BBA 75244

THE UNUSUAL MEMBRANE PERMEABILITY OF TWO HALOPHILIC UNICELLULAR ORGANISMS

MARGARET GINZBURG

Botany Department, Hebrew University, Jerusalem (Israel)

(Received October 14th, 1968)

SUMMARY

1. The permeability of a Halobacterium species and *Dunaliella parva* to several radioactive substances has been measured.

2. The method used was to centrifuge a cell suspension, within which was dissolved the given radioactive substance, and measure the radioactivity of the resultant cell pellet and supernatant. The volume of radioactive substance within the cell pellet was then calculated.

3. Both organisms were found to be impermeable to [^{14}C]dextran (mol. wt. 80 000).

4. Results indicated that the Halobacterium cell was freely permeable to [^{14}C]-sucrose, [^{14}C]inulin, [^{14}C]starch, and even [^{14}C]polyvinylpyrollidone (mol. wt. 30 000–40 000).

5. A region of the *D. parva* cell was permeable to [^{14}C]sucrose and to [^{14}C]inulin.

6. It is argued that the results indicate the existence of large pores in the cell membranes of these two organisms.

INTRODUCTION

The work presented in this paper arose indirectly from an attempt to measure the volume of medium trapped within cell pellets of two halophilic unicellular organisms, the ion metabolism of which was being studied. When the usual probe molecules (*e.g.*, [^{14}C]inulin) were used for the measurement of the 'trapped volume', it was found that unexpectedly high values were obtained, and it appeared that these probe molecules were measuring some additional parameter. A more thorough investigation seemed called for because of the light that it would shed on the membrane permeability of the organisms in question.

The technique employed in this work was to compare a given cell pellet volume with the apparent volume of radioactive probing-solution within the cell pellet. A cell suspension was thoroughly mixed with a small amount of the radioactive solution in question. The suspension was then centrifuged, and the radioactivities of the resultant cell pellet and supernatant were measured. From the radioactivity per unit volume of supernatant could be calculated the volume of radioactive substance within the cell pellet. A comparison was made with the total volume of the cell pellet.

METHODS

Organisms used in experiments

The two halophilic organisms used were both isolated from the Dead Sea (main constituents: NaCl and $MgCl_2$, both at 1.5 M). The first was a red bacterium of irregular rodlike shape, apparently a member of the genus Halobacterium. The second was the green alga *Dunaliella parva*. Both organisms are characterised by the absence of a rigid cell-wall.

Halobacterium was cultured at 37° on a medium of 10% yeast autolysate, 3.5 M NaCl, 150 mM $MgSO_4$ with small additions of Ca^{2+} and Mn^{2+} (ref. 1). *D. parva* was cultured at 32° in a complex salt medium adapted from MACLACHLAN[2], with the addition of 1.5 M NaCl. All experiments were done on growing cultures except where stated.

Measurement of volume of bacterial pellets

A method was adapted from SCHULTZ AND SOLOMON[3]. Cytocrit tubes were used, consisting of a glass bulb of 3–4 ml capacity connected to a fine tube made of precision-bore capillary tubing of 1 mm diameter (Chance Bros., Smethwick, England). A known volume of cell suspension was introduced into the cytocrit tube and was centrifuged in a horizontal centrifuge at 13000 × *g* for 30 min. The horizontal centrifuge was designed and built locally by Mr. E. SOCHATCHEWER. After removing the tube from the centrifuge, the length of the cell pellet formed was read under a binocular microscope by means of a micrometer eye-piece. The volume of the cell pellet was then calculated, and could be expressed as a percentage of the original volume of suspension to be introduced into the tube. The cell pellet consisted of packed cells *plus* a constant proportion of intercellular medium.

Samples were read in duplicates which agreed to within 4%.

Measurement of volume of medium trapped in cell pellets

(*a*) *In cytocrit tubes* (*bacterial pellets*). 4 ml cell suspension labelled with a given radioactive probe molecule were centrifuged in cytocrit tubes in the manner described above. At the end of the period of centrifugation the supernatant was drawn out by means of a syringe and kept for counting. The pellets were removed in one of two ways.

