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ADVERSE EFFECTS OF TRIS HYDROCHLORIDE, A COMMONLY USED BUFFER IN PHYSIOLOGICAL MEDIA

BY J. S. GILLESPIE AND A. T. McKNIGHT*

From the
Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ

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SUMMARY

1. Tris, 10 mM, produced inhibition of motor responses to adrenergic motor nerve stimulation in all smooth muscles studied (rat anococcygeus and vas deferens, perfused rabbit ear artery). Up to 30 mM Tris was without significant effect on those motor responses to cholinergic nerve stimulation examined (guinea-pig ileum).

2. A similar reduction in responses to endogenous motor agonists was seen only in the anococcygeus, while in the rabbit ear artery, responses to noradrenaline (NA) were potentiated.

3. Tris consistently produced increased tone in the rat anococcygeus and rabbit aorta, but in no other tissue. Small reductions in tone were seen with Tris in spiral strips of rabbit ear artery.

4. Inhibitory responses to adrenergic nerve stimulation and to NA in the rabbit jejunum and to inhibitory nerve stimulation in the rat anococcygeus were unaffected by Tris.

5. In the perfused rabbit heart Tris produced a negative inotropic effect and reduced responses to vagal stimulation. Responses to sympathetic nerve stimulation and to ACh and NA were not significantly altered.

6. Up to 40 mM Tris had little effect on responses of the rat diaphragm to motor nerve stimulation.

7. The effects of Tris on the rat anococcygeus were not reduced by increased calcium levels but were reduced at lower incubation temperatures. The negative inotropic effect of Tris on the rabbit heart was also reduced at lower temperatures.

8. The buffer HEPES produced a reduction in responses to stimulation in the rat anococcygeus and vas deferens alone, but at higher

* M.R.C. Scholar.

concentrations than those of Tris. Increases in tone in the anococcygeus and aorta were never seen with HEPES.

9. These results indicate that Tris in concentrations commonly used as a buffer in physiological salines can exert toxic effects on neuromuscular transmission in smooth and cardiac muscle though not in skeletal muscle. The effects are variable, mainly presynaptic and appear to affect in particular motor and especially adrenergic transmission. They may be associated with intracellular metabolic actions of Tris.

INTRODUCTION

Tris (hydroxymethyl) methylamine (Tris) has been increasingly used as a buffering agent in biological experiments since its first introduction for this purpose by Gomori (1946). It is of particular value in experiments where for one reason or another the bicarbonate ion is undesirable. In tissue culture experiments, however, Tris has been found to exert toxic effects (Good, Winget, Winter, Conolly, Izawa & Singh, 1966; Stinson & Spencer, 1968), probably associated with a metabolic action inside the cells which Tris has been shown to penetrate (Ligou & Nahas, 1960; Brown & Goot, 1963; Lambotte, Kestens & Haxhe, 1971). In spite of this information there appears to have been no systematic determination of the possible toxic effects of this agent on mammalian preparations where it continues to be used in various modified physiological salines. The only report we have found in the literature is that of a negative inotropic effect on the perfused heart (Mattiazzi, Cingolani & Gonzalez, 1972).

We have therefore examined the effect of Tris, in the concentration range in which it is usually used as a buffer (10–40 mM), on the responses of innervated preparations of smooth, skeletal and cardiac muscle to nerve stimulation and to the exogenous transmitter substances. Six different smooth muscles were chosen to allow study of effects on adrenergic nerves (rat anococcygeus and vas deferens, perfused rabbit ear artery and Finkleman preparation of rabbit jejunum), cholinergic nerves (guinea-pig ileum transmural stimulation), motor nerves (rat anococcygeus, vas deferens, rabbit ear artery and guinea-pig ileum) and inhibitory nerves (Finkleman preparation of the rabbit jejunum, inhibitory nerves of the rat anococcygeus). The effects on responses to exogenous agonists was studied in these preparations where possible, and also in spiral strips of rabbit aorta and ear artery. The skeletal muscle studied was the isolated innervated rat diaphragm and the cardiac muscle, the isolated innervated rabbit heart.

