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Studies on the Alimentary Tract of the Merino Sheep in South Africa. XVI.—The Fate of Nitrate in Ruminal Ingesta as Studied *in Vitro*.

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Introduction.

Potassium nitrate has been found to be toxic to sheep, producing the effects of nitrite poisoning. This was clearly shown in investigations on tribulosis (geeldikkop) when both the juice of expressed *Tribulus*, which contains a large amount of potassium nitrate, and potassium nitrate itself were dosed to sheep (Quin, 1930; Rimington and Quin, 1933). *In vitro* experiments have shown that potassium nitrate, when added to the ruminal ingesta of sheep, disappears with the production of nitrite under certain conditions (Sapiro, in press). In such experiments the nitrite appeared at a stage when most of the nitrate had disappeared and only when ruminal ingesta from poorly fed sheep was used. It seems logical to conclude that nitrite represents an intermediate stage in the full reduction of nitrate to products of use to the organisms in their metabolism, e.g., ammonia, which has been shown to be formed from urea (Pearson and Smith, 1943).

Apart, however, from its toxicological significance, the conversion of nitrate by ruminal ingesta may be of interest from a nutritional aspect. If potassium nitrate were supplied to sheep in such a manner as to produce fully reduced compounds, without the intermediate formation of an appreciable concentration of nitrite, it might supplement the nitrogen requirement in an otherwise poor diet. In this way nitrate would fulfil the same purpose as urea and other non-protein nitrogenous compounds in supplementary feeding.

In the present work the rate of disappearance of potassium nitrate in ruminal ingesta *in vitro* has been studied, using ingesta from sheep with ruminal fistulae and on different diets. The effect of the presence of varying quantities of glucose on the rate of disappearance of the nitrate has been determined and conditions for the production and persistence of nitrite have been investigated. Furthermore, these results have been partially confirmed by an *in vivo* experiment on sheep.

Technique.

A similar method was used in this study to that applied previously (Sapiro, in press). Tubes, each containing 20 c.c. of ingesta, were maintained at 39° C. in a constant temperature bath; each tube was provided

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with a fine dropper for taking samples. The potassium nitrate was added to the tubes in the form of a 0·1 or a 0·5 per cent. solution, either alone or in conjunction with a measured quantity of glucose solution. The contents were mixed and drops taken from the tubes at regular intervals of time and tested for nitrate with diphenylamine reagent (Feigl, 1935), or for nitrite with sulphanilic acid and naphthylamine reagent (Feigl, 1935).

Experimental.

I. *Conditions for the Production of Nitrite.*

In the experiments mentioned above (Sapiro, in press) comparatively small quantities of nitrate were used. Subsequent investigation, however, has shown that, with larger quantities of nitrate, nitrite is produced when ingesta of either grass hay fed sheep or lucerne fed sheep is used. Furthermore, in such cases nitrite is found to be present very soon after the addition of the nitrate to the ingesta and to persist for some time after the disappearance of nitrate. The following differences were noticeable in the results between the ingesta of well-fed (lucerne) and poorly-fed (grass hay) sheep:—

(i) The ingesta of well-fed sheep accounted for the disappearance of much larger quantities of nitrate.

(ii) In ingesta from well-fed sheep the nitrite produced disappeared again more rapidly than from ingesta of poorly-fed sheep.

In all cases, however, the concentration of nitrite, as judged by the colour produced in the test, was at a maximum at the time of complete disappearance of nitrate.

Typical results are illustrated in Table 1, where ruminal ingesta from sheep 16, a sheep fed on lucerne hay and sheep 14, on a diet of poor quality grass hay, are compared.

Table 1.

Sheep No.	Diet.	Potassium Nitrate added, mg. per 20 c.c. ingesta.	Time in Minutes.				Ratio, Nitrite disappearance time/nitrate disappearance time.
			Nitrate Dis-appearance.	Nitrite Appear-ance.	Nitrite Maximum.	Nitrite Dis-appearance.	
16	Lucerne.......	2·5	14	4	14–16	20	1·43
		5·0	(*a*) 30	4	28–38	48	1·60
		7·5	(*b*) 48	4	38–52	68	1·42
		10·0	80	4	80	115	1·44
		15·0	125	4	130	168	1·34
						Average.	1·45
14	Grass Hay.....	0·4	8	2	12	20	2·50
		0·7	16	2	14–16	34	2·13
		1·0	(*c*) 28	2	26	56	2·00
		1·4	(*d*) 46	2	50	90	1·96
		1·7	65	2	55–65	135	2·08
						Average.	2·13

For each sheep the ratio *nitrite disappearance time/nitrate disappearance time* is found to be constant for different quantities of nitrate added. This ratio will henceforth be referred to as *Constant A*.

The differences, mentioned above, between the ingesta from sheep on different diets are clearly shown by considering the following points in Table 1:—

(i) In 30 minutes (reading *a*), 20 c.c. of ingesta from the lucerne-fed sheep accounted for 5 mg. of potassium nitrate; whereas in approximately the same time, viz. 28 minutes (reading *c*) only 1 mg. of nitrate was reduced by 20 c.c. of ingesta from the grass hay fed sheep. Similarly, readings *b* and *d* indicate a disappearance of 7·5 and 1·4 mg. of nitrate respectively in approximately the same time from these two ingesta. Calculating the time of disappearance of 1 mg. of nitrate, it is found that the average value of this period, over the whole set of readings, is 6·8 minutes for the lucerne sheep and 28·4 minutes for the grass hay sheep.

