TRANS-ACONITATE UTILIZATION BY SHEEP

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Summary

Sheep fed diets containing 3.5 and 7.0% trans-aconitate on a dry weight basis for 5 days appeared normal and maintained normal levels of blood citrate, ketones, and aconitate, but showed large increases in urinary citrate. Calcium and magnesium levels in plasma and urine were not substantially modified. When trans-aconitate was placed in the rumen it disappeared rapidly but did not increase the concentration of rumen volatile fatty acids; blood and urinary aconitate values remained low. trans-Aconitate did not inhibit the fermentation of soluble substrates by rumen microorganisms in vitro. Both cis- and trans-aconitate were fermented slowly.

Intravenously injected sodium trans-aconitate at 1.0 m-mole/kg body weight produced no ill effects. The citrate which subsequently accumulated in blood and urine was not a radiometabolite of [1,5-14C]trans-aconitate, suggesting that it was formed by aconitate hydratase inhibition. Plasma calcium and magnesium values were not depressed by intravenous trans-aconitate administration but urinary calcium excretion increased and urinary magnesium decreased. Under similar conditions of injection, sodium citrate was lethal.

These data are believed to exclude trans-aconitate as a sole cause of lethal aconitate hydratase inhibition or of hypomagnesaemia in sheep.

I. INTRODUCTION

Burau and Stout (1965) have suggested that trans-aconitate may poison cattle by forming magnesium ion complexes which induce hypomagnesaemia, or by competitively inhibiting aconitate hydratase, the enzyme catalysing interconversion of citrate, cis-aconitate, and isocitrate within the tricarboxylic acid cycle. These suggestions were prompted by the observation that high trans-aconitate concentrations occur in herbage from early spring pastures inducing hypomagnesaemia (Burau and Stout 1965). The highest trans-aconitate concentration (4.2% of dry weight) observed in a grass species was found in Phalaris tuberosa L. Symptoms resembling those of Phalaris poisoning in sheep have also been caused by dietary fluoroacetate, namely progressive citrate accumulation, loss of appetite, muscular incoordination, body tremors, tetanic convulsions, and death (Jarrett and Packham 1956). The biochemical basis for fluoroacetate poisoning appears to lie in its enzymic conversion in vivo to fluorocitrate (Peters 1957). Both fluorocitrate (Peters 1957)

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and trans-aconitate (Saffran and Prado 1949) act as competitive inhibitors of mammalian aconitate hydratase. trans-Aconitate has been previously shown to strongly inhibit the respiratory oxygen uptake by sheep rumen epithelium in tissue slices (Pennington and Sutherland 1956). It is therefore desirable to establish whether trans-aconitate can contribute to the Phalaris poisoning syndromes, currently ascribed mainly to the effects of tryptamine alkaloids (Gallagher, Koch, and Hoffman 1966).

Stout, Brownell, and Burau (1967) have found more than 1% trans-aconitate, on a dry weight basis, in 14 of the 30 grass species examined and in 8 of 54 dicotyledonous plants. Though there is clear need to study trans-aconitate metabolism by mammals, the action of trans-aconitate on rumen microorganisms is also obscure. Both inhibition (Pisano, Blahuta, and Mullen 1959) and utilization (Altekar and Rao 1963) have been demonstrated with bacteria. The present paper considers first the utilization of dietary trans-aconitate and then the effects of intravenously injected trans-aconitate on sheep.

II. Methods

(a) Sheep and Materials

The toxicity of trans-aconitate was tested with Merino or Merino × Border Leicester wethers weighing between 37 and 53 kg. Food was withheld from sheep during the 24 hr preceding injection or intraruminal administration experiments.

trans-Aconitie acid for feeding experiments was prepared from citric acid according to Bruce (1961) and recrystallized twice from acetic acid to achieve a melting point greater than 193°C (dec.). [1,5-14C]trans-Aconitic acid was similarly prepared from [1,5-14C]citric acid. trans-Aconitic acid, A grade, for injection experiments was obtained from California Corporation for Biochemical Research, Los Angeles. cis-Aconitic acid was synthesized from trans-aconitic acid according to the method of Deutsch and Phillips (1957). All acids were checked for purity by thin-layer chromatography as described later.

