INFLUENCE OF AZIDE ON THE PERMEABILITY OF RHOEO CELLS

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In the years 1953 to 1956 Bogen and his collaborators published a series of papers containing interesting observations on osmosis and the effects of respiratory inhibitors, azide and DNP, on the osmotic behaviour of different cell types. By means of classic methods of determining permeation and permeability plasmometrically, they advanced the theory that both water and non-electrolytes are taken up and withheld metabolically in the cell vacuoles. Höfler and Url (1958) criticised the interpretations claiming that the results could be explained by ordinary non-metabolic uptake and exudation of solutes, leaving the question open whether the inhibitors influenced the cytoplasmic permeability to non-electrolytes.

Since these inhibitors are widely used to distinguish between metabolic and non-metabolic uptake of compounds, an attempt has been made to study the effect of azide on permeability. Permeability is here used in the strict sense as the capacity of the cytoplasm to allow a non-metabolic passage of diffusible solutes.

The study was undertaken during a visit in 1959 to the Botany Department of Duke University, Durham, N.C., made possible by an invitation from the University and the hospitality of Prof. Paul J. Kramer, for which I wish to convey my sincere thanks. I am also indebted to Dr. Y. Waisel, Jerusalem, for valuable discussions in connection with this study. The results are preliminary in nature, but have led to certain fairly well-supported conclusions.

Materials and Methods

Plant Material

The general procedure involved plasmometric studies on *Rhoeo discolor* epidermal cells, also employed by Bogen. Sections 3 mm. in length were made from the second fourth from the tip of the abaxial side of the mid-vein of the leaf. They were studied under the microscope and the cells measured by means of an eye-piece micrometer. Five sections were taken for each treatment and in each section ten cells showing symmetrical plasmolysis were measured at intervals of time given in the descriptions of the experiments. This means that all values are based on 50 randomly chosen cells. However, the sections were selected for uniformity of the cells, and the standard errors for the measurements did not exceed 2 per cent. All experiments were

repeated. The Rhoeo plants grew in a green-house adjacent to the laboratory.

Measurements

Plasmolysis and deplasmolysis were determined from measurements of the contraction in length of the protoplast in relation to the length of the cell. The ratio protoplast length/cell length is called G. In the plasmometry literature this symbol denotes the relative protoplastic volume or the degree of plasmolysis—a low figure denoting a high degree of plasmolysis. Our G-values do not denote volumes, but owing to the shape of the cells and the uniform cell material they can be used as relative figures for the degree of plasmolysis. In one instance an exact estimation was made of the volume from surface of protoplast and cell, and a G-value of $0{\cdot}45 \pm 0{\cdot}01$ was obtained. With the length measurement, G was estimated to $0{\cdot}41 \pm 0{\cdot}01$. It is obvious, however, that the deviations must be greater at higher values of G owing to the tapering cell ends. The rate of deplasmolysis was computed from changes in G during 15–45-minute intervals, and recorded as $\triangle$ G, or the change in G per hour. The $\triangle$ G values are thus not directly comparable to those of the literature either.

All experiments were carried out at room temperature, which varied ordinarily between 25 and 30° C. Temperatures around and above 35° C., which frequently occurred, increased the toxicity of azide, and experiments had to be discarded in such instances.

Solutions

Distilled water and unbalanced salt solutions change the charge of the cytoplasmic surface and probably also the permeability in a not easily predicted way. For this purpose all solutions employed contained 10^{-3} *M* K-Na-phosphate buffer of pH 5·0 and $CaCl_2$ 10^{-3} *M*. This basal solution is included in all solutions mentioned below, unless otherwise stated.

Sucrose 0·3 *M* was employed as a plasmolyticum, in some experiments replaced by mannitol. Deplasmolysis was studied in water, sucrose, or glycerol 10^{-3} *M*, always containing the basal buffer solution. Sodium azide was given in 10^{-4} *M*, or the same concentration as employed by Bogen (1953) on Rhoeo.

In the final experiments, which were repeated and varied in different ways, the plasmolysis and deplasmolysis were measured in the absence and presence of azide. Some properties of the plant material had to be investigated in preliminary experiments.

