PENETRATION OF MANNITOL INTO POTATO DISCS[1]

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This paper deals with a problem which, while minor in itself, may nevertheless loom large when interpreting experiments on the relationship between osmotic values and cell enlargement.

In 1953 Bonner et al (1) published some experiments on the uptake of water by slices of artichoke tuber (*Helianthus tuberosus* L.) which showed that the increase in respiration of this tissue caused by auxin was very much less when in hypertonic solutions of mannitol than when in water. However, Burström (6) gave reasons for believing that the value for the osmotic pressure of the tuber tissue at incipient plasmolysis, given by Bonner et al as 0.12 M, was too low, and reported a number of measurements which yielded values of 0.4 M and up. Further, while Burström's data undoubtedly established that the osmotic pressure of artichoke tissue was higher than had been thought, the data also brought out another, and a surprising, fact. This unexpected observation, which could not be explained at the time, was that when the tuber slices had been immersed in mannitol for 54 hours, the osmotic pressure at incipient plasmolysis had increased to extremely high values, in one case reaching 1 M and, in general, attaining values nearly proportional to the mannitol concentration used.

This great rise could mean one of two things; either mannitol steadily enters the cells, or else the exposure to mannitol causes liberation within the cells of some osmotically active material. Either of these two phenomena would have a most important bearing on studies of the water uptake of tuber slices which, since the pioneer work of Reinders (12), have proven to be useful material for the study of auxin action.

The result was of special interest to us since we had noted in several experiments with mannitol that potato discs, after their growth had been inhibited by high external osmotic pressure, tended to show a partial recovery. Examples of this will be shown below. More important, it was demonstrated (see fig 14 of ref 13) that if water uptake in auxin were restricted for 24 hours by adding 0.3 M mannitol, then on return for 4 hours to plain auxin the sections rapidly took up extra water and essentially recovered all the ground lost during the inhibition period. This was interpreted as evidence that auxin had been continuously modifying the cell walls at about the same rate as though the mannitol had not been present, i.e., that auxin action on cell walls had been uninterrupted. However, if mannitol had been entering the cells, this subsequent rapid water uptake might be the result merely of an increased osmotic value and would not be attributable to the action of auxin. Ordin et al (11) and Cleland and Bonner (7) have described comparable experiments with Avena coleoptile sections; the former authors concluded from plasmolytic measurements that the internal osmotic pressure of coleoptile tissue increases markedly in sucrose but only slightly in mannitol solutions. A further study of the recovery phenomenon in potato tissue therefore seemed important.

In this paper indirect evidence will first be presented which makes it improbable that mannitol enters the cells of potato tissue appreciably, and then direct measurements of mannitol uptake, using C^{14}-mannitol, will be described.

MATERIALS AND METHODS

From selected large potatoes, var. Katahdin, cylinders were cut lengthwise with a cork borer 9 or 15 mm in diameter and then sliced with a hand microtome into discs 1 mm thick[3]. The discs were vigorously washed in fast-running tap water for 15 minutes and then soaked in a shallow layer of distilled water (about 1.5 mm deep), for 24 hours. In a few cases, noted in the text, an osmoticum was used instead of water for this 24-hour period. The method was in all respects the same as that used in our previous studies of metabolism and water uptake of potato discs [see e.g. reference (10) and literature there cited] and very nearly the same as that used in most other laboratories. At 24 hours the slices were blotted with controlled pressure, weighed (initial fresh wt), and placed on plastic nets in petri dishes so that the discs just broke surface of the test solutions (cf. fig 1 of ref 10). The fresh weight after the experimental period was determined in the same way. In some cases a salt mixture comprising $KCl : CaCl_2$: : 3 : 1 was used as osmoticum. Its molarity was arbitrarily calculated assuming total dissociation. For this reason the concentration of the salt mixture, like that of Carbowax (see footnote to table II) is referred to as "M". Naphthalene-acetic acid, NAA, was the only auxin used, since it was found by Hackett and Thimann (10) and confirmed by Brian et al (4) that potato slices respond only very weakly to indoleacetic acid.