(ii) The values of *Constant A* show that, in the case of the lucerne fed sheep, the time of disappearance of nitrite was less than one and a half times that of nitrate; whereas, in the case of the poorly-fed sheep, nitrite disappearance time was more than twice that of nitrate. Statistical analysis of these ratios, giving a value of t (difference/standard deviation), indicates a highly significant difference, viz. $t = 6 \cdot 47$.

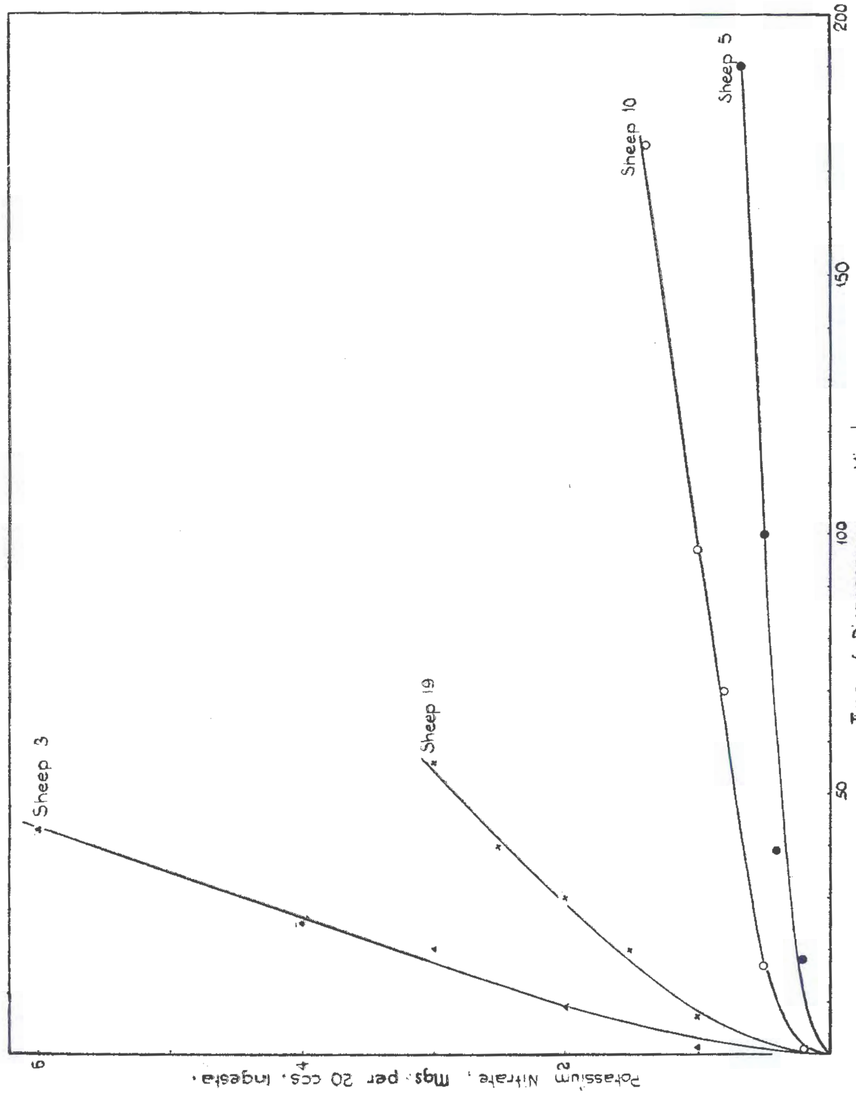
TABLE 2.

Sheep No.	Diet.	Date.	Potassium Nitrate added, mg. per 20 c.c. Ingesta.	Time of Nitrate Disappearance. (Minutes).
10	Grass hay..........................	6/6/47	0·2	<1
			0·5	17
			0·8	70
			1·0	97
			1·4	175
19	Lucerne hay.........................	11/6/47	1·0	7
			1·5	20
			2·0	30
			2·5	40
			3·0	56
5	Grass hay..........................	12/5/47	0·2	18
			0·4	39
			0·5	100
			0·7	190
3	Lucerne hay.........................	19/5/47	1·0	<1
			2·0	9
			3·0	20
			4·0	25
			6·0	43

II. *Graphical Presentation of the Disappearance of Nitrate.*

It was found that a graphical representation of quantity of nitrate reduced/time, took the form of a half parabola. In Table 2 typical values are given for the disappearance of nitrate from the ingesta of four different sheep, while Figure 1 illustrates the curves drawn from these values.

FIGURE 1.



Daily Variation of Rate of Disappearance of Nitrate.

Construction of curves for the nitrate disappearance rate in ingesta from the same sheep showed a daily variation even though the diet remained constant. This variation can be represented graphically: The quantities of nitrate reduced by the ingesta in any arbitrary period of time (45 minutes is a suitable value) are read from the curves of *nitrate disappearance/time;* these values are then plotted against the dates on which the readings were taken. Figure 2 illustrates the variations in Sheep 3, on a lucerne hay diet and Sheep 6, on a diet of grass hay, over a period of ten days.

It is noticeable from the figure that the two sheep, on different diets show a similar daily variation. This phenomenon has been noticed also in the measurement of cellulose digestion and of fermentation of glucose by ruminal ingesta. No satisfactory explanation of these observations can as yet be advanced.

