

SOME METABOLIC IMPLICATIONS OF THE TRIS EFFECT IN BEETROOT TISSUE

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Summary

In freshly sliced tissue tris(hydroxymethyl)aminomethane (Tris) induces a greatly accelerated rate of K^+ uptake and an increased net loss of Cl^- from the tissue.

The induced excess in cation uptake can either be mediated by a mechanism of exchange of H^+ produced in metabolism, or, alternatively, HCO_3^- in the external solution may act as the accompanying ion in cation uptake. In both cases the result would be acidification of the external solution, an increase in malate and citrate content of the tissue, and a drop in the respiratory quotient. Evidence is presented which favours the concept of H^+ ion exchange when excess cation is accumulated.

It is suggested that one aspect of the stimulative capacity of Tris may depend on its capacity to remove excess H^+ at the membrane, thus facilitating exchange for K^+ at the appropriate sites.

I. INTRODUCTION

In the presence of tris(hydroxymethyl)aminomethane (Tris) buffer, freshly sliced beetroot tissue accumulates K^+ from a potassium chloride solution at a greatly accelerated rate and yet Cl^- accumulation does not take place until many hours later when the tissue slices have aged (Van Steveninck 1964). It was suggested that Tris stimulated an existing mechanism of cation uptake while a concomitant anion uptake was not possible until the actual mechanism for anion transport had developed during the process of "aging".

Several pathways by which inequality of cation and anion uptake could be achieved are possible. Attention was paid to the possible role of bicarbonate ions (cf. Hurd 1958, 1959), the Tris carbamate anion ($RNHCOO^-$), and H^+ ion exchange. Changes in the organic acid balance of the tissue and its respiratory quotient were also recorded.

II. MATERIALS AND METHODS

Disks of beetroot tissue, 1 mm thick and 6 mm diameter (for respiratory studies) or 15 mm diameter (for salt-transport studies), were cut and rinsed three times with distilled water to remove cell debris before commencing the experimental treatment, particulars of which are given in the appropriate paragraphs of the experimental section.

Standard Warburg techniques (Umbreit, Burris, and Stauffer 1964) were used for respiratory measurements. Each flask contained 30 small disks (≈ 1 g tissue) and

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3 ml of solution. Organic acids were determined on batches of 200 large disks (≈ 37 g tissue) at appropriate intervals by the extraction and purification procedure of De Kock and Morrison (1958) with some minor changes in quantities employed, and adding a final purification cycle through Zeokarb 225 to remove NH_4^+ from the solution before reducing the volume at 40°C . This was done to improve the running of paper chromatograms.

Determination of total organic acids in aliquots of beetroot extract was carried out according to the method published by Palmer (1955), employing a Dowex No. 1 column (X10, 200–400 mesh). Separation of organic acids was achieved by column chromatography, each column being prepared from 5 g MFC dry silica-gel mixed with 3.5 ml 0.5N H_2SO_4 and slurried with approximately 20 ml of the solvent mixture used for the initial elution of the column. Gradient elution was achieved by employing two reservoirs connected by a siphon. The recipient reservoir was stirred magnetically, and connected to the column with a capillary tube. Mixtures of chloroform and n-butanol were used for elution. Before use, the chloroform was washed with water to remove the ethanol. The mixtures generally employed were from 278 up to 300 ml of butanol–chloroform (10 : 90 v/v) in the recipient reservoir and from 200 up to 222 ml of butanol–chloroform (60 : 40 v/v) in the donor reservoir. Both mixtures were equilibrated with 0.5N H_2SO_4 and passed through filter paper to remove suspended water droplets. Size of fractions collected varied from 2 to 4 ml with the progression of the elution. The titration of fractions was carried out in the absence of carbon dioxide with phenol red as indicator, using a nitrogen gas bubbler to mix solution with titrant (approx. 0.01N NaOH), and a 5-ml self-filling and zeroing burette. If the separation of malic and citric acids was not sharply defined, paper chromatograms were run of small aliquots of the appropriate fractions and the relative amounts of the two acids estimated by the density of their spots.

