0.35–0.70	0.7–1.0
Number of cells in range	40	22	36
Mean value of M_T	0.82	1.40	1.67
Standard error of mean	0.06	0.16	0.15

DISCUSSION

(*a*) *The results from Chara corallina*

When a large batch of Chara cells is used in a single experiment with a short influx period, the resulting values of M_{ov}/M_T have a continuous, but not a gaussian, distribution. There is no sign of a discontinuous distribution centred on values in integral ratios, such as would be predicted by the "quantization" of MACROBBIE[1]. Both M_{ov} and M_{ov}/M_T appear to be correlated with M_T at the uptake time of 10 min. MACROBBIE[2] has observed, at short influx times, a correlation of M_{ov} with M_T, and at long times (*e.g.* 60 min) a correlation of M_{ov}/M_T with M_T. However, each of these correlations was observed in the means of batches of 10 cells, differently treated, and not in the scatter of the similarly treated cells of one batch. Thus there is no clear relationship between her observed correlations and ours.

It is to be noted that since M_{ov} and M_T are both positive and since M_{ov} must be less than M_T, a positive correlation of the two quantities must exist *a priori*.

Our actual results are not very dissimilar from those of MACROBBIE[1,2] though our values of M_{ov}/M_T are higher than are found in Nitella. The upper peak in the distribution (Fig. 3a) does not appear in the distribution for her Nitella experiment NA3 (Fig. 3c).

There is no sign of any quantization of values of M_{ov}/M_T in our results.

(*b*) *The results from Nitella translucens*

The original data of E. A. C. MACROBBIE[1] (personal communication) yield a rather featureless distribution of values of M_{ov}/M_T when they are combined directly without manipulation. Similarly, when the individual experiments are kept separate (Fig. 4), no clustering around integral ratios is apparent. We must therefore examine her manipulations of her data, which lead (i) to correlation coefficients with values near 1.0, and (ii) to histograms which she claimed[1] put the results "in a form in which the quantization is immediately obvious". The following account is based on MACROBBIE[1] and E. A. C. MACROBBIE (personal communication).

For each experiment on 8–42 cells, she ranked the values of M_{ov}/M_T, and chose groups of values, separated by gaps which she says are obvious. Listed below are the

values of M_{ov}/M_T in her expts. T and NA3 (Table I, ref. 2). Commas mark the gaps chosen by MACROBBIE[1] as separating distinct groups of data values:

Expt. T: 0.16 0.17 0.21 0.21 0.22, 0.32 0.33 0.33 0.33 0.35 0.39 0.45 0.47 0.49, 0.65 0.67 0.67 0.69 0.72

Expt. NA3: 0.046 0.047 0.062 0.066 0.070 0.077, 0.097 0.098 0.107 0.108 0.121 0.124 0.126 0.128 0.141, 0.15 0.16 0.17 0.18 0.18 0.18 0.19 0.19 0.19 0.20 0.20 0.21, 0.22 0.22 0.23 0.24 0.25, 0.28 0.28 0.31 0.32, 0.34 0.34 0.34 0.36 0.38 0.40.

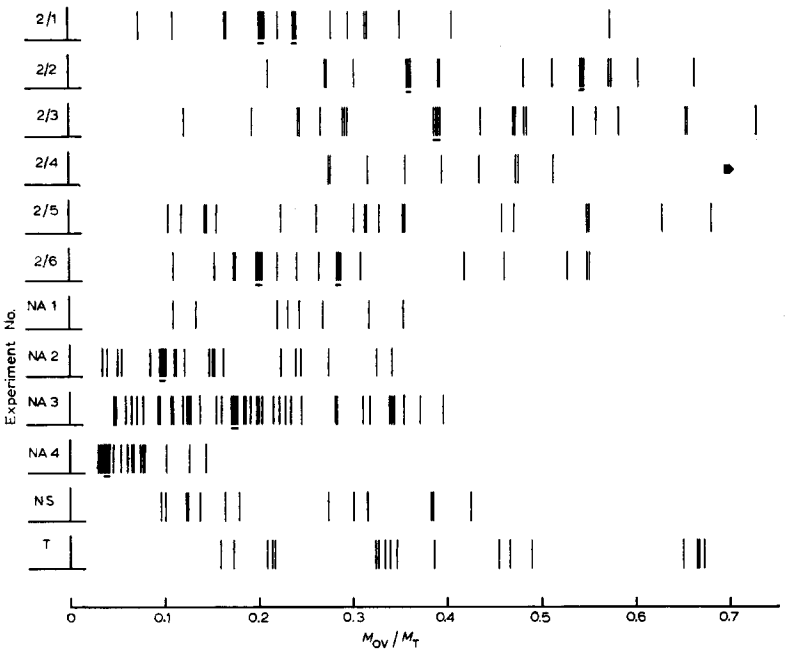

Fig. 4. A distribution diagram of values of M_{ov}/M_T in the experiments of MACROBBIE[1]. Each vertical line represents a single value of M_{ov}/M_T. The horizontal bar under some groups of points indicates that all the points in the group have the same value, the mean of the group. The arrow shown in the tabulation for Expt. 2/4 indicates that the following points are off the diagram: 0.909, 0.909, 0.909, 1.07, 1.11.