The first method was as follows. During centrifugation, cytocrit tubes were closed at the basal end by a tightly fitting teflon plug. After centrifugation, the plug was pulled out and a pellet of cells was extruded by careful application of air pressure at the upper end. The pellet was extruded onto a tared aluminium disc and was weighed on a Misco quartz helix balance. After drying at 90° overnight, the pellet was reweighed. The difference between the two recorded weights gave the amount of water in the pellet. The radioactivity of the pellet was then measured, and the volume of medium trapped within each pellet was calculated. The density of the medium was 1.17 and its dry weight content was 0.24 g per ml of medium. Hence the water content of the medium is 0.93 g per ml medium. Results were expressed as the proportion of supernatant water in the total pellet water. This method was used for [^{14}C]inulin, [^{131}I]polyvinylpyrollidone and serum [^{131}I]albumin.

The second method was simpler. Cytocrit tubes sealed at the base were used. After centrifugation of the labelled suspension, the length of the cell column was

measured and the volume of pellet was calculated. The supernatant was removed as before. The inner surface of the upper part of the tube and bulb was rinsed repeatedly. The final rinsings were checked for radioactivity. None above background was ever found. The cell pellet was then washed out into a 4-ml volumetric flask which was made up to volume with distilled water. It was enough to shake the flask in order to break open the cells, since the membranes require the presence of a high salt concentration for their integrity. The resultant fluid was centrifuged and the supernatant used for counting. Results were expressed as the volume of trapped medium as a proportion of the total pellet column.

This method was used for [^{14}C]dextran.

(*b*) *In microfuge tubes.* Use was made of the technique of differential density separation[4], by which a cell suspension is centrifuged in the presence of an immiscible liquid of density intermediate between that of the cells and that of the supernatant. During centrifugation, the immiscible liquid forms a layer which lies in between the bulk of the medium and the cells accompanied by a small volume of medium.

For bacteria, the immiscible liquid used was dimethylphthalate (specific gravity 1.191). For the alga, a mixture of dimethylphthalate and Octoil-S (Consolidated Vacuum, Rochester, N.Y.) with a final specific gravity of 1.064 was used.

250 μl of cell suspension, labelled with a radioactive marker, were centrifuged in 400-μl polyethylene tubes, each containing 50 μl immiscible liquid. The centrifuge used was a Beckman microfuge. Time of centrifugation was 5 min. It was found that 90% of the cells were separated from the supernatant 15 sec after the start of centrifugation.

Samples were taken at various intervals of time after addition of the radioactive label to the cell suspension, in order to obtain a time-curve which could be extrapolated to zero.

At the end of the period of centrifugation, the supernatant was drawn off with a Pasteur pipette and saved for counting. The tip of the tube, containing packed cells, was cut through the layer of immiscible liquid with a new razor blade and was transferred to a clean glass tube. The immiscible liquid from a number of tubes was pooled and saved for counting. The only compound found in even trace amounts in the immiscible liquid was [^{14}C]dextran (*e.g.*, inulin: 5540 counts/min per μl in supernatant; 0 counts/min per μl in dimethylphthalate. Dextran: 5860 counts/min per μl in supernatant; 2.5 counts/min per μl in dimethylphthalate).

After measurement of radioactivity in the supernatant and in the pellets, the volume of medium trapped within the cell pellets was calculated. Samples of the same cell suspension were centrifuged simultaneously in duplicate cytocrit tubes, and the pellet volumes measured. Results were expressed as the volume of radioactive medium per total pellet volume.

All experiments were performed at 23 $\pm$ 2°.

Measurement of radioactivity

A number of compounds labelled with ^{131}I were used. When cell suspensions had been labelled with ^{131}I, the cell pellets could be measured directly without any extraction of the contents. Cell pellets and aliquots of the supernatant were measured in a Tracerlab well-type scintillation counter.

Pellets from cell suspensions labelled with ^{14}C-labelled compounds were ex-

tracted in 1 ml water and were shaken sufficiently to break the cells. Both types of cell break open in distilled water. After centrifugation, aliquots of the clear supernatant were taken for counting. The amount of salt in the samples was measured, and samples for counting the background were prepared, containing the same amount of salt as did the experimental samples. By means of an external standard, it was checked that there was no significant quenching. Samples were counted in a Packard Tri-carb liquid scintillation counter, using BRAY's solution[5] as liquid scintillator.