Paper chromatography was done either by the ascending method with ether–acetic acid (glacial)–water (15 : 3 : 1 v/v) as main solvent or by the descending method with butanol–90% formic acid–water (100 : 30 : 100 v/v) as main solvent. The acids were identified by means of co-chromatography with known acids. A variety of solvent systems was used [for instance n-propanol– NH_4OH (sp. gr. 0.910) (70 : 30 v/v); phenol–water (80 : 20 v/v) over 2N NH_4OH ; m-cresol–acetic acid–water (50 : 2 : 48 v/v), etc.]. The spray used most successfully was a solution of 0.04% bromocresol purple in equal parts (v/v) of 35% formaldehyde solution and ethanol adjusted to pH 5.0 with 0.1N NaOH. After treatment the chromatograms were incubated over a dish with 3% NH_4OH solution.

Nitrogen determinations were carried out by direct nesslerization of the Kjeldahl digests according to an improved method published by Williams (1964). Each result was based on triplicate or quadruplicate samples.

The radioisotopes $^{42}\text{K}^+$ and $^{36}\text{Cl}^-$ were used to determine fluxes of K^+ and Cl^- (cf. Van Steveninck 1964).

K^+ and Na^+ concentrations of the external solutions or of extracts prepared by boiling disks twice in distilled water for two consecutive 1-hr periods were determined by flame-photometry. Cl^- measurements were made by an electrometric method adapted from Furman and Low (1935).

III. RESULTS

Figure 1 illustrates the magnitude of excess cation uptake which is achieved when fresh beetroot disks are put in a 10^{-2}M Tris hydrochloride buffer (pH 7.8) containing 1 mM KCl solution. The Tris-induced excess cation uptake is mainly due to a greatly increased influx of K^+ and a decrease of the apparent influx of Cl^- compared with the controls.

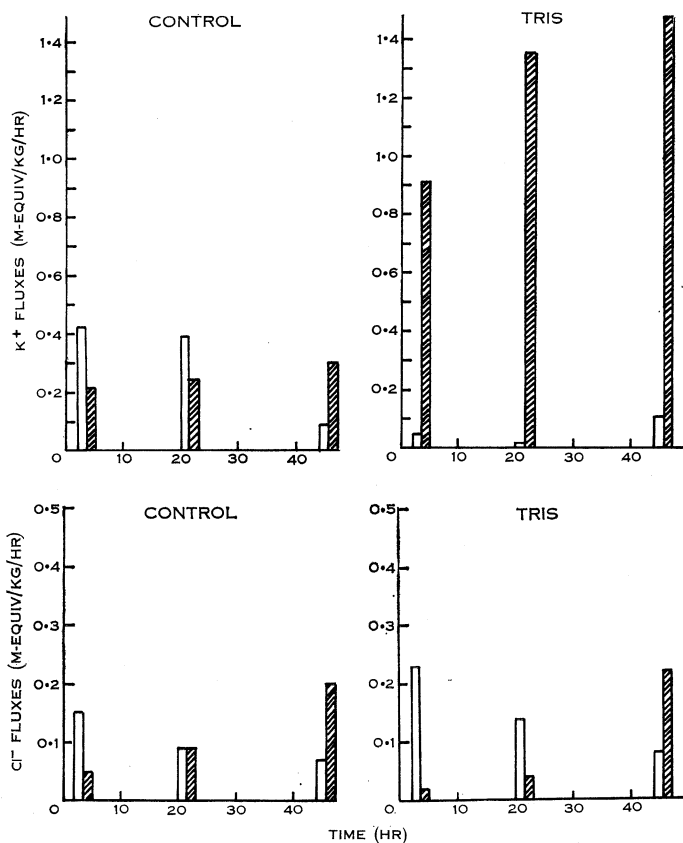


Fig. 1.—Apparent K^+ and Cl^- fluxes measured by means of $^{42}\text{K}^+$ and $^{36}\text{Cl}^-$ during and after the lag phase in disks of beetroot tissue. Disks either in the presence of 10^{-2}M Tris buffer (pH 8) or in controls (pH 7), both solutions containing 1 mM KCl. Cross-hatched columns: apparent influx into the disks; open columns: apparent efflux from the disks into the external solution. Measurements over the periods of 2–4 hr, 20–22 hr (i.e. during the lag phase), and 44–46 hr (i.e. after completion of the lag phase) after cutting the disks.