Nitrate Disappearance as a Test for Ruminal Activity.

From comparative experiments it has repeatedly been shown that the variation of nitrate disappearance rate follows the same trend as the variation of cellulose digestion and of glucose fermentation in any particular sheep. Thus, apart from either its toxicological or nutritional aspects, the disappearance of nitrate from ruminal ingesta constitutes an additional test for ruminal activity.

III. *The Effect of Glucose on the Rate of Nitrate Disappearance.*

Comparatively small amounts of glucose added to ruminal ingesta were found to cause marked acceleration in the disappearance of nitrate. For the study of this effect two types of experiment were conducted: (*a*) where varying amounts of glucose were applied with a fixed quantity of nitrate, and (*b*) where the quantiy of nitrate was varied while using a fixed quantity of glucose.

(*a*) *Variation of the Quantity of Glucose.*

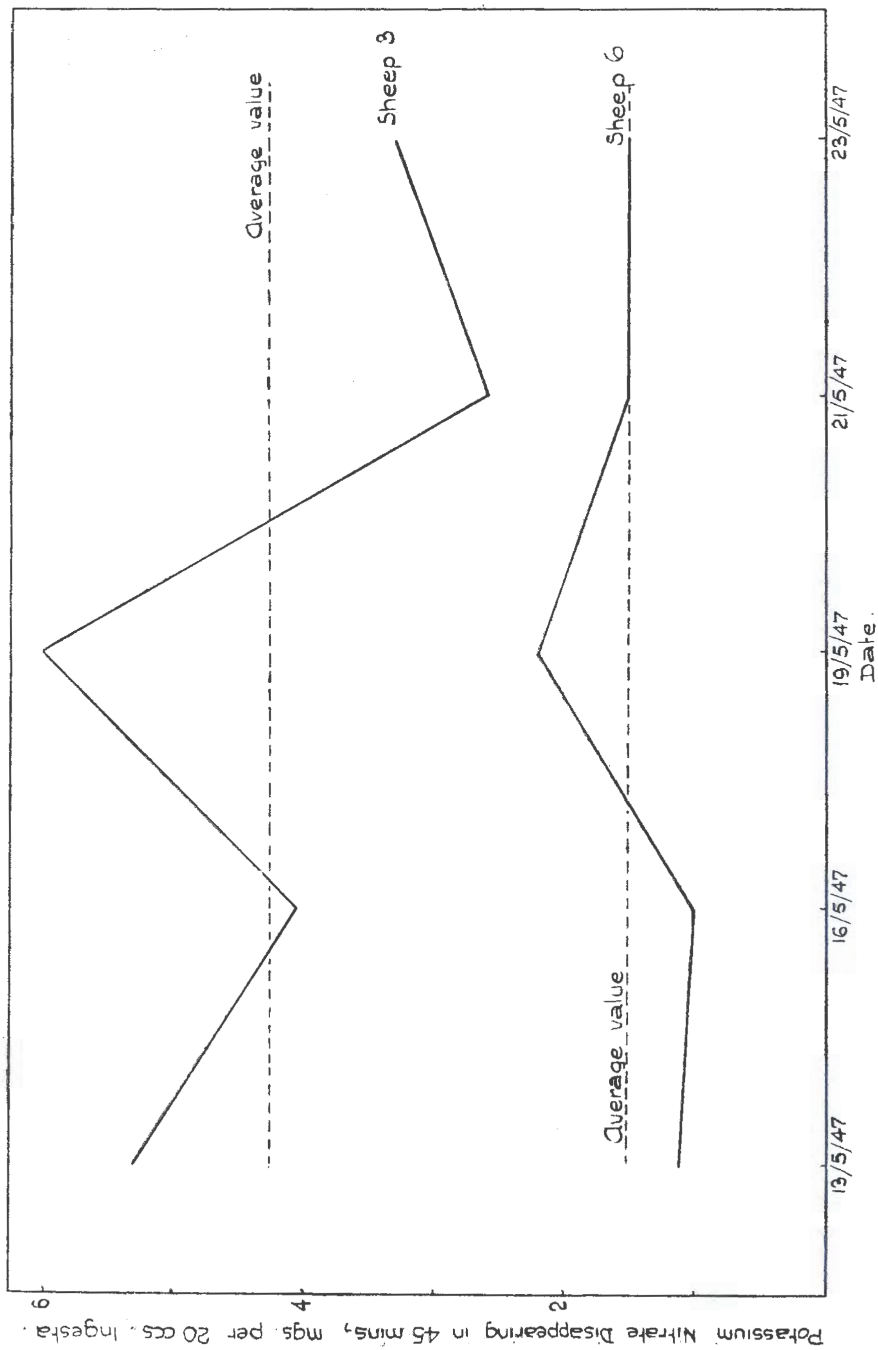
It was found that increasing quantities of glucose caused further reduction in the time of nitrate disappearance until a maximum effect was produced, after which the further addition of glucose had no effect. The maximal depression of time was normally brought about by as little as 15 to 30 mg. of glucose per 20 c.c. of ingesta. The rate of fermentation by ingesta is reduced on the addition of a comparatively large amount of glucose. It was found, however, that the depression of *nitrate disappearance time* was not further affected, after the maximal depression had been reached, by the addition of as much as 2 grams of glucose in 20 c.c. of ingesta.

