TRANS-ACONITATE UTILIZATION BY SHEEP

By G. S. KENNEDY*

[*Manuscript received September* 29, 1967]

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-^{14}C]*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)

* Division of Plant Industry, CSIRO, Canberra; present address: Department of Botany and Plant Pathology, Natural Science Building, Michigan State University, East Lansing, Michigan 48823.

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-Aconitic 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-^{14}C]*trans*-Aconitic acid was similarly prepared from [1,5-^{14}C]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 $KMnO_4$ were decolorized with 1·5% H_2O_2; 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-^{14}C]*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 : 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 9·5 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 micro-organisms *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.

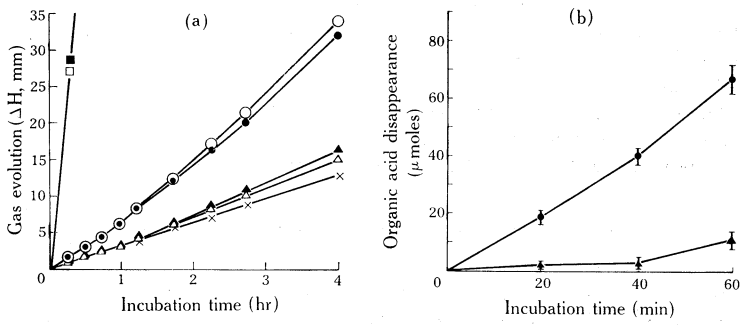

Fig. 1.—(*a*) Effect of *trans*-aconitate on the fermentation of soluble substrates. Gas volume expressed in terms of change in manometric height, ΔH. 0·8 ml rumen fluid incubated with 0·2 ml (20 μmoles) each of *cis*-aconitate (△), *trans*-aconitate (▲), citrate (●), and citrate+*trans*-aconitate (○). × 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.

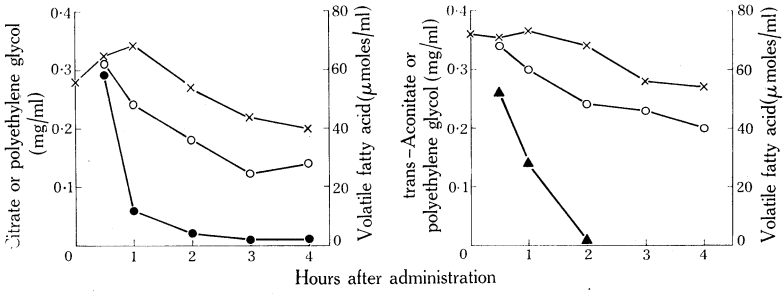

Fig. 2.—Comparison between curves for the disappearance from the rumen of added citrate (●) and *trans*-aconitate (▲) with that of polyethylene glycol (○) *in vivo* with rumen-fistulated sheep. Rumen volatile fatty acids (×) were also measured. Each point is the mean of values from two sheep.

(*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 1

EFFECT OF FEEDING CITRATE OR *trans*-ACONITATE SUPPLEMENTS TO SHEEP FOR 5 DAYS ON LEVELS OF BLOOD AND URINE COMPONENTS

Blood samples taken 1 hr after feeding. Aliquots of 24-hr sample of urine used for analysis. Urine collection commenced on the fifth day after feeding. Mean values are given for the two control sheep and for the four citrate-fed sheep. Separate values are given for each of the two *trans*-aconitate-fed sheep for blood samples and for the urine collected by the two different methods (see text). However, aconitate data for urine collected from metabolism cages are omitted

Daily Dietary Addition (mole)	Whole Blood			Plasma		Urine			
	Citrate (μg/ml)	Aconitate (μg/ml)	Ketone (μg/ml)	Mg (p.p.m.)	Ca (p.p.m.)	Citrate (μg/ml)	Aconitate (μg/ml)	Mg (p.p.m.)	Ca (p.p.m.)
None (control)	19	7	18	17	111	48	103	190	19
trans-aconitate									
0·1	22	12	9	16	118	520		260	9
0·1	24	15	12	17	110	420	180	254	13
0·2	27	17	21	17	113	660		310	15
0·2	24	7	22	18	105	805	240	172	13
Citrate									
0·1, 0·2	17	4	14	19	115	42	61	230	9