For radioactivity studies determinations were made

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TABLE I

PARTIAL RECOVERY OF POTATO DISCS FROM INHIBITION OF WATER UPTAKE BY MANNITOL (EXPT 30–53)

CONC OF NAA mg/l	CONC OF MANNITOL M	CHANGE IN FR WT, AS % OF INITIAL WT		
		2 DAYS	3 DAYS	5 DAYS
0	0	+9.31	+12.55	+12.14
0	0.17	+1.64	+0.82	+3.70
0	0.22	−6.00	−6.40	−2.80
0	0.27	−8.80	...	−3.20
10	0	+10.58	+20.77	+30.95
10	0.19	+2.02	+11.32	+24.65
10	0.24	*−6.42*	*+0.04*	*+15.25*
10	0.29	*−12.90*	*−7.25*	*+2.42*

only on the solution. The discs, usually 10 to 15 in a group, were removed from the experimental vessels and placed on nets as above, in contact with 0.3 M C^{14}-mannitol for various periods[4]. They were then taken out of the mannitol solution, thoroughly blotted and submerged in 10 ml distilled water with stirring; samples of 0.1 ml each were then withdrawn each 1 to 2 minutes for about 20 minutes, spread evenly on planchets, dried, and counted. The error due to self-absorption with minute quantities of substance (< 1 mg/cm² on the planchet) is negligible. When the sections were left in water for an hour or more in order to follow the slow outflow (cf. fig 3) the continued submergence caused general solute leakage to begin and self-absorption became appreciable; for this reason such experiments were not continued. The activity of the mannitol solution varied somewhat from one experiment to another; it was about 10^5 cpm per ml. Counts were made with a gas-flow windowless counter and corrected for background.

RESULTS AND DISCUSSION

I. OSMOTIC RECOVERY WHEN STILL IN OSMOTICUM. The change in weight of potato discs when immersed in concentrations of mannitol around 0.2 M is shown in table I, especially on the last two lines. At above 0.2 M there is a marked loss of water at first, then gain in weight begins. This we refer to herein as "osmotic recovery".

Table I shows that osmotic recovery in solutions without auxin is somewhat erratic and slow; it appears on the 5th day but not on the 3rd. In NAA, however, it appears clearly on the 3rd day in both of the higher concentrations used and is even more pronounced by the 5th day.

It should be pointed out that osmotic recovery was noted (albeit only to a small extent) in the Avena coleoptile in the very first publication on the use of mannitol in growth experiments (15). There the coleoptile sections which had shortened in 0.4 M mannitol in the first 37 hours elongated a little again after 36 hours more. Similar recovery can be detected in the recent data of Cleland (8), in which the irreversible elongation of Avena sections is shown to be promoted by auxin only when the mannitol is below a certain critical concentration. In that paper the different experiments in mannitol were carried out for different lengths of time. By measuring the critical mannitol concentrations carefully from Cleland's figures 2 through 8, which all present comparable experiments in 5 ppm IAA, we find the following:

TIME EXPOSED TO MANNITOL (HR)	CRITICAL CONCENTRATION (M)
3	0.073
4	0.125
6 (3 expts.)	0.113,0.125,0.127
10.5	0.178

The trend to increased critical concentration with increasing time in mannitol is clear, and confirms the fact that Avena sections also show some osmotic recovery.