Not less than 10 000 counts were counted from each sample.

Preparation of compounds for use in experiments

Before use, compounds containing ^{131}I were dialysed overnight in 0.084 M KI and 0.077 M NaCl.

All radioactive compounds were dissolved in small amounts of the medium in which the bacteria or algae were suspended. In order to check that the compound was entirely dissolved, an aliquot was centrifuged in a microfuge tube containing 50 μl of the appropriate immiscible liquid and the cut tip tested for the absence of radioactivity.

Experimental compounds used

[^{14}C]Sucrose, [^{14}C]inulin, [^{131}I]polyvinylpyrollidone, [^{14}C]starch, and human serum [^{131}I]albumin were obtained from the Radiochemical Centre, Amersham, England. [^{14}C]Dextran was obtained from New England Nuclear. The molecular weights of all these substances are shown in Table I. The [^{14}C]starch was derived from tobacco leaf.

RESULTS

Halobacterium sp.

Results are presented in Table I. The smallest volume of radioactive substance, relative to pellet volume, was obtained with the [^{14}C]dextran; this molecule (mol. wt. 80 000) was the largest probe used in the course of this investigation. The volume of 26 % obtained from microfuge tube pellets approximates the theoretical volume of medium trapped between closely packed cells[6]. It is similar to results obtained with *Escherichia coli*[3] and yeast[6]. It is therefore assumed that the dextran volume of 26 % is due exclusively to medium trapped within the cell pellet.

It was found that pellets centrifuged 30 min after mixing [^{14}C]dextran with the bacterial suspension contained the same amount of radioactivity as pellets centrifuged immediately after mixing. This showed that the [^{14}C]dextran was not absorbed by the cells.

It was also checked that the [^{14}C]dextran was not adsorbed by the bacteria. This was done by diluting a concentrated suspension of bacteria containing [^{14}C]-dextran with 10 vol. of nonradioactive medium. On centrifuging this dilute suspension, the radioactivity of the resulting pellets was one-hundredth of those from the concentrated suspension. (Radioactivity of small pellet, 30 $\pm$ 5 counts/min; large pellet, 2996 counts/min.) This was expected, since the cell pellets were one-tenth of their original size, and the radioactivity was one-tenth of the original. However, had adsorption of [^{14}C]dextran onto the bacterial surface occurred, the radioactivity of the

TABLE I

PROPORTIONS OF PELLETS OF HALOBACTERIUM AT EQUILIBRIUM WITH RADIOACTIVE PROBE MOLECULES

Pellets obtained by centrifugation of labelled suspensions as described in text. The results obtained by use of microfuge tubes are extrapolated to time zero.

Substance	*Mol. wt.*	*Pellet water* (%) *Cytocrit, method (a)*	*Pellet vol.* (%)	
			Cytocrit, method (b)	*Microfuge*
[^{14}C]Sucrose	342			110
[^{14}C]Inulin	5000–5500	110 ± 12		100
[^{131}I]Polyvinylpyrollidone	30 000–40 000	105.1 ± 2.2		
[^{14}C]Starch*	20 000–40 000			100
Serum [^{131}I]albumin	65 000	56.1 ± 0.9**		
Serum [^{131}I]albumin	65 000	34.5 ± 2.2***		
[^{14}C]Dextran	80 000		30.4 ± 2.2	25.9 ± 2.8

* Tobacco leaf starch.
** Early logarithmic-phase culture.
*** Late logarithmic-phase culture

pellets from the diluted suspension would have been higher than expected, since in addition to the radioactivity of the trapped supernatant, ^{14}C would have been adsorbed onto the bacterial surface.

The volume of 34.5 ± 2.2 % obtained with serum [^{131}I]albumin in late logarithmic-phase cultures is not significantly different from the dextran volume of 30.4 ± 2.2 % (pellets from cytocrit tubes). The higher value (56.1 %) of the serum [^{131}I]albumin with younger cultures could have been due to digestion of the protein by the bacteria which are known to be proteolytic.