Since the evidence from tissue culture studies associated the toxicity

of Tris with its ability to penetrate cells and further suggested that the buffer *N*-2-hydroxyethylpiperazine-*N'*-2'-ethane sulphonic acid (HEPES) which is unable to penetrate cells was less toxic (Good *et al.* 1966), we have also examined this compound.

A preliminary account of these experiments has been published (Gillespie & McKnight, 1975).

METHODS

Anococcygeus muscles were removed from 200–300 g male rats, drawn through Ag/AgCl ring electrodes for field stimulation and suspended in 10 ml. baths containing Krebs saline at 37° C and gassed with 95 % O_2 + 5 % CO_2 as previously described (Gillespie, 1972). Vasa deferentia from rats were set up in a similar fashion. Contractions were measured with Grass FT 03C isometric transducers and displayed on a Grass polygraph. Field stimulation was by 1 msec pulses of supramaximal voltage for 10 sec at 30 Hz. When the inhibitory responses of the anococcygeus were studied these were uncovered by blocking the adrenergic motor response with phentolamine 10^{-6} M and raising the muscle tone by adding carbachol 10^{-4} M.

Isolated rabbit ear arteries were removed after killing the animals either with an I.V. overdose of Nembutal or by a blow on the neck and bleeding. The preparations were set up as described by de la Lande & Rand (1965) and perfused with Krebs saline at 37° C delivered at a rate of 4–6 ml./min by a constant output Watson–Marlow pump. This flow rate gave mean perfusion pressures of about 60 mmHg and these and the changes in pressure as a result of periarterial nerve stimulation were recorded with a Statham P23AC pressure transducer. Periarterial nerve stimulation was by ring electrodes of platinum wire embedded in Araldite epoxy resin and the stimulation parameters were the same as those for the anococcygeus and vas deferens. In this preparation Tris or HEPES was added to both the outside fluid and the perfusion solution. Noradrenaline (NA) was injected as a bolus into the perfusion fluid through a length of self-sealing rubber tubing close to the arterial cannula.

The rabbit abdominal aortic and the rabbit ear artery strips were prepared from 3 cm lengths of artery after the method of Furchgott & Bhadrakom (1953) and these also were suspended in 10 ml. baths of Krebs saline at 37° C. 3 cm lengths of guinea-pig ileum were set up in 50 ml. baths of Krebs saline at 37° C and arranged for stimulation with co-axial silver electrodes in the manner described by Paton (1955).

Finkleman preparations of rabbit jejunum were set up by removing 3 cm lengths of intestine together with a fan of mesentery containing a major mesenteric artery. The mesentery and artery were drawn through a pair of platinum electrodes similar to those used for the perfused rabbit ear arteries and similar stimulation parameters were used.

To study effects on cardiac muscle, rabbit hearts were isolated with the vagi or sympathetic nerves still attached and set up as Langendorff preparations as described by McEwan (1956) and Hudović & Muscholl (1962). Male rabbits 1·5–3·0 kg in weight were anaesthetized with a halothane, nitrous oxide, oxygen mixture and both vagi or cardiac sympathetic nerves exposed in the neck and cleared of fat and connective tissue as far as the aortic arch. Once the thorax was opened the animal was placed on artificial positive pressure ventilation. The animal was then heparinized (1000 i.u./kg) and killed by cutting through the abdominal aorta. The entire thoracic contents were then removed and transferred to a dish

containing chilled Krebs saline. The lungs, thymus, oesophagus and the major part of the trachea were removed with care to avoid damage to the nerves. The aorta was then cannulated and perfused with double glucose Krebs to which Tris could be added. In tying in the cannula we found that if the ligature was applied, as is usual, round the aorta close to its exit from the left ventricle, then the response to vagal stimulation was poor, presumably as a result of damage to the post-ganglionic fibres. The cannula was, therefore, tied well away from the heart and the carotid and subclavian arteries tied separately to prevent leakage. The perfusion apparatus was constructed to permit injection of acetylcholine (ACh) or NA directly into the arterial cannula close to the heart. The vagus or sympathetic nerves were threaded through ring electrodes embedded in Araldite (Ciba) which incorporated a small plastic Y-piece. The nerves ran through the stem and one arm of the Y and the other arm was used to superfuse the nerves with Krebs saline. The nerves were stimulated with 1–2 msec pulses of supramaximal voltage at either 10 Hz (vagi) or 30 Hz (sympathetic) for 10 or 15 sec. The mechanical response of the heart was measured, with the preparation hanging freely, by connecting the apex of the left ventricle to a Grass FT 03C isometric transducer by a length of thread and a small metal clip. Contractions of the left atrium were measured in a similar fashion. With the heart secured in this way, rotational movements were markedly reduced and no other steps were taken to remove them (see Beckett, 1970).