Our first thought was of course that mannitol enters the cells and thus raises their suction pressure (DPD). A search was therefore made for another osmotic substance, sufficiently soluble in water to give hypertonic solutions, yet with a molecular weight so large that penetration into the cells could be ruled out. A satisfactory material was found in Carbowax. This polyethylene glycol is available in a series of molecular weights and is miscible with water in almost all proportions. One preparation was found toxic, but another, Carbowax 1500, was not. Its average molecular weight is 1,500 (indicated by the name) although the osmotic weight data below indicate that it contains an appreciable fraction of lower molecular weight. Nevertheless its entry into the cells seemed most improbable. Table II summarizes a growth experiment with this substance, in the presence and absence of NAA. Instead of being rinsed

TABLE II

PARTIAL RECOVERY OF POTATO DISCS FROM INHIBITION OF WATER UPTAKE BY CARBOWAX 1500 (EXPT 50–53)

CONC OF NAA mg/l	CONC OF CARBOWAX "M"*	CHANGE IN FR WT AS % OF INITIAL WT		
		AFTER 1 DAY	3 DAYS	4 DAYS
0	0	+7.1	+16.7	+20.9
0	0.156	*−4.6*	*+3.2*	*+6.0*
0	0.196	*−7.4*	*−3.2*	*−1.8*
10	0	...	+19.1	+36.8
10	0.156	*−5.5*	*+7.8*	*+13.2*
10	0.196	*−10.7*	*−2.8*	*−0.9*

* The concentration is given on the basis of a molecular weight of 1,500, but the fact that its osmotic effectiveness exceeds that of mannitol shows that a considerable fraction of it has a lower molecular weight.

[4] The mannitol was kindly supplied by Dr. H. S. Isbell of the National Bureau of Standards, Washington, D. C.

with water for 24 hours as usual, the discs were rinsed for only 15 minutes and then placed in the solutions shown. The osmotic recovery is shown best in the values underlined. Both with and without NAA it is clear that the tissues steadily recover the water content which they initially lost, the recovery being again improved by the presence of the NAA. On the whole, also, the rate and extent of osmotic recovery in Carbowax are not very different from those in mannitol.

II. Behavior of Tissue After Return to Water. Experiments in which tissues were placed in mannitol for some time and then returned to water (both phases containing auxin) were described for pea stem sections in 1951 (14) and for potato discs in 1954 (13). In the former case the mannitol-treated sections never did catch up to the controls; in the latter case they did. An experiment comparable to that given earlier (13), but in which the osmotic inhibition was more complete, is shown in detail in table III. Five groups of carefully matched discs, each group placed in two petri dishes of ten discs each, were prepared as usual; the maximum difference between the weights of these groups was less than 1 %. They were then allowed to grow for 2 days in NAA 10 mg per liter, and reweighed (top line of table III); their water uptake had been virtually identical. Three groups were now transferred to osmotica, one more group placed in fresh NAA solution as a control and the fifth group left dry on the nets but in a nearly saturated atmosphere. After 24 hours all the treated lots had lost weight except the one in the salt mixture, which had made a very small gain; the controls in NAA, however, had gained 190 mg.

All discs were now placed in fresh NAA. After 4 hours the large gain in weight of groups 2 to 5 brought them nearly up to the control level as previously described, and we can conclude, as in 1954 (13), that though growth in the osmotica had been temporarily abolished or nearly so, still the modification of the cell wall under the influence of the auxin had continued. The important point here is, however, that the discs that had been in mannitol or Carbowax did not, in fact, reach the control level exactly and in the subsequent 2 days they lagged considerably behind. By the 5th day the total gains (last line) show that the group that had been exposed to air recovered almost completely, but only those that had been in the salt mixture actually exceeded the controls in their final water uptake. If this be ascribed to a small amount of salt entry, causing an increased suction pressure, then by the same token neither the mannitol nor the Carbowax had entered the cells. Indeed, exposure to these two osmotica seems to have caused a permanent decrease in ability to grow. One explanation of this might be that deposition of cell wall material has continued during the exposure to these two osmotica, making the wall less extensible. However, such a process would be expected to continue also in the salts or in air (columns 4 & 5 of table III). The fact that it does not suggests a more specific explanation, namely that impregnation of the cell wall with mannitol or Carbowax may inhibit the enzyme system which enhances cell wall elasticity. (This might be due to the chemical relationship between these two substances and the carbohydrate of the wall). Cleland and Bonner (7) have also noticed, with Avena coleoptiles, that exposure of more than 3 hours to mannitol interferes with the subsequent growth.