In contrast to the results obtained with [^{14}C]dextran and serum [^{131}I]albumin, the volumes of all the other probe molecules used were more or less equal to the pellet volumes. Alternatively, the weights of pellet water and supernatant water were equal. These high values must be due either to adsorption of the radioactive substance onto the cell surface or to absorption within the cell. The fact that the volumes of the probe molecules and of the pellets are equal to each other seems unlikely to be a coincidence, and indicates that the substances penetrated the cells until equilibration with the external medium was reached. This conclusion may be supported by the results obtained with two concentrations of sucrose, nearly twenty times apart from each other: linear extrapolation of the two curves to time zero yields the same sucrose volume (Fig. 1) and thus implies that the sucrose was absorbed, rather than adsorbed. It is possible, however, that a permease or carrier system was responsible for the penetration of sucrose into the bacteria. This possibility was not investigated.

The time-curves obtained with [^{14}C]sucrose (Fig. 1) and [^{14}C]inulin (Fig. 2) show that equilibration of the probe molecule with the cell interior was attained in less than 1 min.

In conclusion, it is suggested that sucrose, inulin, starch and even polyvinylpyrollidone penetrated into the bacteria.

Dunaliella parva

In four separate experiments, it was found that 27.5 ± 0.9 % of the total volume of algal pellets equilibrated with [^{14}C]dextran, and at the same time, 57 ± 2 % with

[^{14}C]inulin. Both parameters were time-invariant (see Fig. 3). Sucrose was rapidly absorbed by the cells (Fig. 3).

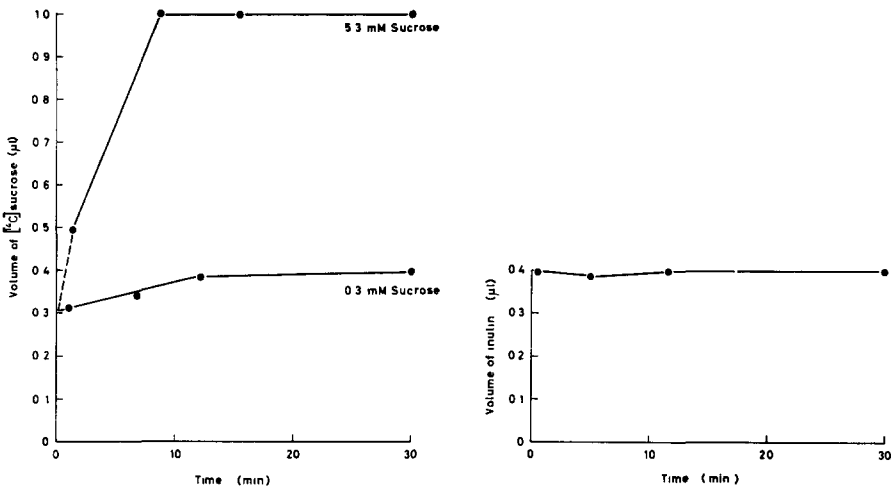

Fig. 1. Measurement of volume of [^{14}C]sucrose at equilibrium with cell pellets of Halobacterium sp. Pellet volume: 0 32 ± 0.03 µl. Abscissa: time interval after mixing [^{14}C]sucrose with bacterial suspension.

Fig. 2 Measurement of volume of [^{14}C]inulin at equilibrium with cell pellets of Halobacterium sp. Pellet volume: 0.36 ± 0.04 µl. Abscissa: time interval after mixing [^{14}C]inulin with bacterial suspension.

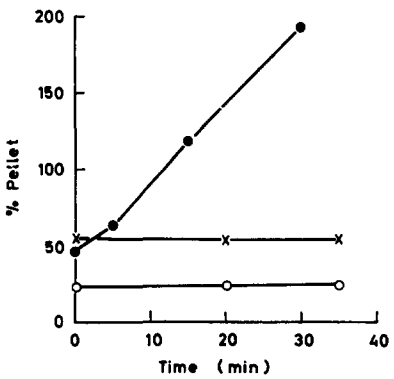

Fig. 3. Measurement of volumes of [^{14}C]sucrose (●—●), [^{14}C]inulin (×—×) and [^{14}C]dextran (○—○) at equilibrium with cell pellets of *D. parva*. Volumes are expressed as percentages of total pellet volume. Abscissa: time interval after mixing ^{14}C-labelled substance with algal suspension.