Results of the organic acid analysis confirmed the Ulrich effect (Table 1), i.e. net synthesis of organic acids when cation uptake is in excess of anion uptake (Ulrich 1941). Decreases in organic acid contents observed during the process of aging indicate that they are utilized in metabolism, but a proportion of the organic acids may have leached into the external solution. The latter seems possible, especially in

TABLE 1
EFFECT OF TRIS BUFFER ($10^{-2}M$, pH 7.9) AND OF ADDED POTASSIUM CHLORIDE ($10^{-3}M$) ON THE ORGANIC ACID CONTENT OF BEETROOT DISKS AND ON THE MOVEMENTS OF K^{+} AND Cl^{-} IONS
212 disks (38.9 g) per 900 ml solution

Treatments	Period of Incubation (hr)	Organic Acid (m-equiv/kg fresh wt.):					Total Organic Acid (m-equiv/kg fresh wt.)	Malate as % of Total	Net Movement* of	
		Malate	Citrate	Lactate	Pyrrolidone Carboxylate	Unknown			K^{+} (m-equiv/kg fresh wt.)	Cl^{-} (m-equiv/kg fresh wt.)
Control	0	16.8	22.2	1.7	2.1	0.5	43.3	39	—	—
Control	48	7.6	16.5	1.1	2.2	0.3	27.7	27	—10.2	—3.7
Control + Tris	48	17.5	28.6	2.9	1.0	0.5	50.5	35	+9.7†	—4.3
Control + KCl	48	9.2	16.5	1.3	0.9	0.3	28.1	33	—7.4	—2.4
Control + Tris + KCl	48	23.4	31.2	3.6	1.6	0.5	60.3	39	+21.4	—5.8
Control + KCl	117	4.2	10.1	0.5	1.2	0.4	16.4	26	+20.2	+22.0
Control + Tris + KCl	117	6.0	17.2	0.5	1.6	0.4	25.7	23	+21.4	+15.8

* + = net uptake; — = net loss from the disks to the external solution.

† Uptake of free space ions released during the first 2 hr of incubation.

fresh disks. Also freshly sliced beetroot tissue is reported to hold an ample supply of sugar (cf. MacDonald and De Kock 1958). Even so, differences in organic acid contents between control and Tris-treated disks showed a satisfactory relationship with their differences in ion movement. For instance, at 48 hr Tris-treated disks showed (per kilogram fresh weight) an excess K^+ over Cl^- uptake of 27.2 m-equiv., whilst controls lost 5.0 m-equiv. more K^+ than Cl^- , the total difference agreeing with the difference in organic acid contents of 32.2 m-equiv. In the absence of potassium chloride, but with Tris present, all K^+ lost from the disks during the first 2 hr was reabsorbed, but not the Cl^- , the difference in ion movement being 14.0 m-equiv. Controls lost an excess of K^+ of 6.5 m-equiv. over Cl^- , the total difference comparing reasonably well with the difference of 22.8 m-equiv. of organic acid between Tris-treated disks and controls. By 117 hours a considerable proportion of malate and

TABLE 2
EFFECT OF TRIS BUFFER ($10^{-2}M$, pH 7.8) WITH AND WITHOUT ADDED POTASSIUM CHLORIDE (1 mM) ON THE OXYGEN CONSUMPTION AND RESPIRATORY QUOTIENT (R.Q.) OF BEETROOT TISSUE AT VARIOUS TIME INTERVALS

