FURTHER ASPECTS OF THE TRIS EFFECT IN BEETROOT TISSUE DURING ITS LAG PHASE

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Summary

The stimulation of K^+ and Na^+ uptake by tris(hydroxymethyl)aminomethane (Tris) in freshly cut beetroot tissue slices was inhibited by D-glucosamine. This effect could be fully reversed by further addition of D-glucose, and the reversal was specific for D-glucose. Tris caused a marked increase in glucose uptake from a dilute glucose solution (5×10^{-4}M). The stimulation of glucose uptake was further enhanced by the presence of Na^+.

Although Tris-stimulated cation uptake appears to depend primarily on the capacity of Tris base (RNH_2) to accept H^+ ions, it is suggested that part of the action may depend on the stimulative effect of Tris on glycolysis which is the predominant respiratory pathway in freshly sliced tissue. Thus the stimulus would result in an additional production of H^+ ions for the cation–H^+ ion-exchange mechanism. D-Glucosamine blockage of cation uptake is ascribed to its action as a competitive inhibitor for a glucose specific hexokinase (ATP : D-glucose-6-phosphotransferase).

I. Introduction

Although freshly sliced beetroot tissue is normally not capable of salt accumulation, it has been possible to stimulate an existing mechanism of cation transport by placing the slices in tris(hydroxymethyl)aminomethane (Tris) buffer at a pH greater than 6 (Van Steveninck 1961, 1962). This K^+ or Na^+ accumulation is mainly the result of a greatly increased apparent influx of these ions, yet Cl^- accumulation does not take place until many hours later when the tissue slices have "aged" (Van Steveninck 1964). Hence, cation uptake is greatly in excess of anion uptake and evidence has been presented that this process is mediated mainly through H^+ ion exchange (Van Steveninck 1966). However, more specific reasons for the stimulation may exist.

Recently, it was indicated that Tris stimulates glucose transport and some early step in the glycolytic pathway in animal tissue (Buse *et al.* 1964). This is substantiated by several reports that Tris induces a condition of hypoglycaemia in a number of animal tissues (cf. Tarail and Bennett 1959; Buse *et al.* 1964) while administration of K^+ enhances its hypoglycaemic effect in dogs (Bennett and Tarail 1961). In fact, Tris buffer has proved to be far from being an inert buffer as claimed by some of the earlier workers (Gomori 1946; Hurd 1958). It has been shown to strongly inhibit glycosidase activities (Dahlquist 1961; Fleming and Pegler 1963), to inhibit the Hill reaction in isolated chloroplasts (Jacobi 1961), and its phosphorylation by alkaline phosphatase has been reported (Dayan and Wilson 1964). It has also been

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shown to act as a competitive inhibitor of K^+ uptake in *Lactobacillus* (McLeod and Onofrey 1954). Mahler (1961) cited examples of five possible ways that Tris could participate or interfere in various reaction systems, either at the substrate or enzyme level, or in its capacity as a chelator of metals such as Ag, Cu, and Zn.

D-Glucosamine which was tested on beetroot mainly because of its similarities in structure with Tris (amine and hydroxyl groups), proved to be a powerful inhibitor of the Tris-induced cation uptake. In contrast to Tris, glucosamine is reported to have a hyperglycaemic effect in animal tissues (Řeřábek 1961; Coulson and Hernandez 1962; Silverman 1963) an effect which is ascribed to its behaviour as a competitive inhibitor of a glucose specific hexokinase (ATP : D-glucose-6-phosphotransferase).

The present work describes the reversal by glucose of the inhibition of Tris-stimulated K^+ and Na^+ transport in beetroot tissue by glucosamine. It was shown that the reversal could be achieved with glucose only.

II. Materials and Methods

Disks of beetroot tissue 1 mm thick and 15 mm in diameter were cut with a hand-microtome and rinsed three times in distilled water over a total period of 1½–3 hr. Either 25 disks (approx. 4·5 g tissue) or 50 disks were incubated in 125–160 ml or 250 ml of aerated solution respectively.