Experimental Results

Adaptation and Plasticity of the Cells

Before commencing the plasmometric tests it was deemed advisable to let the sections adapt themselves to the buffer solutions. This was

done partly in order to rinse the sections from contents of damaged cells, partly in order to establish an equilibrium between solution and the cytoplasmic boundary. This is called pre-washing. In the standard technique this was done for 30 minutes. A pre-washing up to three hours did not influence the value of G in sucrose as shown in Table I, and in other tests it was found that the sections could be left in the solution for seven hours, which actually was necessary in several experiments.

TABLE I

Effect of pre-washing in buffer solution on the degree of plasmolysis (G) in (A) only sucrose 0·3 M, (B) sucrose + buffer solution

Time of pre-washing (min.)	Plasmolyticum	
	A	B
0	0·68	0·63
15	0·65	0·64
60	0·68	0·63
180	0·68	0·64

One interesting observation was made in this connexion, namely, that the epidermal cells are normally never water-saturated and the cell-walls are plastic. This is exemplified in Table II recording experiments in which the sections were at first measured in liquid paraffin, then plasmolyzed, then water-saturated, and finally plasmolyzed again. There is always a large water deficit in the normal leaves, even under extremely humid conditions. On water saturation the cells extend to their full length, and on another plasmolysis they showed a plastic stretching amounting to between 3 and 13 per cent. in length. All these reactions are rapid. It is obviously necessary to saturate the cells before reliable plasmometric determinations can be carried out.

Permeability of the Cells

Its is customary to use sucrose as a plasmolyticum, but the cells regularly deplasmolyze in sucrose at a measurable rate. It is not certain that this depends upon a permeation of sucrose molecules, because it is known that these are easily inverted on the surface of cells (Burström, 1958), and the hexoses ought to both permeate and enter metabolically more easily than sucrose. Comparisons were made with mannitol (example, Table III) and these two sugars generally behaved

TABLE II

Water saturation and tensility of Rhoeo epidermal cells

Lengths in arbitrary units. Standard errors ± 0·10 to ± 0·12. Six experiments (A–F); C–E from rainy days with high air humidity; E and F taken at 9–10 A.M. after watering, leaves dripping wet; E is a young leaf. Consecutive treatments in (*a*) liquid paraffin, (*b*) sucrose, 0·3 *M*, (*c*) water, (*d*) sucrose. All solutions buffered. Differences $a - b$ (elastic extension in normal state, $c - d$ elastic extension water-saturated, $d - b$ plastic extension; relative turgidity in normal state computed with water saturation = 1·00.

Cell length in	Experiments					
	A	B	C	D	E	F
(*a*) Liquid paraffin	8·11	8·35	8·07	8·00	7·78	8·52
(*b*) Sucrose	7·70	7·75	7·82	7·73	7·36	7·95
(*c*) Water	9·06	8·84	8·58	8·45	8·08	8·80
(*d*) Sucrose	8·71	8·28	8·07	7·96	7·86	8·40
Elastic extension:						
Normal state	0·41	0·60	0·25	0·27	0·42	0·57
Water saturated	0·35	0·56	0·41	0·49	0·22	0·32
Plastic extension	1·01	0·53	0·25	0·23	0·50	0·53
Degree of turgidity	0·30	0·55	0·33	0·38	0·58	0·67

TABLE III

Comparison between deplasmolysis in sucrose and mannitol

Plasmolysis in sucrose 0·3 *M*; A left in sucrose, B after 2 hours shifted to mannitol 0·3 *M*. Solutions buffered. Degree of plasmolysis (G).

Time (hours)	A Sucrose	B Mannitol
2	0·55	0·55
4	0·60	0·64
6	0·62	0·64
8	0·68	0·67

similarly. This might indicate an inversion of sucrose, but also that the sugars are not drawn into metabolism, since mannitol is known to be chemically inert in the plant. The inversion error can be reduced by using a large volume of solution, but it cannot be prevented in the space between cell-wall and contracted protoplast. Sucrose can be suspected to give irregular osmotic concentrations, but, on the other hand, the permeability of these sugars is low in comparison with the diosmoticum, glycerol. Representative values are:

for sucrose .. 0·02 G/h ($\triangle$ G)

for glycerol .. 0·45

The uptake of sucrose can be neglected in comparison to that of glycerol, and these figures as well as those of Table I also show that the salt uptake is of no importance for the osmotic conditions. The combination sucrose-glycerol was thus employed in most experiments.

The permeation of both compounds is obviously high compared with data of the literature, as far as such a comparison is possible. This may depend upon the high experimental temperature. Although high, it was in no way abnormal, since the Rhoeo plants were raised under the same conditions.