The mean of the lowest group provided a first estimate of the value of α in the equation $M_{ov} = n\alpha M_T$. All values of M_{ov}/M_T in the list were then divided by this value of α to yield experimental values of $M_{ov}/\alpha M_T$ ($=n'$) and which should approximate to n. This step appears to be necessary because in the Nitella data (Fig. 4) there is often a group of rather low values which is easy to "choose", but no clear grouping among the higher values. The attempt to choose groups among the high values appears to rely heavily on the idea that values of n' must cluster around the integers, *i.e.* in practice the group $n = 3$ in the data consists of those values of n' lying between 2.5 and 3.49. When the allocation of values to groups was complete, a second approximation to α, written $\bar{\alpha}$, was calculated (E. A. C. MACROBBIE, personal communication) from the equation

$$\bar{\alpha} = \frac{\Sigma(M_{ov}/M_T)}{\Sigma n x_n}$$

where x_n is the number of values in the nth group. When $\bar{\alpha}$ was obtained, n' was recalculated, and cells were reallocated if necessary to different groups if their value of n' crossed a boundary at 1.5, 2.5 *etc.*, (ref. 1, p. 338). Further iterative steps are possible.

At this point MACROBBIE[1] used two procedures: (i) a correlation coefficient was calculated for the linear regression of M_{ov}/n on M_T, and (ii) a histogram was compiled showing the distribution of $M_{ov}/\alpha M_T$ in five or six experiments (about 100 cells in all).

This method of data treatment is open to many objections.

The selection of groups in the data is subjective. Thus Expt. T, listed above, was divided into three groups and $\bar{\alpha}$ was calculated as 0.224 (ref. 1). We have divided it into four groups with a value 0.17 for $\bar{\alpha}$ (Fig. 5). The four groups cluster round the integers 1, 2, 3, 4 just as the three cluster round 1, 2, 3 of MACROBBIE[1]. No test known to us will determine which grouping is the "true" one, and we conclude that both are without objective meaning. Data such as that of Expt. NA3 (above) shows no evident grouping, and it may be divided into groups in a variety of ways, each way resulting in a different value of $\bar{\alpha}$. Further, one may impose the same subjective grouping on any list of scattered results: the values of M_T in Expt. T may be grouped with a value of $\alpha = 0.214$ and values of n up to 5.

Since the first steps in the data reduction are subjective rather than objective

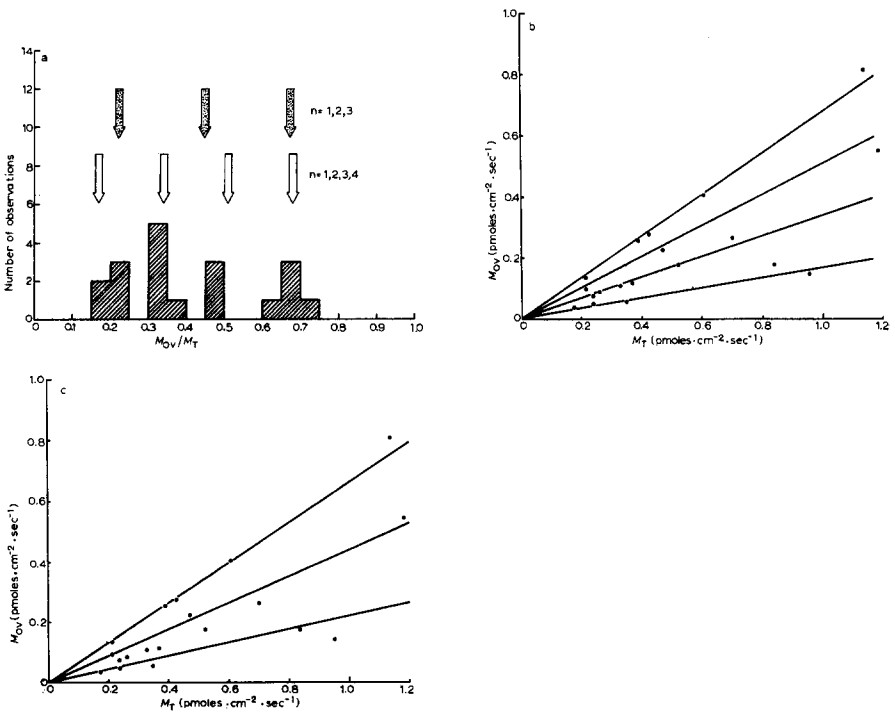

Fig. 5. Aspects of Expt. T with Tolypella[1] showing (a) a histogram of the distribution of values of M_{ov}/M_T with dark arrows indicating the "quantization" of the data of MACROBBIE[1], and light arrows, our "quantization". (b) M_{ov} plotted against M_T, with four lines following from our "quantization", drawn through the points. (c) M_{ov} plotted against M_T, with three lines as drawn by MACROBBIE[1].