Heart rates were measured directly from the experimental records. To assess the effects of nerve stimulation or the exogenous transmitters, the heart rate was measured over a 10 sec period centred on the peak of the effect. Changes in heart rate were expressed in terms of the control rate as a percentage increase or decrease.

The rat phrenic-nerve hemidiaphragm preparations were removed as described by Bülbring (1946) and mounted on a Palmer phrenic nerve electrode assembly in 100 ml. baths containing Krebs at 37° C. Single 1 msec pulses of supramaximal voltage were delivered every 10 sec.

Tris (Sigma) and HEPES (Hopkins & Williams) were dissolved in de-ionized distilled water and the pH adjusted to 7·4 at 30 and 37° C with 1 N-HCl and NaOH respectively and the volume adjusted to give stock concentrations of 250 mM. Further dilutions were in Krebs saline.

Drugs used were acetylcholine chloride (Koch–Light), carbachol (Sigma), (−)-noradrenaline bitartrate (Koch–Light), phentolamine mesylate (Ciba) and doses are given in moles of the base.

Where appropriate, the results are expressed as the mean of the relevant observations ± S.E. of mean. The significance of differences between means was evaluated by Student's t test and those with P values of 0·05 or less were considered significant.

RESULTS

Smooth muscle

The effect of Tris on the response to motor (adrenergic) nerve stimulation and to NA

In each preparation field stimulation was repeated at regular intervals until successive responses became equal. Tris was then added to the bath, stimulation continued and any effect observed was expressed as a percentage inhibition or potentiation with respect to the control response.

In the rat anococcygeus, exposure to Tris 10 mM produced a mean reduction in the response to field stimulation of the adrenergic nerves of $43{\cdot}9 \pm 1{\cdot}02$ % ($n = 6$). The maximum responses to NA and to carbachol were similarly reduced by $46{\cdot}0 \pm 6{\cdot}4$ and $33{\cdot}9 \pm 4{\cdot}7$ % respectively ($n = 6$). These effects developed relatively slowly, reaching a maximum after around 30 min and thereafter remaining fairly constant for periods of exposure of up to 1 hr. The effects were difficult to reverse on returning to normal Krebs saline. In addition to the decline in the motor responses to nerve stimulation or agonist drugs, Tris 10 mM commonly caused a phentolamine-insensitive rise in tone which could be several grams in magnitude. These effects are illustrated in Fig. 1.

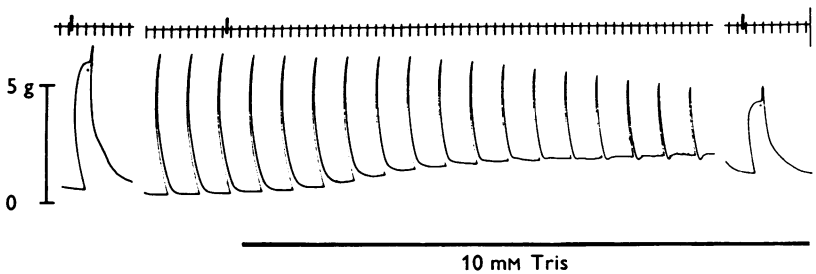

Fig. 1. The effect of Tris 10 mM on the response of the rat anococcygeus at 37° C to NA, 3×10^{-6} M (extreme left and right panels), and to supramaximal stimulation of motor adrenergic nerves at 30 Hz for 10 sec (central panel). Tris is present during the time represented by the dense black bar. Time = 1 min, tension calibration in grams.