Influence of Azide on Deplasmolysis

The typical course of deplasmolysis in sucrose is shown in Table IV with and without azide present. There is no sign of deplasmolysis with azide, but the cells contract slowly. This was also observed by Bogen (1953). After four hours some cells began to die and after eight hours all cells were dead. This dying sets in suddenly and implies a spontaneous contraction of the protoplast to less than one-tenth of its volume, probably a coagulation. This is so rapid that no intermediate

TABLE IV

Plasmolysis and deplasmolysis in sucrose without azide and with azide present during the whole treatment

Solutions buffered. Degree of plasmolysis (G).

Time (hours)	No azide	With azide
1	0·58	0·59
4	0·65	0·53*
8	0·68	†

* Cell of two kinds: (A) with an average G of 0·53, transferred to water they deplasmolyze immediately; (B) at the margins of the sections the protoplasts strongly contracted with irregular granular surface, G estimated to about 0·15, protoplasts obviously coagulated, do not deplasmolyze in water.

† Almost all cells coagulated and decolourized.

stages could be observed. However, until this happens, the cells transferred to water immediately deplasmolyze. This was observed in a number of experiments. A specific example will be mentioned. Sections were plasmolyzed in sucrose + azide. After 4 hours and 50 minutes the cells had attained a G as low as 0·49; they were then divided into two lots, one in water, another in water + azide. Both were completely deplasmolyzed within 5 minutes. Although experiments were arranged in different ways none disclosed a measurable influence of azide on the water uptake.

It was repeatedly found as exemplified in Table IV that azide did not affect the G value attained immediately upon plasmolysis. A slow decrease in G followed preceding the mentioned collapse of the protoplasts. However, this toxicity appeared very irregularly, more commonly at higher temperatures and extended experimental time. It is obviously independent of the regular inhibition of the deplasmolysis. It was thus desirable to shorten the experimental time, which was possible by the use of glycerol as a diosmoticum. Experiments with glycerol were carried out according to different plans and with different times of treatment, but they all gave similar results. Typical values are these: Plasmolysis in sucrose, sections left for 45 minutes, then deplasmolyzed in glycerol, all treatments without and with azide. Obtained △ G for glycerol, no azide 0·54, with azide 0·26. The azide treatment was in this experiment extended up to six hours with virtually the same △ G value as a result.

In order to find out whether azide affects cells differently in plasmolyzed and turgid state the following type of experiment was carried out (Table V). The sections were treated with azide for three hours

TABLE V

Treatment with azide three hours before during, or after plasmolysis in sucrose

Deplasmolysis in glycerol. Solutions buffered. △ G for glycerol computed from 45 minutes deplasmolysis. Two experiments, (A) with G at plasmolysis of 0·62, (B) with G of 0·47.

Treatment	△ G	
	(A)	(B)
No azide ..	0·56	0·39
Azide to turgid cells ..	0·38	0·26
„ During plasmolysis ..	0·32	0·28
„ To plasmolyzed cells .	0·46	0·29

only, either as a pretreatment before plasmolysis (*b*), or during plasmolysis with the sections left for three hours in sucrose + azide (*c*), or plasmolyzed without azide and then left for three hours with azide before deplasmolysis in glycerol (*d*). The decrease in rate of deplasmolysis is nearly the same in all instances. Thus the inhibiting effect of azide remains even after withdrawal of the external addition. This can hardly be called an 'after-effect', since certainly azide remains in the cells. This simplifies the technique and makes it possible to avoid toxicity by reducing the time of treatment with azide. This is probably only a way of lowering the dosage of azide, but it was not possible to investigate the effect of lower external azide concentrations.

All experiments unanimously show that azide delays deplasmolysis, which is interpreted as due to a slow uptake of glycerol. This uptake might be metabolic and not a passive permeation. In order to settle this question the following types of experiments were carried out.

Two treatments were employed: (A) ordinary plasmolysis in sucrose and deplasmolysis in glycerol; the rate of deplasmolysis is assumed to depend upon the endosmosis of glycerol. (B) plasmolysis and deplasmolysis in glycerol, then plasmolysis in an isotonic sucrose solution; the rate of the second plasmolysis is assumed to be a measure of the exosmosis of glycerol. Both types of experiments were carried out with azide added during different steps of the procedure. With both pretreatment and plasmolysis in azide toxicity appeared.