Figures 3 and 4 show graphically the reduction of time of nitrate disappearance due to varying amounts of glucose. Values are given, in Figure 3, obtained with the ingesta of Sheep 15, on a diet of lucerne hay; each tube in the experiment contained 1·5 mg. of potassium nitrate in 20 c.c. of ingesta . Figure 4 represents readings obtained with ingesta from Sheep 10, fed on grass hay; each tube contained 0·6 mg. of potassium nitrate and 20 c.c. of ingesta.

(*b*) *Variation of the Quantity of Nitrate.*

A fixed quantity of glucose had the effect of altering the position of the curve of nitrate disappearance/time, by reducing the *time* values, without affecting the proportionate shape of the curve. The *percentage reduction of time of nitrate disappearance* was found to be constant for varying quantities of nitrate, and is hereafter refrred to as *Constant B.*

FIGURE 2.


Average value
Sheep 3
Sheep 6
Average value
6
4
2
13/5/47
16/5/47
19/5/47
21/5/47
23/5/47
Date.
Potassium Nitrate Disappearing in 45 mins, mgs. per 20 ccs. Ingesta.

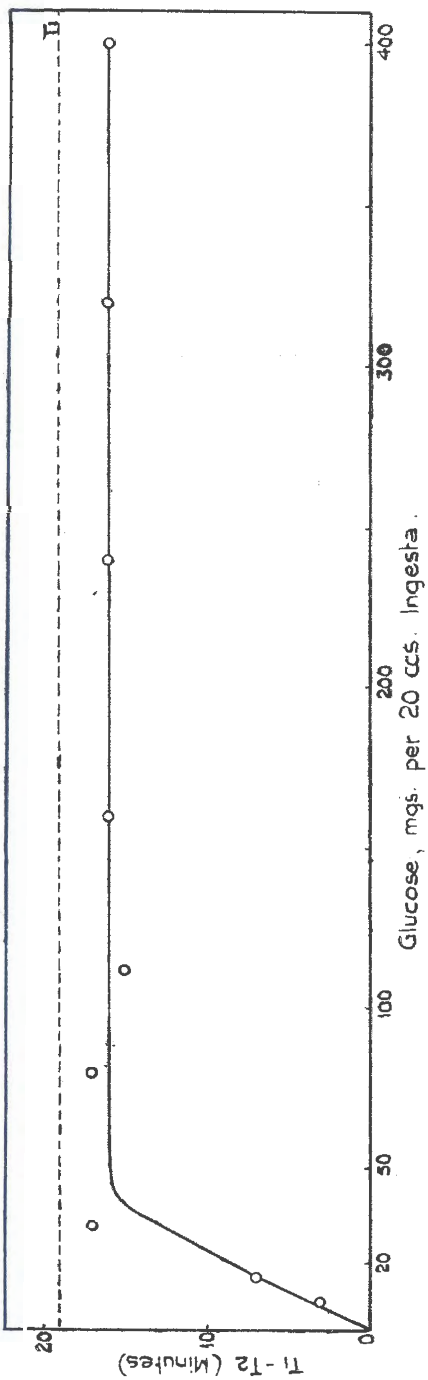


FIGURE 3.

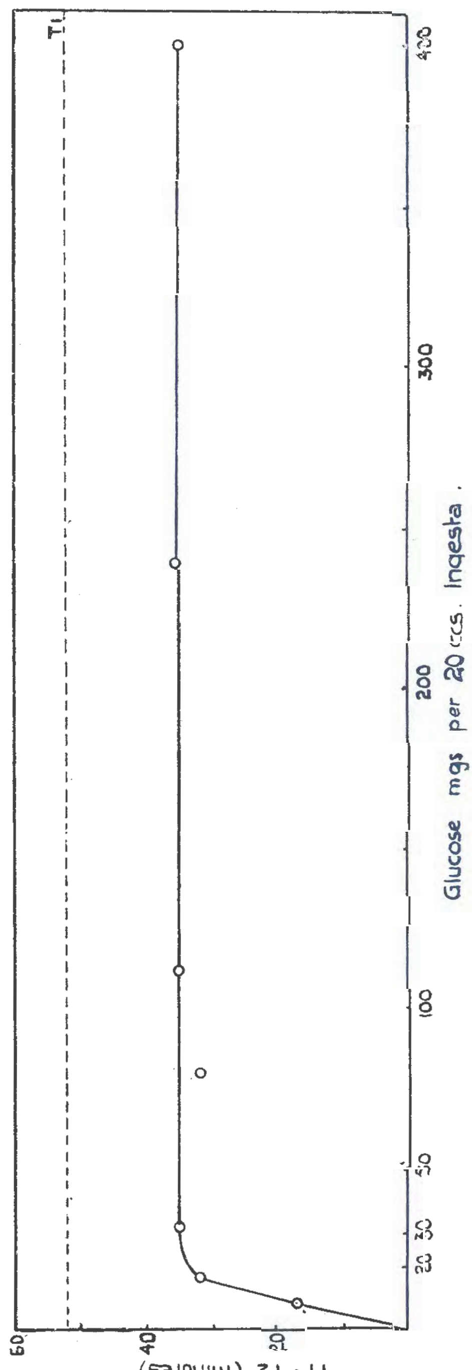


FIGURE 4.

It follows from this observation that the curves illustrated in Figures 3 and 4 would be unaltered in shape or relative proportions for any fixed quantity of nitrate.