In the rat vas deferens the effects of Tris were less; 10 mM Tris reduced the response to motor nerve stimulation by $28{\cdot}3 \pm 5{\cdot}8$ % ($n = 6$) without affecting the responses to NA or carbachol. In this tissue a rise in tone was never seen.

In the perfused rabbit ear artery Tris 10 mM again reduced the response to field stimulation of the periarterial adrenergic nerves, this time by $31{\cdot}7 \pm 3{\cdot}9$ % ($n = 6$), but in this preparation the response to exogenous NA was potentiated by $39{\cdot}0 \pm 9{\cdot}3$ %. No effect on resting perfusion pressure was ever observed (Fig. 2). Unlike the anococcygeus and vas deferens the effects of Tris were readily reversed by washing.

The potentiation of the response to NA and simultaneous depression of the response to adrenergic nerve stimulation prompted us to examine the effect of Tris on another vascular smooth muscle, the aortic strip. The results are shown in Fig. 3. In this tissue the contractile responses to NA are slow to develop so that the total exposure to Tris was considerably longer than in other preparations, often up to 2–3 hr. In spite of this, potentiation of the response to NA by Tris was never seen. In

every preparation, however, Tris 10–20 mM caused a rise in tone similar to that observed in the anococcygeus muscle (Fig. 3) and like it, resistant to repeated washing in Tris-free Krebs or to phentolamine 3×10^{-6} M. The difference between the perfused rabbit ear artery and the aortic strip could represent a difference in the vascular smooth muscle in the two sites, but might also be the result of the nature of the preparation – the perfused intact artery as compared with the spiral strip. To distinguish

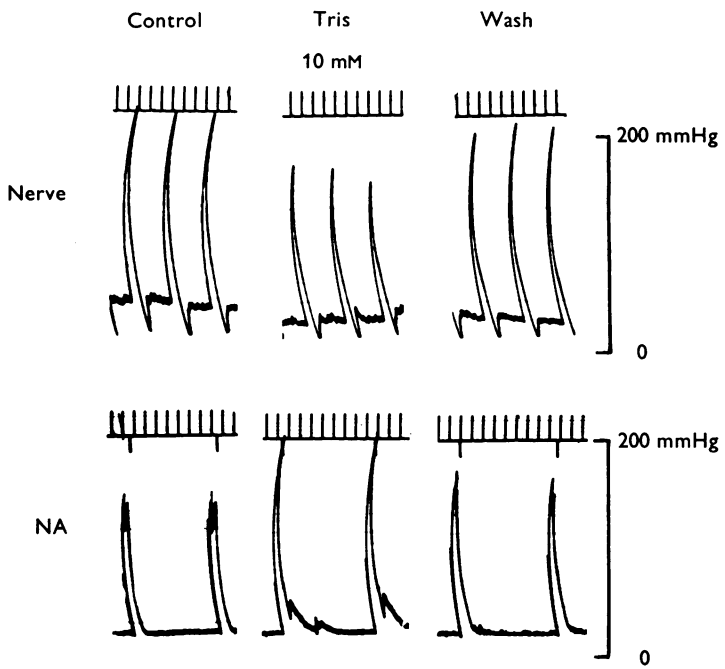

Fig. 2. Responses of the perfused central artery of the rabbit ear to supramaximal stimulation of adrenergic nerves at 30 Hz for 10 sec (top) and to NA 200 ng (bottom) before, and after, the addition of Tris and in the presence of 10 mM Tris. Time = 1 min.

these possibilities rabbit ear arteries were cut into spiral strips and the effects of Tris examined. As Fig. 3 shows, the results were similar to those in the perfused artery. The rise in tone observed in the aortic strip was never seen in strips prepared from ear arteries; rather Tris caused a fall in any intrinsic tone possessed by the smooth muscle. 20 mM Tris produced potentiation of submaximal responses to NA 10^{-6} M which was again reversed on washing (Fig. 3). The maximum response to NA (3×10^{-6} M) was slightly potentiated though this was almost entirely due to the lower base line from which contraction started. The maximum tension achieved was little altered.