Treatments	0 hr		23 hr		46½ hr		70½ hr		94½ hr	
	Oxygen Used ($\mu l/g/hr$)	R. Q.	Oxygen Used ($\mu l/g/hr$)	R. Q.	Oxygen Used ($\mu l/g/hr$)	R. Q.	Oxygen Used ($\mu l/g/hr$)	R. Q.	Oxygen Used ($\mu l/g/hr$)	R. Q.
KCl	—	—	141	1.10	157	1.09	160	1.11	—	—
KCl + Tris	82	0.85	137	0.94	174	0.99	152	0.93	122	0.86
Tris	72	0.84*	—	—	—	—	—	—	102	1.02

* Some K^+ liberated from free space is reabsorbed at an early stage when Tris is present.

citrate was metabolized. The difference in amounts of organic acids between Tris-treated disks and controls diminished as a likely result of K^+ depletion of the external solution by the Tris-treated disks, which continued to accumulate Cl^- in the absence of K^+ (Van Steveninck 1964).

The proportion of malate to total acids indicated that malate was more readily utilized than citrate. Tris-induced cation uptake seemed to favour malate synthesis to some extent because the percentages of malate in Tris-treated disks were higher than those of control disks; at no time, though, did they exceed the level of 39% present in the disks initially.

Respiratory measurements supported the fact that a proportion of the organic acids was metabolized during the process of aging, because the respiratory quotient proved to be greater than 1 in most cases when no Tris was added (Table 2). The respiratory quotient was usually well below 1 in the presence of Tris and potassium chloride during the aging process. In these cases, the rates of excess cation uptake and organic acid synthesis, when related to rates of respiration agreed very well with the magnitude of the depression in respiratory quotient values. In aged tissue, with

the onset of anion accumulation, the depression of these values was much less apparent. Rates of respiration did not seem to be significantly affected by Tris; on the other hand, the presence of potassium chloride caused a distinct increase in respiratory rates.

Hurd (1958) stressed the role of the bicarbonate ion in neutralizing the effects of transfer of unequal charges in aged disks. Experiments with fresh disks indicated that K^+ was indeed more readily taken up from a potassium bicarbonate solution than a potassium chloride solution (Fig. 2). However, the stimulation was not nearly

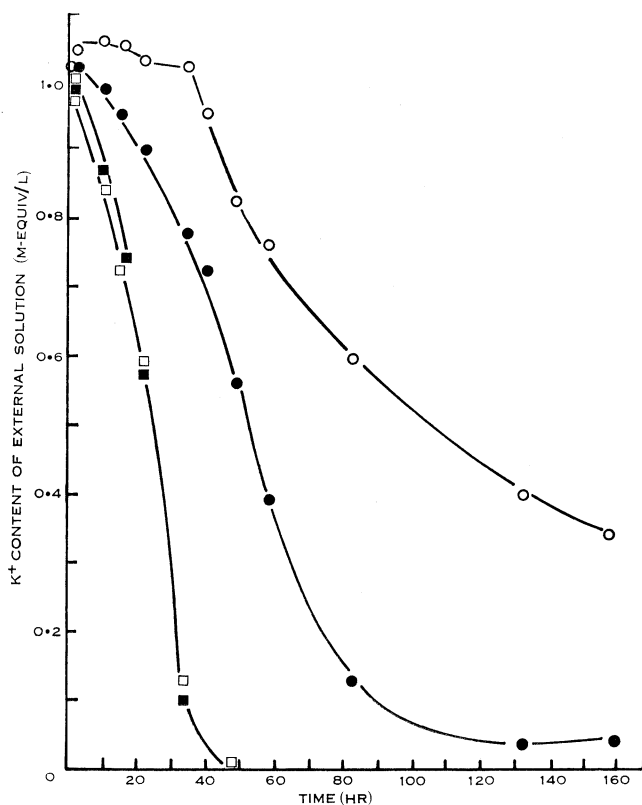


Fig. 2.—Effect of Tris buffer and bicarbonate on net K^+ uptake in disks of beetroot tissue during its lag phase. K^+ concentration of external solution plotted against time. 25 disks (4.4 g) per 160 ml of solution. ○ 1.02 mM KCl; ● 1.02 mM $KHCO_3$; □ 10^{-2} M Tris + 1.01 mM KCl; ■ 10^{-2} M Tris + 1.01 mM $KHCO_3$.