Table 3 gives typical examples of the nitrate disappearance time both with the addition of a fixed amount of glucose and without it. The table shows how closely the values of *percentage reduction of time* approach constancy. Graphs 5 and 6 represent the data in experiments (*b*) and (*c*), respectively, of Table 3.

TABLE 3.

Experiment.	Sheep No.	Diet.	Glucose added, mg. per 20 c.c. Ingesta.	Potassium Nitrate added, mg. per 20 c.c. Ingesta.	Time in Minutes. Nitrate Disappearance, without Glucose.	Time in Minutes. Nitrate Disappearance, with Glucose.	Percentage Reduction of Nitrate Disappearance Time = Constant B.
a	19	Lucerne..........	16	1·0	13	7	46
				1·5	42	18	57
				2·0	70	(17)	(76)
				2·5	100	55	45
				3·0	130	75	42
						Average*	48
b	10	Grass hay.........	16	0·2	<1	<1	—
				0·5	17	10	41
				0·8	70	30	57
				1·0	97	45	54
				1·4	175	97	45
						Average.	49
c	19	Lucerne..........	80	1·0	7	(3)	(56)
				1·5	20	4	80
				2·0	30	6	80
				2·5	40	9	78
				3·0	56	12	79
						Average*	79
d	10	Grass hay.........	80	0·5	30	9	70
				0·7	62	14	77
				1·0	125	30	76
				1·2	—	65	—
				1·2	—	65	—
				1·4	—	80	—
						Average.	74

* Neglecting the bracketed figures, obviously due to poor readings.

Statistical analysis applied to *Constant B* for the different groups in the above table illustrates the following points:—

(i) The reaction is affected to a different extent by the two different quantities of glucose. For deducing this result an *f* value is

FIGURE 5.

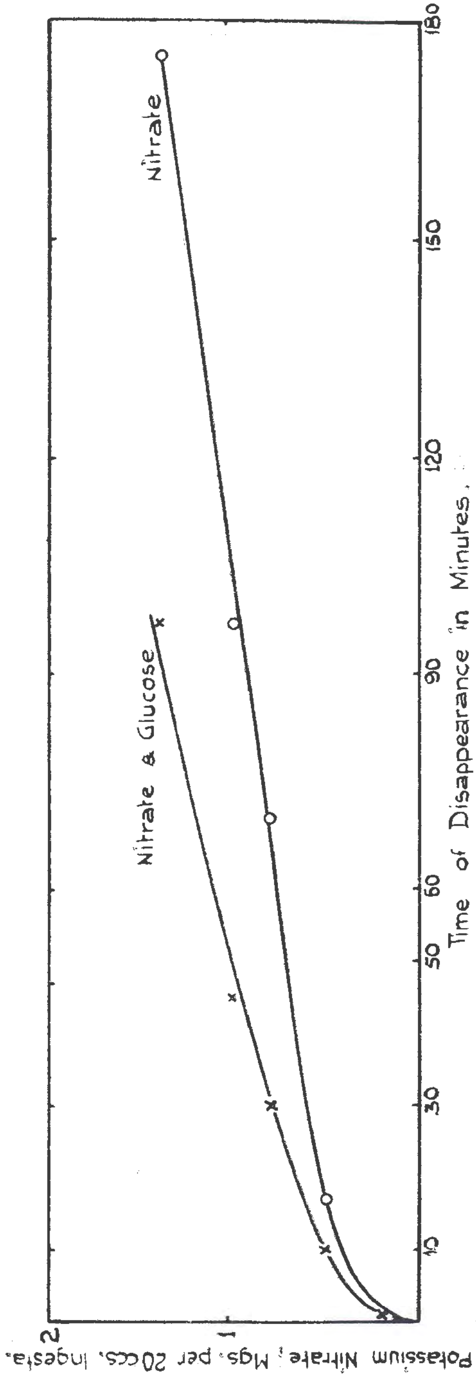
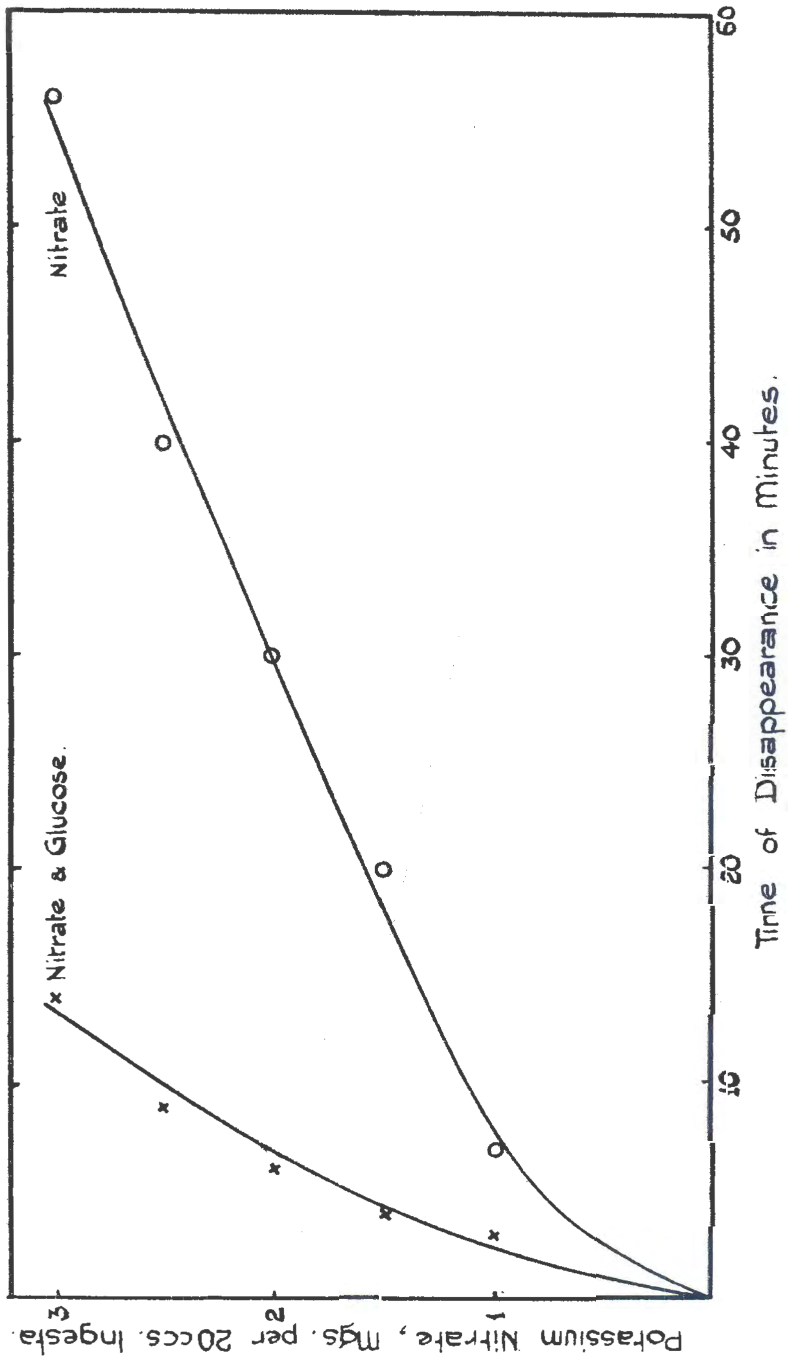