Treatments were carried out mostly in triplicate and sometimes in duplicate and experiments were repeated where appropriate. The experiments were done partly in New Zealand and partly in Australia; different sources of beetroot tissue gave essentially the same results.

K^+ and Na^+ concentrations in external solutions were determined directly by flame-photometry.

Glucose determinations were carried out immediately by an enzymatic method published by Dahlquist (1961). The reagent consisted of 71·5 mg glucose oxidase (Sigma Glucosoxidase II, 1 mg of which oxidized 13 mM glucose to gluconic acid in 1 min), 5 mg horseradish peroxidase (Sigma), 5 ml of a 1% solution of *o*-dianosidine in 95% ethanol, and made up to 1000 ml with 0·5M Tris hydrochloride buffer at pH 7·0. The reagent was stored in the refrigerator, but never for more than 5 days. 1 ml of each solution to be tested was mixed with 6 ml of reagent in a test tube and the tubes placed in a water-bath at 37°C for 60 min. The colour intensity was measured spectrophotometrically at 420 mμ against a blank without glucose, and calibrated with a glucose stock solution consisting of 100 mg glucose +2·7 g benzoic acid per litre.

III. Results

Figure 1(*a*) shows that freshly sliced disks of beetroot when placed in a dilute solution of potassium chloride (1–3 mM) did not accumulate any K^+ until a period of 30–40 hr (commonly referred to as the lag phase) had elapsed (Van Steveninck 1961). The onset of net accumulation was prevented by 10^{-2}M D-glucosamine hydrochloride (neutralized with KOH to pH 7), while *N*-acetyl-D-glucosamine had no significant effect on uptake [Fig. 1(*a*)].

An immediate and rapid accumulation of K^+ was induced by the presence of 10^{-2}M Tris buffer [cf. Fig. 1(*b*)]. Its effectiveness remained equal on further addition of 10^{-2}M *N*-acetyl-D-glucosamine, but was blocked entirely by the addition of 10^{-2}M D-glucosamine. The blockage was not always quite as complete as shown in Figure 1(*b*) due to some variation between different batches of beetroot.

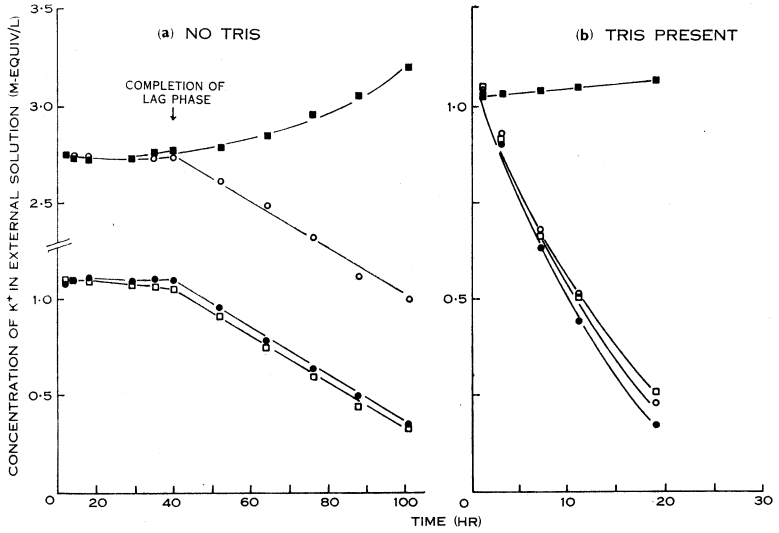

Fig. 1.—Effects of D-glucosamine, *N*-acetyl-D-glucosamine, and D-glucose on net K^+ uptake by disks of beetroot tisse in the absence of Tris buffer (*a*) and when Tris buffer is present (*b*). In (*a*) 25 disks (4·25 g tissue) were used per 160 ml solution; in (*b*) 25 disks (4·7 g tissue) were used per 125 ml solution, all solutions containing 10^{-2}M Tris buffer at pH 7·8. Other additions as follows:

(*a*) ○ Control+1·01 mM KCl
● Control+2·7 mM KCl
□ 10^{-2}M *N*-Acetyl-D-glucosamine +1·06 mM KCl
■ 10^{-2}M D-Glucosamine+2·7 mM KCl