as great as that caused by Tris. Further, there was practically no difference in K^+ uptake from solutions containing either 1 mM potassium chloride or 1 mM potassium bicarbonate in the presence of Tris hydrochloride buffer. Hurd (1956) indicated that Tris buffer at pH 8.5 would contain at least 2 mM HCO_3^- as a result of respiratory and atmospheric CO_2 . This is unlikely because Tris base is reported to combine with CO_2 to form Tris carbamate ($RNH_2 + CO_2 \rightarrow RNHCOO^- + H^+$) at a rate much more rapid than the hydration of CO_2 to H_2CO_3 (Edsall and Wyman 1958).

A carbonic anhydrase could conceivably operate at the surface of storage tissue cells although as yet carbonic anhydrases have been reported to be present in leaf tissue only (Waygood and Glendenning 1950). However, a pure preparation of Diamox (2-acetyl-amino-1,3,4-thiadiazole-5-sulphonamide), a specific inhibitor of carbonic anhydrase (Miller, Dessert, and Roblin 1950), at concentrations ranging from 10^{-5}M to $6 \times 10^{-4}\text{M}$ and in the presence or absence of Tris, did not affect K^+ uptake from solutions containing either 1 mM potassium chloride or 1 mM potassium bicarbonate.

An alternative possibility was that Tris carbamate might act as the accompanying ion in K^+ uptake. Total nitrogen contents were determined on disks which had been allowed to accumulate an excess of K^+ and control disks in which K^+ and Cl^- uptake was approximately equal (Table 3). None of the experiments favoured the idea that Tris is being accumulated as its carbamate or in any other form.

TABLE 3

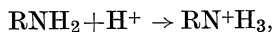
TOTAL NITROGEN PRESENT IN DISKS OF BEETROOT TISSUE* BEFORE AND AFTER TRIS-INDUCED K^+ UPTAKE

Tris concentration 10^{-2}M , pH 7.9 An increase of 0.014 g nitrogen per kilogram fresh weight of tissue is equivalent to an uptake of 1 m-equiv. Tris per kilogram fresh weight of tissue

Expt.	Period of Incubation (hr)	Treatment	Nitrogen (g/kg fresh wt.)	K^+ Absorbed (m-equiv/kg fresh wt.)
A	0	Control	0.94	—
	46	Tris	0.91	27.3
B	0	Control	0.91	—
	49	Tris	0.86	27.4
C	100	Control	1.48	22.4
	100	Tris	1.52	35.8

* cv. Crimson Globe; roots of experiments A and B grown in New Zealand, those of experiment C grown in England.

Close observations of pH changes of the external solution showed that excess cation uptake was always associated with a fall in pH. Since the concentration of Tris is known and its pK_a value is 8.14 (Gomori 1946), it was possible to calculate the amount of H^+ released from the tissue in accordance with the equations:



and

$$\text{pK}_a = \text{pH} + \log\{[\text{RN}^+\text{H}_3]/[\text{RNH}_2]\}.$$

When no potassium chloride was present, the increase in H^+ concentration was 0.51 m-equiv/l while in the presence of 1 mM potassium chloride this amounted to

1.42 m-equiv/l, which would account for about 90% of the amount of K^+ absorbed by the tissue during the same period of time (Table 4).

TABLE 4

EFFECT OF POTASSIUM CHLORIDE ON THE CHANGE IN pH OF CONTROL SOLUTIONS CONTAINING TRIS BUFFER DURING THE COURSE OF AN EXPERIMENT WITH DISKS OF BEETROOT TISSUE
Potassium chloride concentration 1 m-equiv/l. Amount of tissue 4.5 g per 160 ml solution

Time (hr)	pH		Ionic Tris Concentration* (m-equiv/l)		Amount of K^+ Absorbed (m-equiv/l)	
	Control	KCl Added	Control	KCl Added	Control	KCl Added
2	7.90	7.90	6.35	6.35	0	0
18	7.90	7.85	6.35	6.61	0.03	0.09
42	7.85	7.70	6.61	7.34	0.06	0.89
67	7.80	7.60	6.86	7.78	0.06	1.02

* Calculated from equation given on p. 277.