Regardless of this small subsequent inhibition, however, there is certainly no indication in the present data of penetration into the cell by mannitol or Carbowax.

The rapid uptake of water on transference from the osmotica to water suggested that the suction pressure of the discs could be thus determined, by using a narrow range of osmotic solutions instead of water.

Table III

Behavior of Matched Potato Discs During & After 4 Hours in Osmotica or in Moist Air

Group	1	2	3	4	5
Wt on day 2	1.420	1.425	1.425	1.415	1.420
Next 24 hr in:	NAA	NAA + Carbo 0.17 "M"	NAA + Mann. 0.20 M	NAA + Salts 0.25 "M"	Moist Air
Wt on day 3	1.610	1.410	1.385	1.463	1.312
Wt after 4 hr in NAA	1.632	1.570	1.520	1.557	1.542
Wt on day 5	2.037	1.858	1.840	2.073	2.021
		% changes in wt			
Day 2–3, in osmotica	+13.6	−0.5	−3.8	+3.5	−7.4
In next 4 hr, in plain NAA	+1.5	+10.7	+10.4	+13.7	+16.2
In following 36 hr	+28.4	+20.1	+22.6	+29.4	+33.6
Total gain	43.5	30.3	29.2	46.6	42.4

* Note the complete return to control level after treatment with salts or after drying, but only partial return after treatment with mannitol or Carbowax. NAA 10 mg per liter in all solutions. Percentage change in weight calculated from weights on day 2. (Expt 46–53).

Indeed, this is essentially the standard classroom experiment. Accordingly a group of 5 discs after 3 days in osmoticum were blotted, weighed, and placed for a few hours in a series of salts or mannitol solutions of graded concentration, and then reweighed. Similar experiments have been used by Brauner and Hasman (2, 3) to compare potato discs which have been held in sugar and in Ca salts, or in air and in nitrogen. Unfortunately these workers (3) used for auxin IAA, which as mentioned above (see 10) has only very little effect on potato discs. NAA is a much more powerful auxin for this material. Nevertheless our results agree with Brauner's in principle. Figure 1A shows an example. The discs had been for 3 days on 0.25 M salt mixture, with or without NAA 10 mg per liter. In the first place the 1.5 hour measurement (solid line) shows that the solution in which the discs neither lose nor gain in weight (i.e., whose osmotic pressure is equal to the suction pressure) is considerably more concentrated for discs that have been on NAA than for the controls. The values are 0.282 and 0.230, respectively; hence *NAA has increased the suction pressure by 23 %*. Similarly, in figure 1B, in which the discs had been 3 days in Carbowax (0.12 "M") with Carbowax also used as the test osmoticum, the values are 0.151 and 0.126, i.e., *NAA has increased the suction pressure by 20 %*. The increase due to the auxin is thus nearly the same with two different osmotica.

Second, the 1.5 hour measurements in both figures show that the slopes of the lines are clearly steeper for discs kept in NAA than for controls. This is true for both gain and loss in weight. This demonstrates that