the next steps must be suspect. The calculated regression coefficients for M_{ov}/n on M_T can have no meaning (despite their high values) since the selection of groups based on the slope M_{ov}/M_T automatically produces a good correlation of M_{ov} with M_T within each group. Since the groups are chosen around the integers it follows that a combined regression of M_{ov}/n on M_T will produce high values of r. To check this, normally distributed random values of dummy data of M_{ov} and M_T were produced (IBM Scientific Subroutine package: GAUSS subroutine) and used to calculate values of M_{ov}/M_T. They were grouped as described above, with the following results: G1. Values of M_{ov}/M_T: 0.23 0.26 0.27, 0.39 0.42 0.44 0.54 0.56 0.58 0.62 0.62 0.66, 0.74 0.75 0.80 0.83, 1.00 1.05. $\bar{\alpha} = 0.265$; $r = 0.921$; slope of regression line, 0.250; intercept, 0.011. G2: 0.09 0.24 0.24, 0.29 0.30 0.39 0.40 0.40, 0.48 0.51 0.53 0.61, 0.65 0.69 0.73 0.73, 0.89 0.90, 1.06 $\bar{\alpha} = 0.177$; $r = 0.937$; slope 0.189; (intercept, -0.011. These regression coefficients are as high as some obtained by MACROBBIE[1] for her data.

The histograms seem to be open to fewer objections than the regression coefficients, since the values of each experiment are divided only by a single constant $\bar{\alpha}$, before being combined. Since the value of $\bar{\alpha}$ is not uniquely determinable in any one experiment, the shape of the histogram is obviously not unique either, and no conclusions can be drawn from it.

The gap in the histograms at about $n = 1.4$ seems to correspond to a common tendency in the Nitella data for a group of low values to be separate from the higher values. This may be a significant feature of the results, but this would need further tests; it might be a product of experimental technique or represent some real group of cells.

(d) Experimental errors

An estimate of random experimental errors in such experiments as these can be made. The fluxes M_{ov} and M_T are given by the following expressions, omitting constants:

$$M_{ov} \propto Y_{ss} \cdot V_0 \cdot c_0 \cdot t^{-1} \cdot Y_0^{-1} \cdot v_{ss}^{-1}$$

$$M_T \propto Y_T \cdot V_0 \cdot c_0 \cdot t^{-1} \cdot l^{-1} \cdot d^{-1} \cdot Y_0^{-1}$$

where Y_{ss}, Y_T and V_0 are the radioactivities of the sap sample, the whole cell, and the aliquot of solution, respectively, V_{ss} is the volume of the sap sample, V_0 is the volume of the aliquot of solution, c_0 is the concentration of Cl^- in the solution, t is the influx period, and l, d are the length and diameter of the cell.

All these measurements are subject to errors, the most serious being counting errors in samples of low activity. Counting errors are stated by MACROBBIE[1] to have been usually 6–10%, but up to 20%. In the present work the errors in counting the sap samples were less than 7%, and in the other samples 3%.

The total estimated errors are $\pm 8\%$ in M_{ov} and $\pm 7\%$ in M_T in these experiments, and $\pm 10\%$ and $\pm 8\%$ in the experiments of E. A. C. MACROBBIE (personal communication). However, to her errors should be added a further amount, not easily estimated, since she has used uptake times varying from 5 to 15 min. The fraction in the vacuole may well vary by some 15% in this time (Fig. 4, ref. 2).

(e) Gaps in data

The grouping of data of MACROBBIE[1] seems to rely on the existence of gaps in the distribution of values in an experiment. No objective criterion exists for the

size of a credible gap in experimental data. However, if the expected experimental error is $\pm$ 10% we can scarcely believe in gaps whose size is less than 20% of their median value. On this basis, if we examine her choice of gaps in her data, we find that of 35 gaps chosen to separate data groups, about 19 are greater than or equal to 20% of their median value, and 15 are less. Of gaps in her data not chosen to separate data groups, 9 are greater than or equal to 20% of their median value. This illustrates the subjective nature of the choice of group boundaries, and suggests again that it was biased towards the production of groups round the integers.

CONCLUSIONS

"Quantization" is hardly a feature of the data which leaps to the eye of the observer. It must be considered as an hypothesis subject to experimental test.

The grounds on which hypotheses for test are normally set up appear to be two: (i) that, in accordance with Occam, they are the simplest unfalsified hypotheses available, or (ii) that they follow from some reasonable argument on *a priori* grounds. Such an hypothesis may win some acceptance when tests which might well have falsified it can be shown to have failed to do so.

Looking at the quantization hypothesis in these terms one concludes, first, that it is far from being the simplest available, second, that no argument known to us suggests it.

There seems no reason to accept its existence as a phenomenon or its usefulness as an hypothesis. If its usefulness as an hypothesis were accepted, it would appear that its testing would require much more evidence than is available to date, or more sophisticated tests.

It does seem clear that there may be unexpected features in the distribution of values of M_{ov}/M_T. No further conclusion seems justified at the present.

ACKNOWLEDGEMENTS

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