TABLE 5

EFFECT OF TRIS BUFFER AND K^+ IONS ON CHANGES IN pH OF SOLUTION IN WHICH DISKS OF VARIOUS TISSUES HAVE BEEN IMMERSSED

Arrows indicate gradual fall or rise towards minimum or maximum pH, respectively

Tissue	Time (hr)	pH of External Solution	Concn. of K^+ in External Solution (mM)	Tissue	Treatment	pH of External Solution:	
						After 30 min	After 76½ hr
Beetroot (25 disks, 4.4 g, placed in distilled water)	½	6.15	0.100	Artichoke	25 disks of each tissue placed in 160 ml $10^{-3}M$ KCl	6.10	→ 7.20
	4	6.20	0.230	Swede		6.00	→ 7.10
	8	6.50	0.270	Carrot		6.05 → 6.85(42)*	→ 6.60
	17	6.55	0.281	Parsnip		6.00 → 6.70(42)*	→ 6.45
	30	6.55	0.295	Beetroot		5.95 → 6.55(30)*	→ 5.40
	41½	6.30	0.220	Artichoke	25 disks of each tissue placed in 160 ml $10^{-3}M$ KCl plus $10^{-2}M$ Tris	7.95	→ 8.0
	47	6.00	0.182	Swede		7.90	→ 8.0
	65	5.90	0.060	Carrot		7.95	→ 7.55
	89	5.60	0.024	Parsnip		7.95	→ 7.50
				Beetroot		7.90	→ 7.70

* Time of pH measurement when net uptake commenced.

† No Tris effect: K^+ absorption not stimulated.

‡ Tris effect: K^+ absorption greatly stimulated.

It should be pointed out that tissues which respond to Tris treatment in the same fashion as beetroot showed a characteristic fall in pH at the completion of the lag phase (cf. Table 5) when disks were incubated in distilled water. The initial rise in pH

was due to an excess loss of cations over anions. Other tissues such as swede and artichoke which do not respond to Tris treatment (Van Steveninck 1960) did not show the characteristic fall in pH but a continued rise. This rise indicated an excess loss of cations over anions during the lag phase, and an excess of anion uptake over cation uptake during the accumulatory phase of those tissues which do not respond to Tris treatment.

IV. DISCUSSION

Fresh disks of beetroot tissue can be made to accumulate a large excess of K^+ over Cl^- from a potassium chloride solution by adding Tris buffer. Evidently, the fundamental problem concerns the mechanism of charge separation which allows excess ion movement, and the actual ion movements which initiate and equilibrate the charge separation (Robertson 1960). Figure 3 represents two alternative

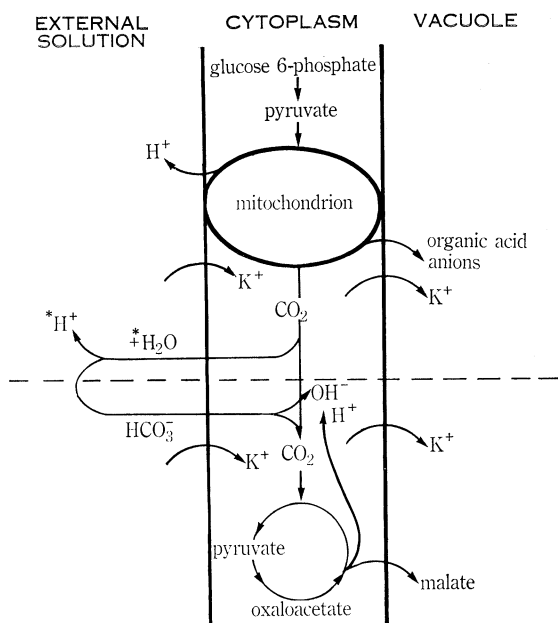


Fig. 3.—Diagrammatic representation of two alternative mechanisms which could explain excess cation uptake in beetroot tissue during its lag phase.