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

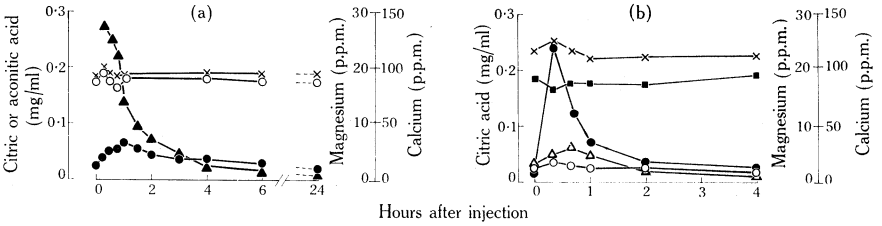

Fig. 3.—(*a*) Effect of intravenous *trans*-aconitate injection on blood citrate (●), *trans*-aconitate (▲), plasma magnesium (○), and plasma calcium (×) levels. Each point is the mean of values from three sheep. (*b*) Blood citrate levels following citrate (●), *cis*-aconitate (△), and saline (●) injections.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

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

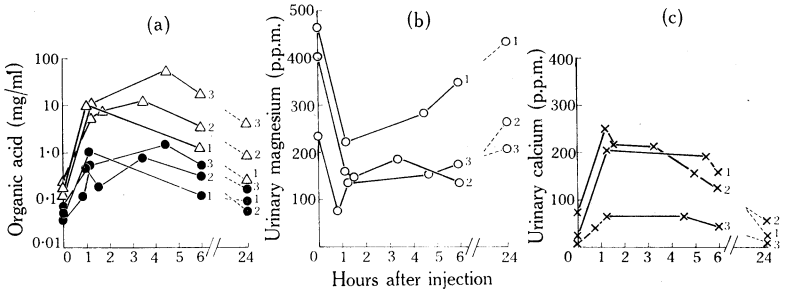

Fig. 4.—Effect of intravenous *trans*-aconitate injection on the urinary concentration of citrate (●), *trans*-aconitate (△), magnesium (○), and calcium (×) levels. Sheep 1 and 2 were fed lucerne pellets, sheep 3 poor quality *Phalaris* straw.

[1,5-^{14}C]*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

TABLE 2

RADIOACTIVITY OF *trans*-ACONITATE AND CITRATE IN BLOOD AND URINE FOLLOWING INTRAVENOUS INJECTION OF [1,5-^{14}C]*trans*-ACONITATE TO SHEEP 2

Specific activities, expressed as disintegrations per minute per 100 μg, given in parenthesis

Time after Injection (hr)	*trans*-Aconitate in Blood (μg/ml)	*trans*-Aconitate in Urine (μg/ml)	Citrate in Blood (μg/ml)	Citrate in Urine (μg/ml)
0·7	206(4,031)		85(0)	
1·0	95(3,537)		56(31)	
3·5		12,800(3,621)		794(28)
6·0		3,400(3,745)		305(41)

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

TABLE 3

URINE COMPOSITION FOLLOWING SODIUM CITRATE INJECTION

1·0M sodium citrate, pH 7·4, was injected into two sheep at the rate 0·3 m-mole/kg body weight

Time from Injection to Sampling	Volume (ml)	Citrate (μg/ml)	Ca (p.p.m.)	Mg (p.p.m.)
Sheep 1				
Preceding 24 hr	1016	38	19	235
0·25–0·5 hr	180	2800	216	128
0·5–3·0 hr	50	2400	70	105
Sheep 2				
Preceding 24 hr	1160	43	32	235
0·3–1·2 hr	60	1500	80	121
1·2–2·2 hr	100	814	67	108
23–25 hr	90	30	30	95

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-^{14}C]*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 aconitate 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 fluoroacetate 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 hypomagnesaemia 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

Atomic absorption determinations of calcium and magnesium were very kindly performed by Dr. D. J. David. Acknowledgment is made of the advice of Dr. M. Freer, Mr. K. R. Christian, and Professor C. H. Gallagher. Messrs. M. D. Holgate and T. Shepherd gave skilled technical assistance.