Nitrate
Nitrate & Glucose
Time of Disappearance in Minutes.
10
30
50
60
90
120
150
180
2
1
Potassium Nitrate, Mgs. per 20ccs. Ingesta.

FIGURE 6.


Nitrate
Nitrate & Glucose.
Time of Disappearance in Minutes.
10
20
30
40
50
60
1
2
3
Potassium Nitrate, Mgs. per 20ccs. Ingesta.

calculated, where f is the ratio of variances for two groups. A comparison of the group where 16 mg. of glucose was added (*a* and *b*) with the group in which 80 mg. of glucose was used (*c* and *d*) gives f 103·2, a highly significant value. This value indicates that at least one of the quantities of glucose (viz. 16 mg.) is below that necessary to give the maximum effect.

(ii) The total effect of glucose was similar for the lucerne-fed and hay-fed sheep. In this case the data for lucerne sheep (*a* and *c*) were compared with the data for sheep on grass hay (*b* and *d*) and an insignificant value of f, viz. 1·4, was obtained. It was noted, however, that within the group in which 80 mg. of glucose was added (*c* and *d*) a *t value* is obtained which shows a significant difference between the hay-fed and the lucerne-fed sheep, viz. $t = 2{\cdot}61$.

IV. *The Effect of Glucose on the Rate of Disappearance of the Nitrite Formed.*

It was found that the presence of glucose accelerated the disappearance of the nitrite formed from the nitrate in the same manner as it accelerated nitrate disappearance. The *percentage reduction of time (from zero) of nitrite disappearance* was constant for a fixed quantity of glucose; this value is referred to as *Constant C*.

Defining *nitrite persistence* as the interval of time between the disappearance of nitrate and of nitrite, it can be deduced mathematically, from the constancy of *A*, *B* and *C*, that the *percentage reduction of nitrite persistence* due to a fixed quantity of glucose is also constant for varying amounts of glucose. This constant is designated *Constant D*. Experimental results show the constancy of *D* within reasonable limits of error, and thus provide further evidence of the constancy of *A*, *B* and *C*.

Table 4 quotes experimental values to illustrate the above points.

Statistical analysis of these figures, by calculation of values of t for the corresponding constants of the two sheep, illustrates the marked difference in two of the constants, viz. *B* and *C*, between the lucerne-fed and the hay-fed sheep. The following values are obtained:—

Constant B (lucerne)/Constant B (grass): $t = 9{\cdot}33$, highly significant.

Constant C (lucerne)/Constant C (grass): $t = 12{\cdot}02$, highly significant.

Constant D (lucerne)/Constant D (grass): $t = 2{\cdot}12$, insignificant.

A graphical confirmation of the constancy of *C* is provided in Figure 7, constructed from the values in the second part of Table 4. It will be noted that the curve for nitrite disappearance/time is affected in the same manner by the addition of glucose, as is the curve for nitrate disappearance/time.

The constancy of *D* is more difficult to demonstrate graphically, as the values for nitrite persistence in the presence of glucose are very small and of the same order as the experimental errors.

TABLE 4.