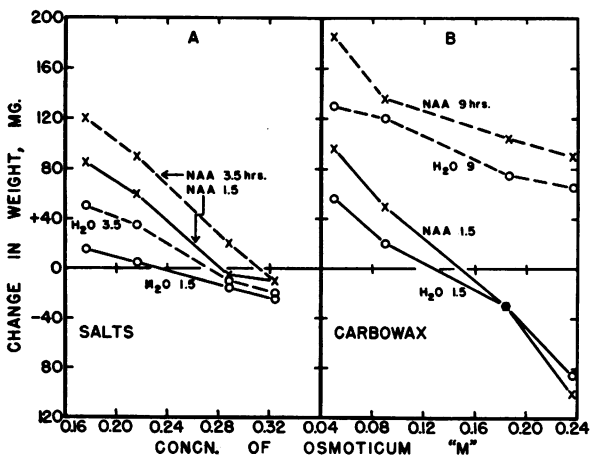


FIG. 1. Suction pressure determinations (gain or loss in wt) of groups of 5 potato discs. A: After floating for 3 days on 0.25 M KCl-$CaCl_2$ solution (3:1) with or without NAA 10 mg per liter. Solid lines, values after 1.5 hours in the test osmoticum; dashed lines, after 3.5 hours. The test osmoticum was the same as that for pre-treatment, i.e., KCl: $CaCl_2$: : 3:1. B: After floating similarly on 0.12 "M" Carbowax 1500. Solid lines, after 1.5 hours in the test osmoticum; dashed lines after 9 hours. The test osmoticum here was Carbowax 1500.

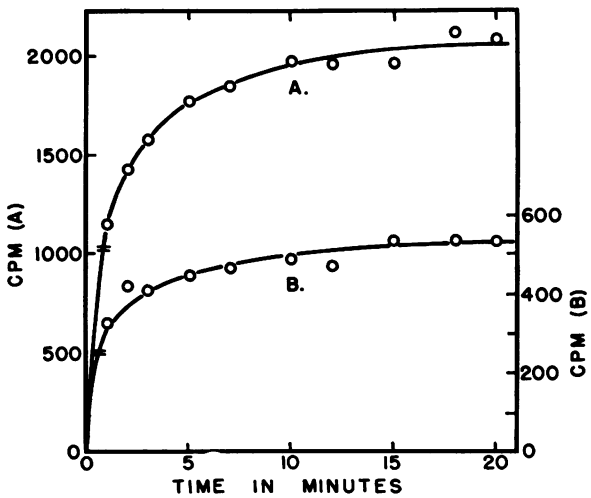


FIG. 2. Outflow into water of C^{14} from potato discs that had been floated on C^{14}-mannitol for 3 days (curve A) or 1½ hours (curve B). NAA, 10 mg per liter, was present in both cases Abscissa: isotope content in the water. The concentration of isotope in the mannitol was not the same in the two experiments.

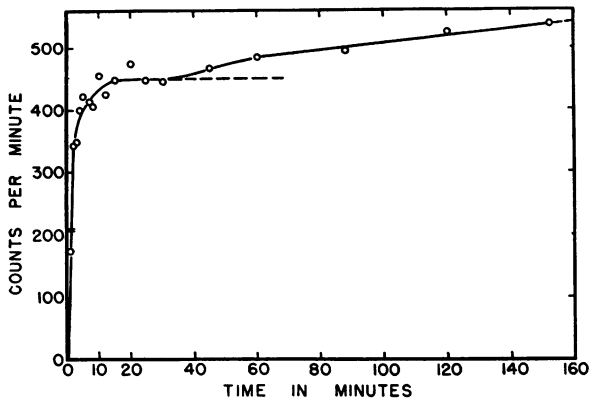
the elastic extension of the cell walls has been increased by the auxin. This result is particularly clear-cut because the discs during the 3-day treatment with osmoticum had grown very little; thus the increased elasticity can hardly be the *result* of growth.

In the third place, the figures show the osmotic recovery phenomenon very simply. The discs remain in the test osmoticum, except for being quickly blotted and weighed from time to time. Yet, as the dashed lines show, after 3.5 hours (fig 1A) or 9 hours (fig 1B) the discs which had previously lost weight now gained it. The change is in each case greater with NAA than without it (cf. tables I & II).

It may be well to emphasize that there appear to be three different responses involved: A, osmotic recovery, B, the effect of NAA in promoting this, C, the effect of NAA on cell wall elasticity.