mechanisms for excess cation uptake. One (upper half) is based on an exchange of metabolically produced H^+ for K^+ at the plasmalemma followed by secretion of K^+ and organic acid into the vacuole. The other mechanism depends on the presence of HCO_3^- in the external solution, the concentration of which would be determined by the pH of the external solution and a possible conversion of respiratory CO_2 to bicarbonate at the outer surface of the membrane. Bicarbonate ions then would accompany the K^+ ions across the plasmalemma and, in the acid environment of the cytoplasm, pyruvate would fix the resulting CO_2 by Wood-Werkman reaction to form malate which in turn would be secreted together with K^+ into the vacuole. Both mechanisms would account for the observed acidification of the external solution. However, in the first mechanism H^+ ions are metabolically produced and must per force negotiate the plasmalemma, while in the second mechanism the H^+ ions find their source in the external solution and remain there.

Hurd (1959) stressed the importance of HCO_3^- ions in explaining excess cation uptake despite many reports that HCO_3^- has deleterious effects on plant growth (cf. Stolwyk and Thimann 1957; Hurd 1959; and others). Hurd therefore suggested that the increased content of cations or organic acid anions or both in the cells could be responsible for the deleterious effects observed on growth. It is the opinion of the present author that at least in *fresh* disks of beetroot the first alternative of H^+ exchange is of much greater importance than the HCO_3^- mechanism proposed by Hurd. This opinion is based on the following points in evidence:

- (1) Freshly prepared disks develop an anion accumulatory system only after a period of aging (Van Steveninck 1964), hence it is unlikely that an exclusive HCO_3^- accumulatory system would exist in fresh tissue.
- (2) Tris cations (RN^+H_3) might facilitate the approach of HCO_3^- ions near the membrane by shielding the negative charges at the membrane surface. However, this is not likely since Tris base (RNH_2) is the active form of Tris, the ionic form (RN^+H_3) being practically inactive (Van Steveninck 1965).
- (3) HCO_3^- is not present as such in an alkaline Tris solution because Tris base combines with CO_2 to form Tris carbamate (RNHCOO^-) much faster than the hydration of CO_2 to form bicarbonate (Edsell and Wyman 1958).
- (4) It was shown that the Tris carbamate anion is not accumulated during the period that excess K^+ uptake is induced.
- (5) Possible activity of a carbonic anhydrase system operating at the cell surface to form bicarbonate required to balance excess cation uptake was investigated, as carbonic anhydrase has been reported to be active in other plant parts (Waygood and Glendenning 1950). However Diamox at concentrations ranging from 10^{-5}M to $6 \times 10^{-4}\text{M}$ was found to be without effect on K^+ uptake. Furthermore, Samiy *et al.* (1961) found that Tris did not affect carbonic anhydrase activity.
- (6) Organic acid analysis showed that the total amounts of malate and citrate increase at approximately the same rate while excess cation uptake is taking place. However, malate seems to be metabolized more readily than citrate, and the present evidence, although not in favour of malate-dependent secretion of K^+ into the vacuole, cannot be regarded as conclusive evidence against it.
- (7) It is most likely that Tris base (RNH_2) acts as an H^+ ion acceptor in producing the Tris effect. Part of its stimulative capacity is most likely due to an efficient removal of H^+ at the membrane level, allowing K^+ to take the place of this H^+ and thus promoting the existing mechanism of K^+ for H^+ exchange at the cell surface. However, it was shown previously that the presence of Tris buffer caused a marked decrease in the amount of K^+ associated with the negative charges in the Donnan free space (Van Steveninck 1965). Unless some special relations exist between the Tris molecule and the specific anionic surface charges involved in K^+ transport, it would seem likely that Tris would compete with K^+ for these anionic charges rather than promote the K^+ for H^+ exchange.

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