(b) Analytical

Unless otherwise stated, aconitate determinations were made according to Saffran and Denstedt (1948). Thin-layer chromatography on silica gel, using chloroform–methanol–formic acid (90 : 16 : 8 by volume) with p-dimethylaminobenzaldehyde as detecting agent (Smith 1960), was used for qualitative checks of trans-aconitate occurrence. Citrate was determined by a pentabromoacetone method (Stern 1957), modified in that the last traces of excess KMnO4 were decolorized with 1.5% H2O2; interference by trans-aconitate was negligible. Blood ketones were measured as acetone according to Bakker and White (1957). Polyethylene glycol was determined by the turbidimetric method of Hyden (1956), taking the maximum reading after the addition of trichloroacetic acid reagent. Total volatile fatty acids were titrated after distillation in a Markham still (McClymont 1951).

Following intravenous injection with [1,5-14C]trans-aconitate, blood and urinary organic acids were purified by partition on silica gel (Swim and Utter 1957), then applied in bands to 2-mm silica gel thin-layer chromatography plates and developed as described previously. After detection with a light spraying of p-dimethylaminobenzaldehyde reagent, the zones were scraped into scintillation fluid consisting of ethanol, dioxan, toluene, water (47 : 77 : 12 by volume) containing 80 g/l naphthalene and 5 g/l 2,5-diphenyloxazole, and counted in a scintillation spectrometer.

Calcium and magnesium were determined in urine collected into 1·0N HCl, and in blood plasma prepared and deproteinized as described by Wootton (1964). The determinations were carried out by atomic absorption spectroscopy methods (David 1960), modified in that ammonium
chloride was present in both sample and standard solutions. Strontium chloride (1500 p.p.m. Sr) in both sample and standard was used to suppress interferences in the determination of calcium.

(c) In vitro Fermentation

Samples of rumen fluid were obtained through rumen fistulae from three sheep, filtered through four layers of surgical gauze, and used either immediately or after periods of up to 2 hr at 38°C bubbled with carbon dioxide. Gas exchange accompanying organic acid fermentation was measured manometrically as described by McBee (1953) with 0.2 ml (20 μmoles) substrate and 0.8 ml rumen fluid. Flasks and manometers were flushed with carbon dioxide. The correction factor to overcome variation in flask volume was (gas volume of flask + manometer)/(gas volume of smallest flask + manometer). In vitro metabolism was further studied with rumen material from the same three rumen-fistulated sheep, which were fed 500 g lucerne pellets, pellets plus 0.2 mole citrate, or pellets plus 0.2 mole trans-aconitate respectively daily for 3 days. Incubations were made at 38–40°C in test tubes containing 0.5 ml (100 μmoles) substrate and 0.5 ml rumen fluid, bubbled with water-saturated carbon dioxide. Aconitate was determined spectrophotometrically, essentially as described by Racker (1950), on samples deproteinized with 0.1N sulphuric acid and centrifuged at 10,000 g for 30 min. Further measurements of aconitate disappearance during the course of in vitro fermentation were obtained, this time incubating the samples in McCartney bottles which were flushed with carbon dioxide, stoppered, and shaken at 38–40°C; aconitate was determined by the method of Saffran and Denstedt (1948).

(d) Intra-ruminal Administration of trans-Aconitate and Citrate

Approximately 500 ml rumen fluid was withdrawn, mixed with 2.5 g polyethylene glycol, 0.1 mole citrate or trans-aconitate (1.0M, one-third neutralized with NaOH), and returned to the sheep through a fistula. The experiment was done twice using only two sheep; thus each sheep received both trans-aconitate and citrate administration. Samples for volatile fatty acid, polyethylene glycol, and organic acid determinations were taken by withdrawing approximately 400 ml fluid by rumen pump, mixing, and sampling. Unused fluid was returned to the rumen. The sheep were fed lucerne pellets, both during the experiment and for several months beforehand.