The effect of Tris on the response to motor (cholinergic) nerve stimulation and to carbachol

The effect of Tris on the motor responses of smooth muscle to cholinergic nerve stimulation was examined in the transmurally stimulated guinea-pig ileum preparation. In this preparation Tris in concentrations up to 30 mM had no effect in ten out of twelve preparations on the response

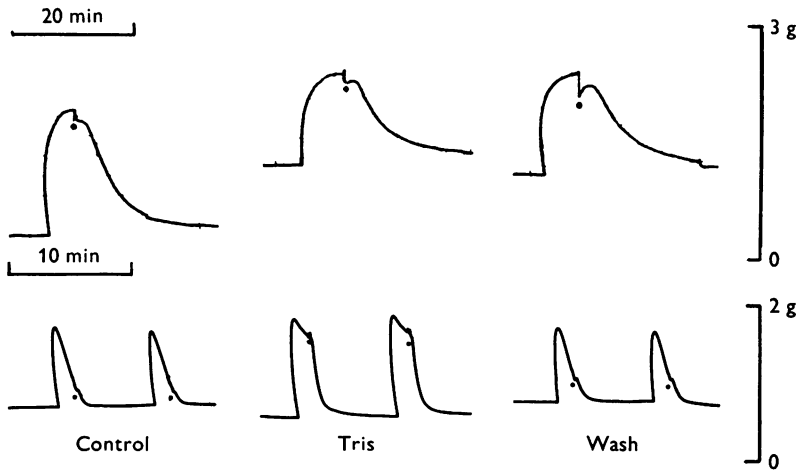

Fig. 3. Responses of spiral strips of rabbit abdominal aorta (top) and central ear artery (bottom) to NA, 3×10^{-5} and 10^{-6} M respectively, before and after the addition of Tris and in the presence of 20 mM Tris. Tension calibration in grams.

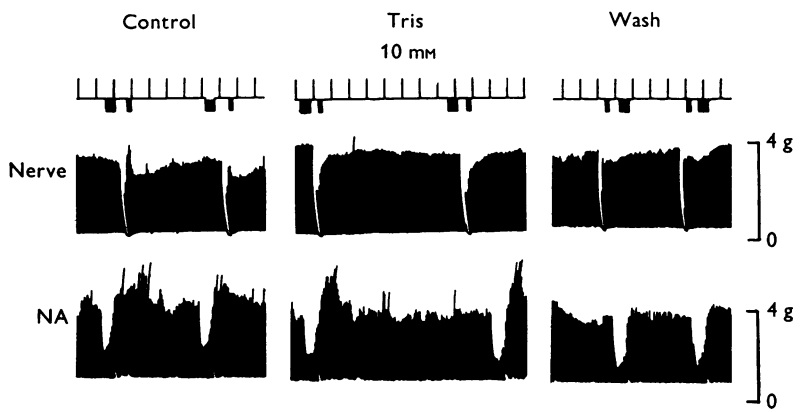

Fig. 4. Responses of the Finkleman preparation of rabbit jejunum to supramaximal stimulation of periarterial sympathetic nerves (top) and to NA 10^{-6} M (bottom) before and after the addition of Tris, and in the presence of 10 mM Tris. Time = 1 min, tension calibration in grams.

to transmural stimulation. In the remaining two preparations there was a small reduction (10 %). The responses to carbachol 10^{-5} M were also little affected by even the highest concentration of Tris (12·13 ± 3·57 % reduction, $n = 4$). These effects were rapidly reversed on washing.