Sheep.	Diet.	Nitrate added mg. per 20 c.c. Ingesta.	Time in Minutes.				Percentage Reduction in Time.		
			Without Glucose.		With Glucose.		Of Nitrate Dis-appear-ance (Con-stant B.)	Of Nitrite Dis-appear-ance (Con-stant C.)	Of Nitrite Persis-tence (Con-stant D.)
			Nitrate Dis-appear-ance.	Nitrite Dis-appear-ance.	Nitrate Dis-appear-ance.	Nitrite Dis-appear-ance.			
					(i)	(i)			
16	Lucerne......	2·5	14	20	6	0	57	—	—
		5·0	30	48	14	16	53	67	89
		7·5	48	68	20	26	58	62	70
		10·0	80	115	30	45	62	61	57
		15·0	125	168	50	60	60	64	77
					Average	s.......	58	63	73
					(ii)	(ii)			
14	Grass hay....	0·4	8	20	0	4	—	80	—
		0·7	16	34	4	6	75	82	89
		1·0	28	56	6	8	79	86	93
		1·4	46	90	8	16	83	82	82
		1·7	65	135	14	24	78	82	86
					Average	s.......	79	82	88

(i) Quantity of glucose: 64 mg. per 20 c.c. ingesta.
(ii) Quantity of glucose: 48 mg. per 20 c.c. ingesta.

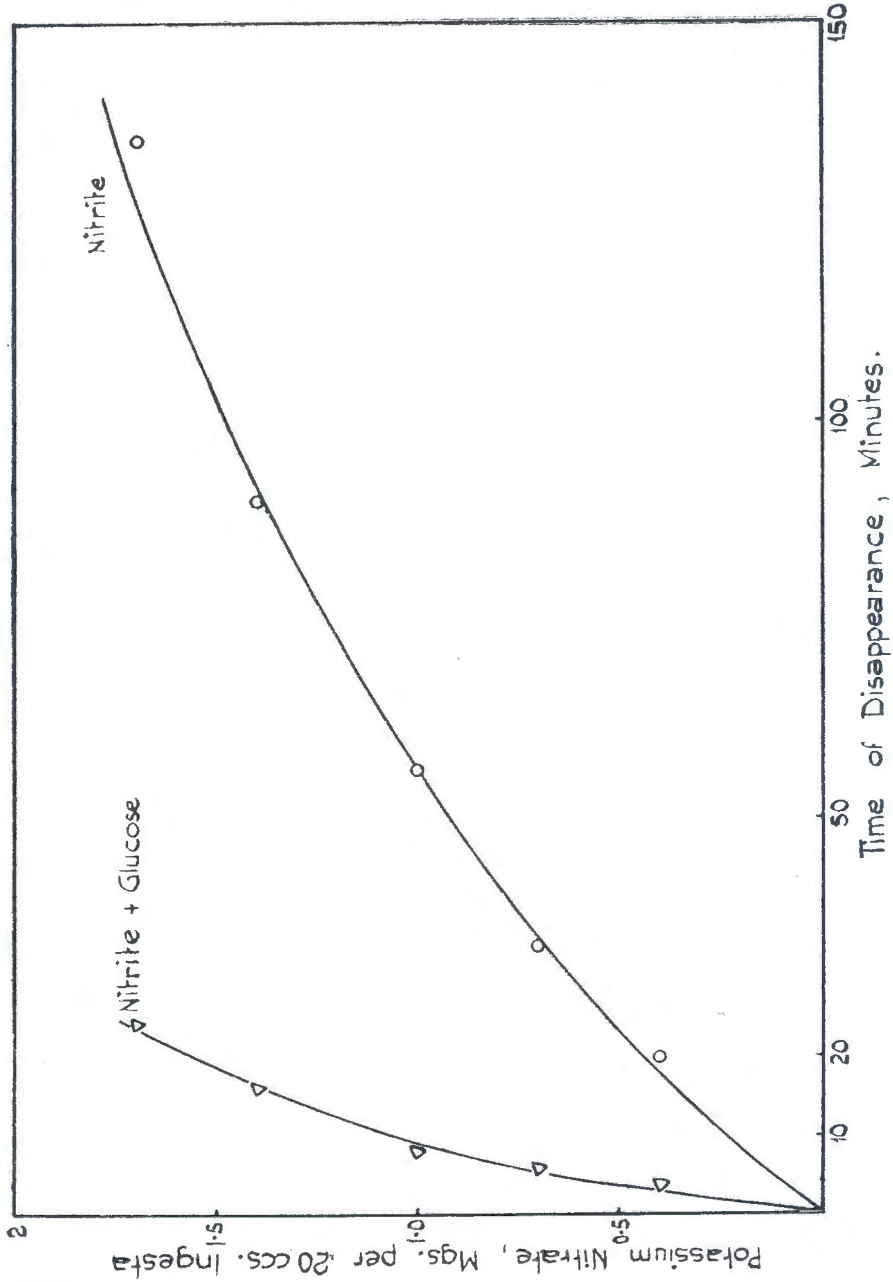
V. In Vivo *Experiment.—The Effects of Diet and the Dosing of Sugar on the Toxicity of Nitrate.*

Rimington and Quin (1933) showed that methaemoglobinaemia could be caused in sheep by dosing watery extracts of plants belonging to the genus *Tribulus*. This effect was proved to be due to the action of inorganic nitrite produced from pre-existing nitrate by a plant enzyme oxidation—reduction system.

The *in vitro* experiments here described have shown (*a*) that nitrite formed from nitrate persists longer in ingesta from a poorly-fed sheep than in that from a well-fed animal and (*b*) that the addition of sugar greatly accelerates the disappearance of the nitrite in either type of ingesta. These findings suggest that both the nature of the previous diet and the presence of sugar in the rumen would exert an effect on the tolerance of sheep to nitrate.