The plasmolysis and deplasmolysis in B without azide were, of course, very rapid. After 19 minutes G amounted to 0·81 and after 32 minutes full deplasmolysis was attained (G = 1·00). The following computations were made from experiments with no toxicity:—

Experiment type	Glycerol	Azide	△ G
A	in	—	0·45
	in	+	0·16
B	out	—	0·41
	out	+	0·17

The experiments of types A and B were not carried out simultaneously and the good agreement between them may be a mere coincidence. The treatments with and without azide are directly comparable and show that azide decreases the passage of glycerol in both directions, into and out of the cell according to the diffusion gradients. The results are only qualitative, since the change of G with time is not linear, but even so the results are clear-cut. This seems to be a good indication that azide affects the permeability of the membranes, because both endosmosis and exosmosis can hardly be due to active mechanisms.

COMMENTS

The results of this study are in good agreement with those of Bogen (1953). Deplasmolysis has been interpreted in the conventional way as a result of endosmosis of plasmolyticum or diosmoticum. Höfler and Url (1958) emphasize that also Bogen's results can be explained on the same basis. This does not prove that the interpretations are correct, or disprove a metabolic uptake of non-electrolytes in the sense of Bogen's. The very strong inhibition of deplasmolysis by azide would indicate, if Bogen's explanation is correct, either that more than one-half of the water uptake is metabolic, or that less than one-half of the glycerol uptake depends upon passive permeation.

The first possibility can be definitely ruled out. An effect of azide on water permeation could not be demonstrated, although several experiments were devised for this purpose. The water permeability was so great under the experimental conditions that no determinations could be made of its rate, but it was at least ten times higher than the deplasmolysis, with azide present in both instances.—The second possibility is at least unlikely.

On the other hand, the azide-inhibited plasmolysis in the system with isotonic external sucrose and internal glycerol speaks decidedly against Bogen's interpretation. If glycerol was withheld metabolically, azide ought to increase its exudation and the ensuing plasmolysis, whereas the rate is decreased. This indicates that the permeability is decreased by low additions of azide. The results do not exclude the possibility that a metaosmotic uptake of solutes occurs, but such an assumption is not necessary, and the change in permeability offers a simpler explanation.

A plausible cause of the change in permeability is that metabolism is required for the maintenance of the normal cytoplasmic structure and thus the normal permeability. This is a reasonable assumption, if there is a continuous turn-over of cytoplasmic constituents. A similar conclusion was reached by Blinks and his collaborators (1938), who found that oxygen deficiency reduced the membrane potentials in Halicystis, although their data do not give any information about the permeability.

The irreversible poisoning by azide at prolonged treatment under coagulation of the cytoplasm, on the contrary, involves its destruction and loss of selective permeability. The slow contraction of the protoplast occurring after plasmolysis in sucrose, also observed by Bogen, may be a first indication of an increased permeability, as assumed by Höfler and Url, but another possibility should also be considered. Sucrose is inverted on the surface of cells of different kinds with an accumulation of hexoses in the external solution (Burström, 1958). This might lead to both a slow protoplasmic contraction and an increased deplasmolysis by endosmosis of hexose. The inversion is

certainly temperature-dependent and should cause a temperature sensitivity of the osmotic equilibration, which might be mistaken for a temperature-dependent metabolic uptake of solutes or water. The inversion is decreased by divalent cations and ought to be low in our experiments, but higher with the experimental conditions of Bogen's. This is a source of error to which attention should be paid in accurate plasmometric determinations.

The conclusion that azide in low additions decreases the passive permeation is of special interest because azide and other respiratory inhibitors are employed for identifying metabolic reactions, *e.g.*, distinguishing a metabolic transport of compounds from a passive diffusion or a migration by means of a mass movement of solutions. Such interpretations must obviously be made with some caution, if it holds good that not only azide but respiratory inhibitors in general also affect the passive permeability. A passive permeation of the types mentioned may indirectly be regulated by metabolism.

Summary

It has been shown by means of plasmometric determinations on Rhoeo that azide decreases the permeability for a diosmoticum, glycerol, and entirely suppresses that for sucrose. The implications of this result for the distinction between metabolic and non-metabolic uptake of solutes are emphasized.

It is also pointed out that initial plasticity of cell-walls and inversion of sucrose used as a plasmolyticum ought to be considered in plasmometric determinations.

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