(e) Dietary Administration of trans-Aconitate and Citrate

This experiment employed 10 sheep. Dietary additions were made by applying aqueous solutions of trans-aconitic acid or citric acid (one-third neutralized with NaOH) to lucerne pellets which were then dried at 70°C. The trans-aconitate additions, each administered for 5 days to two sheep, were 0.1 mole/day (approximately 3.5% trans-aconitate on a dry weight basis) and 0.2 mole/day (7.0% trans-aconitate). Another four sheep received similar treatments, except that citrate was substituted for trans-aconitate addition. Control analyses (Table 1) refer to analyses from the remaining two sheep which were each fed the same quantity of untreated pellets as the above sheep. Urine was collected with the aid of metabolism cages for one replicate of this experiment and then, to avoid food debris, into containers strapped to the sheep for the other replicate. Urinary aconitate values in Table 1 were obtained using the latter collection method. Urine was collected into 100 ml 1.0N HCl or into 20 ml 8% (w/v) thymol in isopropanol. Blood samples were taken by syringe from the external jugular vein and sodium heparin used throughout as an anticoagulant.

(f) Intravenous Administration of Citrate and cis- and trans-Aconitate

Intravenous injections were made with acids in 1.0M aqueous solution adjusted to pH 7.4 with NaOH. Injections were made over a 10-min period into an external jugular vein and post-injection blood samples withdrawn through a polyethylene catheter in the corresponding vein on the opposite side. trans-Aconitate at 1.0 m-mole/kg was administered to five sheep, cis-aconitate and citrate to one sheep each. A lower level of citrate, 0.3 m-mole/kg, was administered over 20-min periods to two sheep.
III. Results

(a) In vitro Utilization

The manometric readings in Figure 1(a) represent not only the carbon dioxide released from substrate and buffer, but also the production of other gases, chiefly methane. Glucose and citrate showed rapid rates of fermentation by rumen microorganisms in vitro, which were not depressed by trans-aconitate [Fig. 1(a)]. Under these conditions cis- and trans-aconitate were fermented slowly. Citrate fermentation did not show a strongly developed initial lag phase, of the type observed by Clarke and Meadow (1959) to accompany citrate fermentation by Pseudomonas aeruginosa and attributed to adaptive permease formation.

![Figure 1](image)

Fig. 1.—(a) Effect of trans-aconitate on the fermentation of soluble substrates. Gas volume expressed in terms of change in manometric height, ∆H. 0.8 ml rumen fluid incubated with 0.2 ml (20 μmoles) each of cis-aconitate (△), trans-aconitate (▲), citrate (●), and citrate + trans-aconitate (○). × No substrate. Results for similar experiments with glucose (□) and glucose + trans-aconitate (■) are also shown. (b) In vitro fermentation of citrate (●) and trans-aconitate (▲) by rumen fluid obtained from sheep fed diets supplemented with 3.5% citrate or 3.5% trans-aconitate for 3 days. 9.5 ml rumen fluid incubated with 0.5 ml (100 μmoles) substrate.

Mean values plus standard error of means from six determinations are given.

Citrate, but not cis- or trans-aconitate, was substantially broken down during in vitro incubation with rumen fluid for 1 hr [Fig. 1(b)]. There appeared to be considerable variation in the rate of citrate breakdown by rumen fluid obtained from different sheep. In this experiment, disappearance rates were also measured at daily intervals over a 3-day period when sheep were fed organic acid supplements at 0.2 mole per day. The rate of trans-aconitate fermentation remained at the low level shown in Figure 1(b). However, the citrate breakdown on successive days, under similar conditions to those specified for Figure 1(b), was 44, 39, 56, and 66 μmoles per hour. A slow change of this type could be caused by altered microbial populations in the rumen, or adaption by existing types.