(*b*) ○ Control+1·03 mM KCl
● 10^{-2}M D-Glucose+1·02 mM KCl
□ 10^{-2}M *N*-Acetyl-D-glucosamine +1·01 mM KCl
■ 10^{-2}M D-Glucosamine+1·01 mM KCl

In the particular experiment illustrated in Figure 1(*b*) 10^{-2}M D-glucose accelerated uptake to a small degree over and above the Tris-effect. In the majority of experiments the addition of D-glucose had little influence on K^+ uptake; however, D-glucose nearly always stimulated Na^+ uptake significantly, especially during the early stages of the lag phase (cf. Fig. 2). This phenomenon is interesting in view of the fact that beetroot may contain up to 20 g/kg fresh weight of reducing sugars ($\simeq$ 0·1M when taken over the whole of the tissue). The amounts present, however, fluctuate greatly during the lag phase (cf. MacDonald and De Kock 1958).

The D-glucosamine inhibition of Tris-stimulated cation uptake was completely reversed on addition of an equimolar concentration of D-glucose (Fig. 2). In fact the rates of uptake of Na^+ may exceed those of the control treatments. The reversibility in the case of Na^+ was usually more pronounced than with K^+. However, the reversal did not remain complete for more than 24 hr as the external glucose concentration

diminished due to uptake by the tissue. A further addition of glucose at 41 hr reversed the inhibition by D-glucosamine which became apparent again through loss of glucose from the external solution (Fig. 2).

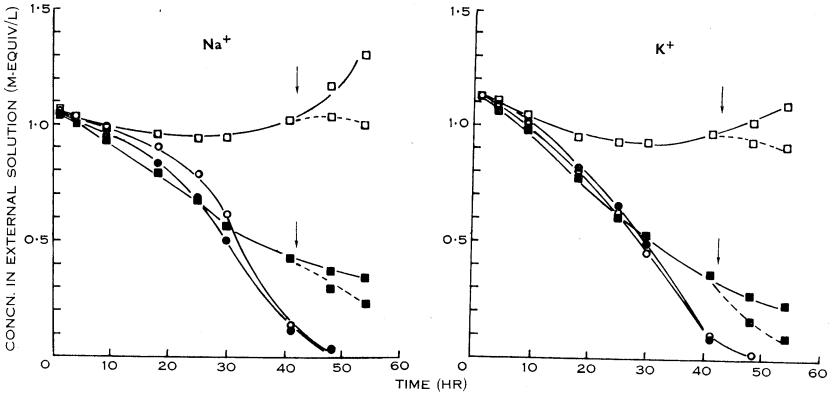

Fig. 2.—Reversal of the inhibition by D-glucosamine of Na^+ and K^+ uptake, respectively, by D-glucose. 50 disks (9·98 g) of beetroot tissue per 250 ml solution, all solutions containing 10^{-2}M Tris buffer at pH 7·8 and either 10^{-3}M NaCl or 10^{-3}M KCl respectively. Other additions as follows: ○ Control. ● 5×10^{-3}M D-Glucose. □ 5×10^{-3}M D-Glucosamine. ■ 5×10^{-3}M D-Glucosamine $+5\times10^{-3}$M D-glucose. ↓ D-Glucose equivalent to a concentration of 5×10^{-3}M added at $42\frac{1}{4}$ hr.

Results presented in Table 1 clearly indicate that only D-glucose could completely reverse the inhibition by D-glucosamine of Na^+ uptake. D-Galactose,

TABLE 1

EFFECTIVENESS OF GLUCOSE AND OTHER SUGARS IN REVERSING THE INHIBITION BY GLUCOSAMINE OF TRIS-INDUCED Na^+ UPTAKE BY DISKS OF BEETROOT TISSUE

Glucosamine and all sugars at 5×10^{-3}M concentration in 10^{-2}M Tris buffer, pH 7·8, plus 1 mM NaCl. 50 disks (either 9·85 or 9·80 g tissue) per 250 ml solution. Rates of Na^+ uptake measured over periods of 1–42 hr (expt. A) or 1–24 hr (expt. B)