III. OUTFLOW OF ISOTOPE AFTER EXPOSURE TO C^{14}-MANNITOL. The procedure of these experiments is described under Materials and Methods. After the discs had been kept for 3 days on NAA plus labeled mannitol, 0.3 M, they were carefully blotted and placed in stirred distilled water for 20 minutes, during which time 0.1 cc samples were removed every 1 to 2 minutes, dried down, and counted. In this way the outflow of absorbed mannitol into the solution could be followed. A typical result is plotted in curve A of figure 2. Equilibrium was reached within 20 minutes and removal to fresh solution at this time yielded no additional counts in another 20 minutes. The time taken to reach the half-maximum value was 0.9 minutes in figure 2 curve A, and in two similar runs the values found were 1.1 and 1.5 minutes, respectively.

The fact that so rapid an equilibration took place after 3 days in mannitol suggested that little real pene-



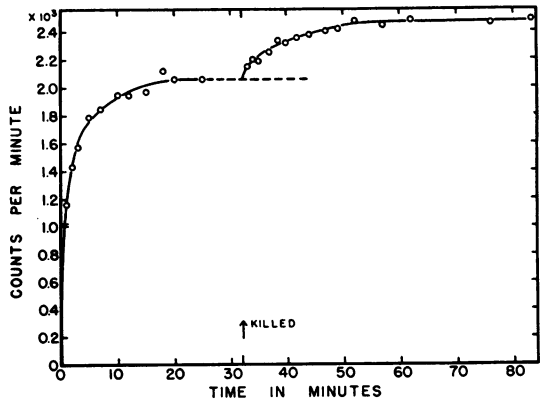


FIG. 3 (*above*). Outflow experiment similar to figure 2 but continued to 2½ hours. The discs had been 3 days on C^{14} mannitol containing NAA. Note the slow recommencement of outflow after 40 minutes' immersion.

FIG. 4 (*below*). Experiment similar to figure 3 but tissue killed by adding acetone after 32 minutes.

tration could have taken place. This was confirmed by exposing the discs to mannitol for shorter times. After 1 day in C^{14} mannitol (plus NAA) the outflow half-time was still 1.5 minutes. The outflow after only 1½ hours in C^{14} mannitol (again plus NAA) is shown in curve B of figure 2. The half-time was now about 0.5 minute, which may not be significantly different, the shape of the curve was the same, and again equilibration was complete in about 15 minutes.

The above experiments appear to indicate a one-compartment system for mannitol uptake, in which the mannitol is apparently taken up very quickly and is free to return rapidly to the external solution. However, if the discs remained in distilled water for more than 40 minutes it was found that a slow further release of isotope could be detected, indicating a small second compartment. Such behavior, in discs which had been 3 days on C^{14}-mannitol plus NAA, is shown in figure 3. Since the second compartment showed no sign of reaching equilibrium in 2½ hours, the cells in a comparable experiment were killed by adding acetone[5] after attaining their first equilibrium. Figure 4 shows that there was now a rapid release of isotope from the second compartment, the time for half-maximum value being about four minutes. The final activity of the solution was increased 430 cpm above the value of 2,050 cpm reached at the first equilibrium.

From the activities measured and the total volume of the discs (0.20 cc each) and of the solution (10.0 cc), the fraction of the volume of the disc into which the mannitol solution had entered can be calculated. Table IV summarizes these effective volumes for the two compartments in experiments of different duration. It may be assumed that the first compartment is the Apparent Free Space in the sense of Briggs (see 5) but that the acetone-sensitive compartment lies within the cells. It is evident that the effective volume of the free space does not change markedly with the time of exposure to mannitol, while the effective volume of the second compartment does. Thus the mannitol enters the free space very rapidly and the interior of the cells very slowly. The maximum concentration of mannitol reached in the tissue, as the sum of that in both compartments, in no case exceeds 0.09 M; that in the second compartment after one day does not exceed 0.008 M.

[5] Although acetone is not perhaps the ideal agent to destroy permeability its use was preferred to that of chloroform because of the undesirability of having a non-aqueous phase present.