(b) Intra-ruminal Administration of trans-Aconitate and Citrate

Both citrate and trans-aconitate disappear rapidly from the rumen (Fig. 2). Citrate, as has been previously reported by Packett and Fordham (1965), increased the volatile fatty acid content of the rumen; trans-aconitate did not (Fig. 2). Follow-
ing administration of 0·1 mole of trans-aconitate into the rumen, the level of blood aconitate and citrate increased slightly from base values of 9 and 19 µg/ml respectively to achieve maximum values of 23 and 27 µg/ml 1 hr after administration.

Fig. 2.—Comparison between curves for the disappearance from the rumen of added citrate (●) and trans-aconitate (△) with that of polyethylene glycol (○) in vivo with rumen-fistulated sheep. Rumen volatile fatty acids (×) were also measured. Each point is the mean of values from two sheep.

(c) Dietary Administration of trans-Aconitate and Citrate

Sheep fed trans-aconitate appeared normal and had blood citrate and ketone values approximating those of control sheep (Table 1). However, urinary citrate

<table>
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<td>Aconitate (µg/ml)</td>
<td>Ketone (µg/ml)</td>
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<td>7</td>
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</tr>
<tr>
<td>0·1</td>
<td>24</td>
<td>15</td>
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<td>0·2</td>
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<td>0·2</td>
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was greatly increased by the administration of trans-aconitate (Table 1). Subsequent trials with sheep fed 0·1 mole trans-aconitate per day have shown that within 24 hr the urinary citrate reached high values (approximated by those in Table 1) which
were maintained over the duration of the feeding trials. On the other hand, concentrations of aconitate in the urine were low (Table 1) and the urinary recovery was calculated as less than 1%. There was no marked influence on either plasma or urinary calcium and magnesium values (Table 1).

(d) Intravenous Injection

No abnormal behaviour was observed in the five sheep injected intravenously with trans-aconitate at 1·0 m-mole/kg body weight. These injections caused varying increases in the blood citrate level [Fig. 3(a)]. The blood volume was not measured but, if it is assumed to be approximately 3 litres (from the data of Panaretto 1964), then only 9% of the injected trans-aconitate can be accounted for in the blood volume 20 min after the commencement of injection. The level of trans-aconitate was observed to fall rapidly [Fig. 3(a)] and there appeared to be rapid renal clearance into the urine (Fig. 4). The blood and urinary citrate was not a metabolite of

![Graph](image)

Fig. 3.—(a) Effect of intravenous trans-aconitate injection on blood citrate (●), trans-aconitate (▲), plasma magnesium (○), and plasma calcium (●) levels. Each point is the mean of values from three sheep. (b) Blood citrate levels following citrate (●), cis-aconitate (△), and saline (●) injections. Plasma magnesium (■) and calcium (●) levels following citrate injection are also shown.

![Graph](image)

Fig. 4.—Effect of intravenous trans-aconitate injection on the urinary concentration of citrate (●), trans-aconitate (△), magnesium (○), and calcium (●) levels. Sheep 1 and 2 were fed lucerne pellets, sheep 3 poor quality Phalaris straw.

[1,5-14C]trans-aconitate (Table 2). Approximately 40% of the injected trans-aconitate was recovered in the urine of the five sheep over a period of 24 hr.
Whereas the injection of 1·0 m-mole/kg of trans-aconitate did not result in any abnormal behaviour, injection of citrate at the same level killed one animal within 7 min. Injections of citrate at 0·3 m-mole/kg over a 20-min period appeared to be close to the toxic dose and caused high urinary citrate and calcium excretion without marked change in urinary magnesium excretion (Table 3). A similar but less pronounced rise in calcium content was apparent in the urine of sheep injected with trans-aconitate (Fig. 4); plasma magnesium and calcium levels remained approximately constant following injection [Fig. 3(a)]. From stability constants (Sillen and Martell 1964) it can be calculated that at pH 7·4 citrate has a much higher binding power for Ca$^{2+}$ than for Mg$^{2+}$ and Mn$^{2+}$ ions. Following citrate injection, plasma calcium levels were not greatly modified [Fig. 3(b)].
IV. Discussion

Sheep fed trans-aconitate supplements appeared normal and had blood citrate values within the normal range. The slow rates of trans-aconitate metabolism observed in vitro, coupled with rapid trans-aconitate disappearance from the rumen in vivo, suggest rapid trans-aconitate absorption through the rumen epithelium. Subsequently, blood and urinary aconitate values remained low, further indicating that trans-aconitate was utilized mainly within sheep tissues. The unexpectedly low urine recoveries of trans-aconitate observed in these experiments suggest a need to study the probable metabolism of trans-aconitate by animal tissues, where appropriate enzymes attacking this compound have not so far been found.