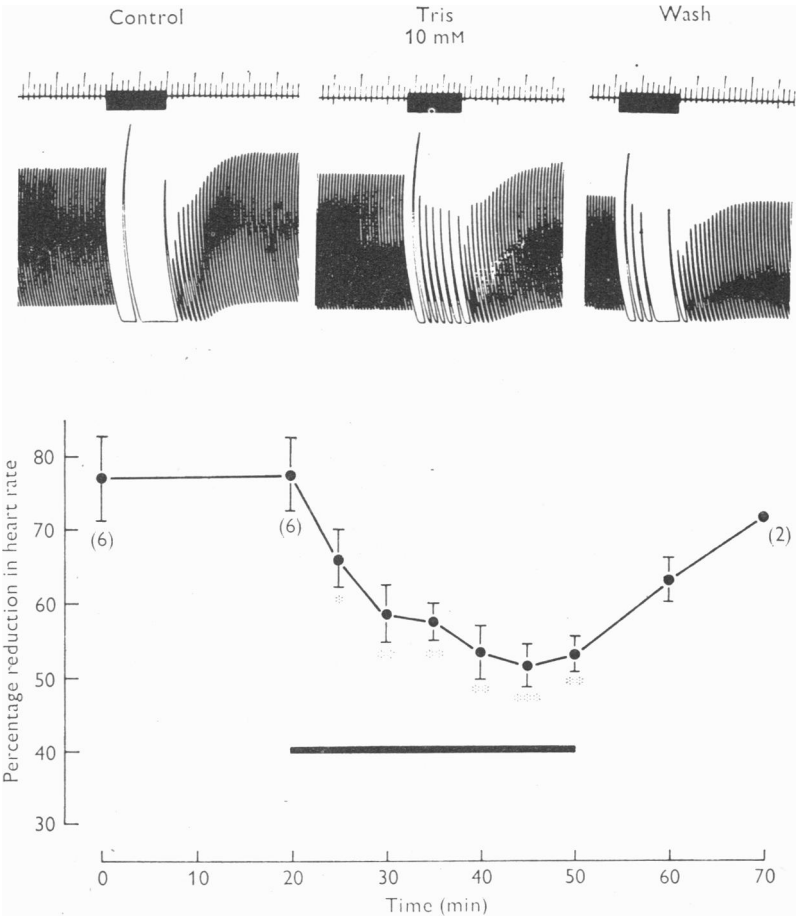

Fig. 5. The effect of Tris on the response of the isolated perfused rabbit heart at 30° C to supramaximal vagal stimulation (10 Hz, 2 msec, for 10 sec). The top panels illustrate in a single experiment the effect of vagal stimulation on isometric contractions of the left ventricle before, during and after perfusion with Krebs containing 10 mM Tris. Time = 1 sec. The graph shows mean responses to vagal stimulation expressed as a percentage reduction in heart rate. Tris is present in the perfusion fluid during the period represented by the dense bar. I-bars are ± 1 S.E. of mean. Numbers in parentheses show the number of observations, otherwise, $n = 4$. * = $0{\cdot}05 > P > 0{\cdot}01$, ** = $0{\cdot}01 > P > 0{\cdot}001$, *** = $0{\cdot}001 > P$.

The effects of Tris on the response to inhibitory nerve stimulation

These were studied on two preparations of smooth muscle, first the Finkleman preparation of rabbit jejunum stimulating adrenergic nerves, and secondly in the rat anococcygeus where the inhibitory transmitter is unknown.

In the rabbit jejunum Tris 10–20 mM had no effect on either the spontaneous pendular movements or tone or on the inhibitory response to periarterial nerve stimulation or NA (10^{-6} M) (Fig. 4). In the rat anococcygeus, Tris in these same concentrations had no effect on the response to inhibitory nerve stimulation.

Cardiac muscle

The effect of Tris on the response to nerve stimulation, to NA or to ACh

In the isolated heart the addition of 10–20 mM Tris to the perfusion fluid caused a negative inotropic effect from which there was no recovery on return to Tris-free Krebs (Fig. 5). The inhibitory responses to vagal stimulation were significantly reduced (Fig. 5), whereas the mean response to exogenous acetylcholine was increased from a reduction of $39{\cdot}9 \pm 2{\cdot}5$ to $45{\cdot}2 \pm 4{\cdot}9\%$, though the difference between these values was not statistically significant. The inhibition of the vagal response by Tris was partially reversed by returning to Tris-free Krebs (Fig. 5).

The effect of Tris on the positive chronotropic response to cardiac sympathetic nerve stimulation was less consistent but overall Tris produced no significant change either in the response to nerve stimulation or to the injection of NA.