These results indicate that a proportion of the algal pellet, identified with supernatant trapped between the cells, is occupied by [^{14}C]dextran. A compartment of the cell is available to inulin and sucrose, but not to dextran. This compartment may tentatively be identified with the cell cytoplasm outside the chloroplast. In any case, the outer cell membrane is permeable to inulin.

DISCUSSION

There is good agreement between the proportion of both bacterial and algal pellets which equilibrated with the [^{14}C]dextran (25.9% and 27.5%, respectively).

These volumes have been identified with intercellular medium trapped between the cells. In contrast to dextran and possibly serum [^{131}I]albumin, all the other molecules proved to be permeable to the cells tried. This held for sucrose (mol. wt. 342) and inulin (mol. wt. 5000–5500). Starch, and even polyvinylpyrollidone with a molecular weight of 40 000 were permeable to the Halobacterium membrane. Other work in this laboratory has shown *D. parva* to be extraordinarily permeable to H^+ and to Tris and phosphate buffers (to be published elsewhere). Thus the permeability properties of the two halophilic organisms studied, differ from those of most other organisms on which similar work has been done[7]. Certainly, the 'typical' animal or plant cell living in moderate conditions is totally impermeable to inulin, and largely so to sucrose. On the other hand, some highly permeable cells do exist. For instance, the blue-green alga Beggiatoa, studied between 1910 and 1951 by Ruhland, by the method of deplasmolysis, was shown to allow the penetration of molecules up to the size of lactose[8]. This method is a reliable one, even though it does not measure permeability directly[9]. Certain diatoms were shown to be permeable to sucrose by Marklund and by Elo (quoted by COLLANDER[7]).

The unusual permeability of the two halophilic organisms studied here, as well as of Beggiatoa and certain diatoms, may be attributed to the existence of large pores traversing the cell membrane. If the molecular weight of the penetrating molecule is any indication of the size of pore, the pores of the halophile studied here which allowed polyvinylpyrollidone (mol. wt. 40 000) to penetrate, must be considerably larger than those of Beggiatoa, impermeable to raffinose. The postulation of large pores poses a difficult question in that it is hard to see how small essential molecules such as amino acids and nucleotides are retained within the cell. Perhaps the membrane is asymmetric: leaky in one direction only. The answer to this question needs further research.

It is suggested that a highly permeable membrane is of adaptive value to halophilic organisms, since it may enable them to withstand sudden changes in the salt concentration of the surrounding medium.

ACKNOWLEDGEMENTS

I wish to thank Professor AHARON KATCHALSKI and Doctor BEN-ZION GINZBURG who contributed their continued advice and help to the advancement of this work. Dr. ELDAD GIBERMAN first demonstrated to me the Beckman microfuge and the technique of differential density separation. He also devised the test for adsorption described on p. 373. Mrs. MARU BING and Miss TAMAR KLEIN performed some of the technical work. This work was executed under Contract No. 14-01-001-521 awarded by the Office of Saline Water, U.S. Department of the Interior.

REFERENCES

1 V. MOHR AND H. LARSEN, *J. Gen. Microbiol.*, 31 (1963) 267.
2 J MACLACHLAN, *Can J Microbiol.*, 6 (1960) 367.
3 S G SCHULTZ AND A K SOLOMON, *J. Gen Physiol.*, 45 (1961) 355
4 R. BALLENTINE AND D. D. BURFORD, *Anal. Biochem.*, 1 (1960) 263.
5 G. A BRAY, *Anal. Biochem*, 1 (1960) 279
6 E J CONWAY AND M DOWNEY, *Biochem J*, 47 (1950) 347
7 R COLLANDER, in F. C STEWARD, *Plant Physiology*, Academic Press, New York, 1959, p 3
8 S SCHÖNFELDER, *Planta*, 12 (1931) 414.
9 O KEDEM AND A. KATCHALSKI, *Biochim. Biophys Acta*, 27 (1958) 229.