TABLE IV

RATES AND VOLUMES OF OUTFLOW OF C^{14}-MANNITOL FROM POTATO DISCS AFTER DIFFERENT PERIODS OF EXPOSURE TO 0.3 M MANNITOL*

DURATION OF EXPOSURE TO MANNITOL	HALF-TIME IN MIN	EFFECTIVE VOLUME (AS % OF INITIAL VOL)			
		1ST COMPARTMENT*		2ND COMPARTMENT*	
3 days	1.1	27	25%	5.4	5.5 %
	1.5	21		5.7	
	0.8	27		5.5	
1 day	1.5	18	16.5 %	2.5	2.4 %
	...	15		2.2	
1.5 hr	0.5	18	18 %	0.75	0.75 %

* First compartment, outflow from intact tissue into water; second compartment, outflow after subsequently killing the tissue with acetone.

In a few of the experiments the discs which had been in contact with C^{14}-mannitol were kept in a closed vessel over KOH for an hour, but no significant quantity of $C^{14}O_2$ could be detected. This indicates that virtually no mannitol could have been dehydrogenated to fructose in the tissue, since otherwise this would have given rise to CO_2.

It can be concluded that no entry of mannitol takes place into potato tissue in a quantity sufficient to account for the osmotic recovery observed. It seems therefore that diminished turgor must give rise to the liberation of osmotic material within the cell, an effect strongly enhanced by auxin. This presumably means hydrolysis of a polymer, perhaps a reserve polysaccharide. It is at least a suggestive coincidence that another large polymer, namely RNA, has been reported to disappear when leaves are exposed to hypertonic solutions of mannitol (9). The significance of such a "manostatic" system, as a possible means of protection against the damaging effects of external osmotic changes, merits consideration in other cases where plants resist rapid changes in solute concentration.

Summary

Washed potato discs floated on hypertonic solutions of mannitol or a high molecular weight polymer of ethylene glycol (Carbowax 1500) lost weight at first and then, after periods between 3 hours and 3 days, gained weight again. This *osmotic recovery* is promoted by naphthalene-1-acetic acid, NAA.

That osmotic recovery could be due to entry of the osmotic material into the cells is improbable because: A, mannitol is known to enter plant cells excessively slowly, and the molecular weight of Carbowax, close to 1,500, should make its entry even slower; B, the subsequent enlargement of the discs in pure water does not indicate any permanently increased osmotic content, while, in contrast, discs that have been in a salt solution do show a slightly increased osmotic content.

A modification of the standard osmotic method for suction pressure determination shows that NAA increases the suction pressure of potato discs which have been exposed to hypertonic solutions. The same procedure allows a clearcut demonstration that NAA increases the elastic extension of the cell walls. This is further confirmation that one effect of auxin is exerted on the mechanical properties of the cell wall.

If the mannitol is labeled with C^{14}, the isotope taken up rapidly leaves the discs when they are returned to plain water, the time to reach half-equilibrium value being of the order of 1 minute, whether the discs have been on C^{14} mannitol for 3 days, 1 day, or 1½ hours. There is also a second, very slow, release of mannitol, which can be made rapid by killing the cells with acetone. The amount of mannitol in this "second compartment" does increase with increasing time of exposure to the mannitol, but even after 3 days it is barley 20 % of that which rapidly leaves the discs in water. After 1 day in 0.3 M mannitol a maximum of 0.008 M had entered the cells.

It is concluded that the mannitol in the first compartment is in the Apparent Free Space, which it can enter and leave rapidly, while that in the second (acetone-sensitive) compartment is within the cells. This result confirms those obtained by the osmotic methods in showing that very little externally-applied mannitol enters potato discs. The increase in the suction pressure of the discs, which appears as osmotic recovery, must therefore be due to a temporary liberation in the cells of osmotic material. This liberation, which is probably due to hydrolysis of a stored polymer, is caused in some way by the reduced turgor, and markedly promoted by auxin. The effect is separate from the increased cell wall elasticity caused by the auxin.

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