The sheep responded to dietary trans-aconitate by increased urinary citrate excretion. The citrate excreted following injection of [1,5-14C]trans-aconitate was not a radiometabolite of the latter (Table 2) and is therefore suggested to have been formed by aconitate hydratase inhibition, probably in tissue not metabolizing trans-aconitate. It should be noted, however, that Pennington and Sutherland (1955) have shown that the respiratory oxygen uptake by rumen epithelium was stimulated by cis-aconitate and the other Krebs cycle acids at a concentration (10 μg/ml) which permitted trans-aconitate to exert powerful inhibition. Failure of trans-aconitate, in present circumstances, to cause a lethal metabolic block can be partly attributed to weak aconitase inhibition (Thomson et al. 1966), rapid urinary clearance (Fig. 4), or perhaps failure to reach mitochondrial aconitase hydratase.

It is difficult to account for the way dietary trans-aconitate increased urinary citrate excretion (Table 1). The low citrate levels in the blood (Table 1) contrast with the high levels observed in sheep poisoned with fluoroacetate (Jarrett and Packham 1956). Data are not available concerning the urinary citrate excretion of fluoroacetate-poisoned sheep. It is known, however, that urinary citrate remains unchanged despite greatly increased tissue and mitochondrial citrate in fluoracetate-poisoned rats (Crawford 1963). Conversely, intravenously injected analogues of Krebs cycle acids have caused approximately 25-fold increases in the urinary citrate of the dog (Orten and Smith 1937), without increasing blood, liver, or muscle citrate (Orten and Smith 1939). Thus, in the present instance, trans-aconitate may have induced a renal, rather than a whole body response, resulting in rapid citrate elimination in the urine. The mechanisms controlling renal citrate disposal are ill defined but acid-base balance, active reabsorption, or other causes could be invoked. The kidney is also particularly active in citrate synthesis (Orten and Smith 1939) and utilization (Herndon and Freeman 1958). Possibly trans-aconitate may preferentially accumulate in renal tubular cells and cause enhanced aconitase inhibition there. Citrate levels are normally extremely high in kidney tissue following fluorooacetate poisoning (Peters 1957).

In the present experiments added citrate disappeared from the rumen fluid in vitro (Figs. 1 and 2). Citrate may also have passed through the rumen epithelium but, if this were so, the low values obtained for blood and urinary citrate indicate rapid citrate utilization in the body, perhaps via the citrate cleavage enzyme recently thought to be associated with lipogenesis (Ballard and Hanson 1967). The toxicity of citrate injected intravenously has been long known (Salan and Wise 1916) and
can be abolished by calcium salt injections (Krebs, Savlin, and Johnson 1937),
suggesting that citrate binds calcium ions, causing hyperirritability and, ultimately,
heart block. The present data indicate that citrate injection causes rapid calcium
excretion without raising urinary magnesium levels. On the other hand, comparatively
high doses of intravenously injected trans-aconitate modify plasma calcium and
magnesium values only slightly [Fig. 3(a)]; nor do these high doses induce hypo-
magnesaemia but, like citrate injections, increase the urinary calcium level without
increasing that of magnesium (Fig. 4; Table 3).

Tolerance of the sheep towards trans-aconitate was so great that, despite
the short duration of these experiments, it is highly unlikely that trans-aconitate, by
itself, could be a major factor inducing hypomagnesaemia or lethal metabolic
inhibition in sheep.

V. Acknowledgments

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VI. References

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