Expt. No.	Treatments	Rate of Na^+ Uptake (m-equiv/kg fresh wt./hr)	Treatments	Rate of Na^+ Uptake (m-equiv/kg fresh wt./hr)
A	Control (no sugars)	0·37	D-Glucosamine	0·02
	D-Glucose	0·43	+D-glucose	0·39
	D-Fructose	0·43	+D-fructose	0·12
	D-Xylose	0·27	+D-xylose	0·09
	L-Arabinose	0·24	+L-arabinose	0·03
	D-Galactose	0·15	+D-galactose	0·12
B	Control (no sugars)	0·48	D-Glucosamine	0·08
	D-Glucose	0·52	+D-glucose	0·51
	D-Mannose	0·15	+D-mannose	0·16

D-mannose, and D-fructose caused a small reversal of inhibition while the pentoses D-xylose and L-arabinose were the least efficient. Therefore, the large difference in

effect between glucose and the rest of the sugars indicated a high degree of specificity for the reversal mechanism.

When glucosamine is not added, both fructose and glucose caused some stimulation of Na^+ uptake in addition to the Tris effect; xylose, arabinose, mannose, and galactose inhibited Na^+ uptake, increasingly in that order.

Experiments were carried out with phloridzin (3×10^{-3}M) and with dinitrofluorobenzene (DNFB, 10^{-3}M), both compounds having been reported to inhibit glucose transport (cf. Fridhandler and Quastel 1955; Le Fevre *et al.* 1964, respectively); however, the results were inconclusive. Phloridzin caused an inhibition of Tris-induced cation uptake by approximately 40% in the absence of added glucose and of 69% in the presence of 3×10^{-3}M glucose. Phloridzin completely repressed

TABLE 2

EFFECTS OF ADDED SODIUM CHLORIDE AND TRIS BUFFER ON THE AMOUNTS OF GLUCOSE ABSORBED BY DISKS OF BEETROOT TISSUE, AND THE CONCURRENT RATES OF NET UPTAKE (+) OR LOSS (−) OF K^+ AND Na^+ IONS

Fresh disks, 3 hr old; 9·8 g tissue per 250 ml solution containing 5×10^{-4}M D-glucose; period of measurement 1–17 hr from incubation; sodium chloride concentration 10^{-3}M; Tris buffer concentration 10^{-2}M

Treatments	Final pH	Glucose Uptake (m-equiv/kg fresh wt./hr)	$K^+ + Na^+$ Uptake or Loss (m-equiv/kg fresh wt./hr)
Control	6·2	0·19	−0·62
Control + sodium chloride	6·2	0·24	−0·63
Tris buffer	7·8	0·33	+0·19*
Tris buffer + sodium chloride	7·8	0·49	+0·25
Tris buffer	6·2	0·26	−0·47
Tris buffer + sodium chloride	6·2	0·40	−0·50

* Reabsorption of K^+ and Na^+ lost from the tissue during the first hour of equilibration.

the glucose-induced reversal of inhibition by glucosamine and did not change the effect of glucosamine alone. These effects, however, were produced over the period 19–45 hr after the administration of phloridzin, which was practically ineffective during the first 19 hr. When in another experiment phloridzin was added 17 hr after the addition of disks to the glucose or glucosamine or both solutions, phloridzin was not effective during any stage of the experiment.

The only experiment with DNFB was discontinued as the compound proved to have a severe toxic effect, causing pigment to leak from the cells followed by death of cells within 9 hr. It was interesting that the presence of 3×10^{-3}M glucosamine afforded a high degree of protection for the tissue cells against the toxic effects of DNFB. This protective effect of glucosamine was almost completely lost in the presence of glucose.

The addition of both sodium chloride and Tris buffer caused a substantial increase in the rate of glucose uptake from dilute glucose solutions (Table 2). The effects were additive. Tris buffer at pH 7·8 stimulated glucose uptake to a higher degree than at pH 6·1.