Skeletal muscle

In the rat phrenic nerve diaphragm preparation, Tris 10 mM had no effect on either muscle tone or the response to stimulation of the motor nerve. Even in the high concentration of 40 mM a reduction in the response to phrenic nerve stimulation was observed in only three out of ten preparations. With this same high concentration a small increase in tone was observed but again in only four experiments.

The effect of calcium concentration and temperature

The inhibition of adrenergic motor responses in the rat anococcygeus and vas deferens and the rabbit ear artery by Tris could be due to a reduction in the levels of ionized calcium available for post-synaptic excitation–contraction coupling or presynaptic excitation–secretion coupling. This possibility was investigated in the rat anococcygeus muscle by examining the effect of raised calcium on the action of Tris. Fivefold

increases in the extracellular calcium concentration neither prevented nor reversed the inhibitory effect of Tris on the response to adrenergic nerve stimulation, nor prevented the rise in tone.

Since Tris is known to affect adversely oxygen utilization and respiratory activity in isolated mitochondria (Stinson & Spencer, 1968) it was of interest to investigate whether the inhibitory effect of Tris would be lessened in conditions where oxygen requirements were reduced. The effects of lowering temperature were therefore examined in the rat anococcygeus muscle and the perfused rabbit heart.

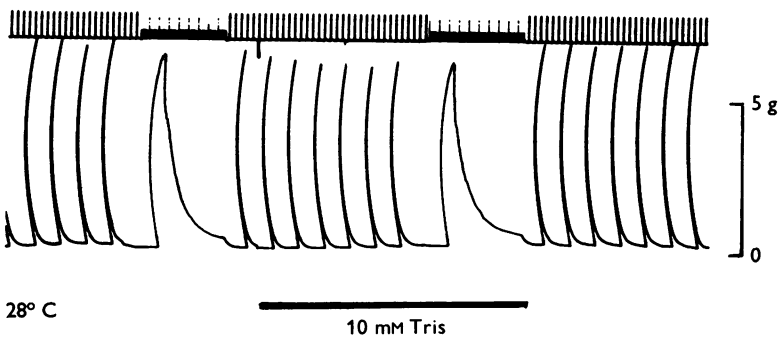

Fig. 6. The effect of Tris on the response of the rat anococcygeus to supramaximal field stimulation of motor nerves at 28° C. Time = 1 min except in the two portions of trace where the chart speed has been increased; here large inflexions are 5 sec. Tension calibration in grams.

At 30° C the negative inotropic effect of Tris on the isolated perfused rabbit heart was reduced, or often absent, but responses to vagal stimulation were still reduced. At 28° C the effect of Tris on the rat anococcygeus was similarly reduced. A rise in tone was seldom seen and the inhibition of the response to adrenergic motor nerve stimulation was slight and rapidly reversed on washing (Fig. 6).

The effect of HEPES

The effect of this buffer compound was examined in five smooth muscle preparations. In those preparations unaffected by Tris (guinea-pig ileum, rabbit jejunum) HEPES was also without effect. In the rabbit aortic strip and the rat anococcygeus the increases in tone observed with 10–20 mM Tris were never seen with up to 30 mM-HEPES. In the three tissues in which the response to motor adrenergic nerve stimulation was reduced by Tris (rat anococcygeus, rat vas deferens and the perfused rabbit ear artery) HEPES consistently reduced the response in the

anococcygeus and vas deferens only at the relatively high concentration of 20–30 mM and even this concentration had no effect on the rabbit ear artery (Fig. 7).