VI. References

Altekar, W. W., and Rao, M. R. R. (1963).—*J. Bact.* **85**, 604–13.
Bakker, A., and White, R. R. (1957).—*N.Z. Jl Sci. Technol.* B **38**, 1001–8.
Ballard, F. J., and Hanson, R. W. (1967).—*J. Lipid Res.* **8**, 73–9.
Bruce, W. F. (1961).—"Organic Syntheses." Coll. Vol. 2. p. 12. (John Wiley & Sons: New York.)
Burau, R., and Stout, P. R. (1965).—*Science, N.Y.* **150**, 766–7.
Clarke, P. H., and Meadow, P. M. (1959).—*J. gen. Microbiol.* **20**, 144–55.
Crawford, M. A. (1963).—*Biochem. J.* **88**, 115–20.
David, D. J. (1960).—*Analyst, Lond.* **85**, 495–503.
Deutsch, D., and Phillips, R. E. (1957).—"Methods in Enzymology." Vol. 3. pp. 424–5. (Academic Press Inc.: New York.)
Gallagher, C. H., Koch, J. H., and Hoffman, H. (1966).—*Aust. Vet. J.* **42**, 279–86.
Herndon, R. F., and Freeman, S. (1958).—*Am. J. Physiol.* **192**, 369–72.
Hyden, S. (1956).—*K. LantbrHögsk. Annal.* **22**, 139.
Jarrett, I. G., and Packham, A. (1956).—*Nature, Lond.* **177**, 580–1.
Krebs, H. A., Savlin, E., and Johnson, W. A. (1937).—*Biochem. J.* **32**, 113–17.
MacLennan, D. H., and Beevers, H. (1964).—*Phytochemistry* **3**, 109–13.
McBee, R. H. (1953).—*Appl. Microbiol.* **1**, 106–10.
McClymont, G. L. (1951).—*Aust. J. agric. Res.* **2**, 158–80.
Orten, J. M., and Smith, A. H. (1937).—*J. biol. Chem.* **117**, 555–67.
Orten, J. M., and Smith, A. H. (1939).—*J. biol. Chem.* **128**, 101–7.
Packett, L. V., and Fordham, J. R. (1965).—*J. anim. Sci.* **24**, 488–93.
Panaretto, B. A. (1964).—*Aust. J. agric. Res.* **15**, 771–9.
Pennington, R. J., and Sutherland, T. M. (1956).—*Biochem. J.* **63**, 353–61.
Peters, R. A. (1957).—*Adv. Enzymol.* **18**, 113–59.
Pisano, M. A., Blahuta, G. J., and Mullen, G. A. (1959).—*J. Bact.* **78**, 146.
Racker, E. (1950).—*Biochem. biophys. Acta* **4**, 211–14.
Saffran, M., and Denstedt, D. F. (1948).—*J. biol. Chem.* **175**, 849–55.
Saffran, M., and Prado, J. L. (1949).—*J. biol. Chem.* **180**, 1301–9.
Salan, E., and Wise, L. E. (1916).—*J. biol. Chem.* **28**, 27.

SILLEN, L. G., and MARTELL, A. E. (1964).—"Stability Constants of Metal Ion Complexes." pp. 474, 477–81. (Spec. Publ. No. 17, The Chemical Society, London.)

SMITH, I. (1960).—"Chromatographic and Electrophoretic Techniques." Vol. 1. p. 272. (Heineman: London.)

STERN, J. R. (1957).—"Methods in Enzymology." Vol. 3. pp. 426–8. (Academic Press, Inc.: New York.)

STOUT, P. R., BROWNELL, J., and BURAU, R. G. (1967).—*Agron. J.* **59**, 21–4.

SWIM, H. E., and UTTER, M. F. (1957).—"Methods in Enzymology." Vol. 4. pp. 586–9. (Academic Press, Inc.: New York.)

THOMPSON, J. F., NANCE, S. L., BUSH, K. J., and SZCZEPANIC, P. A. (1966).—*Arch. Biochem. Biophys.* **117**, 65–74.

WOOTTON, I. D. P. (1964).—"Microanalysis in Medical Biochemistry." pp. 50, 74–5. (J. & A. Churchill, Ltd.: London.)