It is instructive to compare the rates of glucose uptake with those of net movements of K^+ and Na^+ over the same period (Table 2). As expected for tissue during the lag phase, disks showed a net loss of Na^+ and K^+ in controls, but also in Tris buffer at pH 6·1. At this pH 99·1% of the Tris is present in the ionic form ($pK_a = 8·14$). There was a net uptake of K^+ and Na^+ in Tris buffer at pH 7·8 (68·6% of Tris in ionic form).

IV. Discussion

It has been previously established that Tris buffer stimulates an existing mechanism of cation uptake during the lag phase in some storage tissues. It does this as a result of its capacity to accept H^+ ions, thus facilitating exchange of K^+ or Na^+ for H^+ at the appropriate sites at the cell surface (Van Steveninck 1966). This phenomenon is not general, since potato, swede, turnip, and artichoke tissue failed to respond to Tris buffer in contrast with carrot, parsnip, and beetroot tissue (Van Steveninck 1961); it is suggested that there are different mechanisms for cation uptake in these two groups of tissue.

Unless special relations exist between the Tris molecule and the specific anionic surface charges involved in K^+ or Na^+ transport, Tris cations (RN^+H_3) may be expected to compete with K^+ or Na^+ for these anionic charges rather than promote the K^+ or Na^+ for H^+ exchange (Van Steveninck 1965).

From evidence presented in this paper and other evidence in the literature (cf. Introduction), it appears now that Tris stimulates the uptake of glucose from a dilute solution of glucose as well as some early step in the glycolytic pathway. It is suggested that in freshly sliced storage tissue respiration depends largely on glycolysis with a gradual shift taking place towards the pentose phosphate pathway during the process of aging (ap Rees and Beevers 1960). In this respect it seems significant that Tris stimulates cation uptake in fresh beetroot disks to a much greater extent than in aged disks.

The reasons for stimulation may be twofold. The first and probably most important aspect may be that the accelerated rate of glucose breakdown will provide an additional source of H^+ ions for exchange with K^+ or Na^+. The other much more speculative possibility could be that glucose phosphate acts as a carrier for K^+ or Na^+ transport. Thus the stimulation of cation uptake and glucose transport might be linked. However, it was evident from the results (Table 2) that Tris base (pH 7·8), must be present to make net cation uptake possible, while glucose transport was stimulated at both pH 7·8 and pH 6·1, the latter pH precluding net cation uptake. Yet, it may be significant that glucose transport was stimulated to a higher degree at pH 7·8 than at pH 6·1.

Added Na^+ increased the rate of glucose uptake in every instance (Table 2) but the effect was more pronounced in the presence of Tris and depended on the disks being freshly cut (aged disks produced an opposite result, Na^+ causing an inhibition of glucose uptake—Van Steveninck, unpublished data). This finding seems to correspond with Bennett and Tarail's (1961) observation that added K^+ enhanced the hypoglycaemic effects of Tris. Unfortunately, freshly cut disks always lose a small amount of K^+ and Na^+ to the external solution during the first hour of equilibration, hence

it was not possible to ascertain whether Tris would have stimulated glucose uptake in complete absence of external K^+ and Na^+. However, convincing evidence that Na^+ and K^+ uptake in fresh tissue is closely linked with glycolysis is provided in the fact that D-glucosamine, a competitive inhibitor of hexokinase (cf. Introduction), inhibited Tris-stimulated cation uptake completely. This inhibition was entirely reversed by the further addition of an equimolar amount of glucose.

Inhibitors of glucose transport gave inconclusive results. The action of phloridzin (Fridhandler and Quastel 1955) was according to expectation but showed a lag of about 20 hr, while phloridzin applied 17 hr after the addition of glucose or glucosamine solutions to the disks was not effective. The lag in effect indicates that the action of phloridzin is indirect and possibly the result of a breakdown product.

Dinitrofluorobenzene showed a curious interaction with glucosamine. It had a reverse toxic effect causing leakage of pigment which was alleviated in the presence of glucosamine, while the protective effect of glucosamine was almost completely lost through the further addition of glucose. Obviously, the three compounds interacted with each other. However, the site of interaction seemed to have an important bearing on the maintenance of the integrity of the membrane systems.

V. Acknowledgments

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