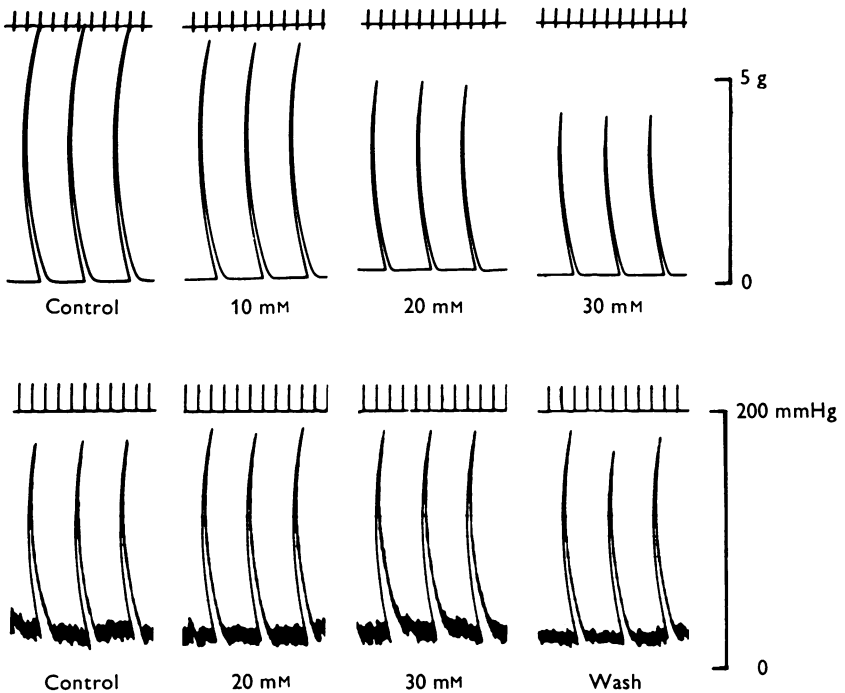

Fig. 7. The effect of increasing concentrations of HEPES on responses to supramaximal motor nerve stimulation in the rat anococcygeus (upper records) and the perfused rabbit ear artery (lower records). Time = 1 min, tension calibration in the upper records in grams.

DISCUSSION

It is difficult in considering these effects of Tris to see any pattern either in the site of action or in the mechanism involved. As far as the site is concerned the results in the smooth muscle preparations suggested that it was predominantly motor adrenergic responses that were inhibited (anococcygeus, vas deferens, rabbit ear artery) and that this could be either a presynaptic action as in the rabbit ear artery where the response to NA was potentiated or post-synaptic as in the rat anococcygeus, in which the responses to NA and to carbachol were also inhibited. Inhibitory responses to nerve stimulation in the rat anococcygeus and rabbit jejunum were unaffected, as was the response to cholinergic nerve stimulation in the guinea-pig ileum. Such pattern as exists in

these results is further weakened in considering the results in the rabbit heart. There the responses to cholinergic nerve stimulation were reduced by a presynaptic action and the adrenergic excitatory responses were little affected.

Two positive observations may have a bearing on the mode of action of Tris. The first is that the inhibitory effects of Tris were less at low temperature and the second that HEPES, which is less able to penetrate cells (Good *et al.* 1966), was less toxic on all the preparations tested. Tris is known to penetrate cells (Ligou & Nahas, 1960) and at intracellular pH some 90% will be ionized (Nahas, 1962) and thus able to exert its buffering action. An increase in intracellular pH has been shown to produce activation of some smooth muscle contractile systems (Schädler, 1967). The ability of Tris to interfere with oxidative metabolism in isolated mitochondria (Stinson & Spencer, 1968) and to inhibit lactate dehydrogenase (Mahler, 1961), the enzyme catalysing the conversion of pyruvate to lactate, might also contribute to an increasing cytoplasmic alkalinity. If changes in intracellular pH are involved in the inhibitory effects of Tris it may be that this is exerted through changes in the availability of the calcium ion, though against this was the ineffectiveness of raised calcium levels to antagonize the inhibitory effects. Changes in calcium ion distribution might also explain the rise in tone produced by Tris in the aortic strip and anococcygeus muscle.

The most practical outcome of these investigations is the demonstration that Tris can quite clearly exert toxic effects on a variety of mammalian preparations, as well as on tissue culture cells, and does so in the range of concentrations commonly employed in mammalian buffer systems. These effects are particularly noticeable on adrenergic motor responses where the effect is mainly presynaptic. In the heart the direct negative inotropic effect reported by Mattiazzi *et al.* (1972) is confirmed and again there is a presynaptic inhibitory action on the vagal response. No effect on skeletal muscle was detected. The effects may be due to an intracellular buffering action of Tris involving cell metabolism.

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