In order to test this hypothesis, eight sheep were divided into two groups, four receiving lucerne hay *ad lib.*, and 300 grams each of maize daily, while the other four were fed on poor quality grass hay only. After a preliminary of three weeks on the above diets, the animals were dosed with varying amounts of potassium nitrate in solution, given either alone or in conjunction with sugar (sucrose). The dose of nitrate was calculated for each sheep individually on the basis of body weight. On the day of

FIGURE 7.


Nitrite
Nitrite + Glucose
Time of Disappearance, Minutes.
10
20
50
100
150
Potassium Nitrate, Mgs. per 20 ccs. Ingesta
0·5
1·0
1·5
2

dosing, all food was removed at noon and dosing carried out at 3 p.m. The animals were kept without food till 9 a.m. the following morning, when they were bled for methaemoglobinaemia examination. An interval of a week was allowed between each of the four trials.

Table 5 gives a summary of the results obtained. The symbols given denote the extent of methaemoglobinaemia found on spectroscopic examination of the blood. The gradation from + to + + + + is based on the degree of dilution at which the methaemoglobin bands were visible.

TABLE 5.

Trial.	Potassium Nitrate Dosed, gm./100 lb. Live Weight.	Sucrose Dosed, Grams.	SHEEP.							
			HAY FED.				LUCERNE FED.			
			1	2	3	4	5	6	7	8
1	20	0		+	+			–	–	
	20	30	–			–	–			–
2	30	0	+++	+++	+++	+++	–			–
	30	30	+	(Died)	+	+		–	–	
3	40	0					–			–
	40	30						–	–	
4	50	0					++	++		
	50	30							+	–

As will be seen from this table, a dose of 20 grams of potassium nitrate per 100 lb. live weight (Trial 1) caused a demonstrable methaemoglobinaemia in both the sheep fed on grass hay. The addition of 30 grams of sucrose to the above dose of nitrate prevented the development of methaemoglobinaemia in the other two animals of this group. When, however, the dose of nitrate was increased to 30 grams per 100 lb. body weight (Trial 2), a very severe methaemoglobinaemia followed in all four of the hay-fed sheep and resulted in the death of one. When the remaining sheep were subsequently dosed with the same amount of nitrate but with sugar added, the methaemoglobinaemia produced was definitely less severe.

Turning to the sheep on the lucerne and maize diet, it will be seen from the table that a dose of 50 grams of potassium nitrate per 100 lb. body weight was required to cause methaemoglobinaemia even when given without sugar. As shown in the case of poorly-fed sheep, the sugar also exerted a marked protective action in these animals.

To sum up these results, it can be stated that a dose of 20 grams of potassium nitrate caused a more severe methaemoglobinaemia in the poorly-fed sheep than did a dose of 50 grams in the well-fed animals. This is in accordance with the *in vitro* experiments, in which the nitrite was found to persist much longer in the ingesta from poorly-fed sheep. Furthermore, the findings that the addition of sugar to ingesta *in vitro* accelerated the disappearance of nitrite formed, was likewise confirmed by the protective action of sugar in preventing methaemoglobinaemia.

Summary.

1. *In vitro* experiments have been conducted on the disappearance of potassium nitrate in ruminal ingesta from sheep on different diets.

2. Nitrate disappears more rapidly from ingesta of lucerne-fed sheep than from that of sheep on grass hay.

3. Nitrite is formed from the nitrate. It appears very soon after the addition of nitrate to the ingesta, reaches its maximum concentration at the moment of complete disappearance of the nitrate and subsequently itself disappears. Nitrite thus formed represents an intermediate stage in the conversion of nitrate to more fully reduced compounds.

4. The ratio nitrite disappearance time/nitrate disappearance time is constant for varying quantities of nitrate (*Constant A*).

5. The nitrite formed also disappears more rapidly from ingesta of lucerne-fed sheep than from that of sheep on grass hay.

6. The disappearance of nitrate from ingesta is represented graphically by plotting *quantity of nitrate* against *time of disappearance.* The resulting curve is a half parabola, with values varying for different ingesta. Daily variation for ingesta can be plotted from a series of such curves.

7. Nitrate disappearance rate can be used as an additional measure of ruminal activity.

8. The addition of glucose shortens the time of nitrate disappearance; the maximum effect for a fixed quantity of nitrate is produced by as little as 15 to 30 mg. of glucose per 20 c.c. of ingesta.

9. The "nitrate disappearance curve" is shifted in position, but remains unaltered in relative proportions, by the addition of a fixed quantity of glucose to the tube in each reading. The *percentage reduction of time of disappearance* of different quantities of nitrate due to a fixed quantity of glucose is a constant (*Constant B*).

10. The time of disappearance of nitrite formed from the nitrate is similarly affected by the presence of glucose. The *percentage reduction of nitrite disappearance time* due to a fixed quantity of glucose is also constant (*Constant C*).

11. "Nitrite persistence" is defined as the interval of time between the disappearance of nitrate and of nitrite. The *percentage reduction of nitrite persistence* due to a fixed quantity of glucose constitutes a fourth constant (*Constant D*).

12. An *in vivo* experiment confirmed the *in vitro* findings on the effects of nitrate, supplied either alone or in conjunction with sugar, on the ruminal ingesta of sheep. It demonstrated clearly the effect of diet and of sugar on nitrate persistence and therefore on nitrite poisoning.

13. Further work on this subject, both from the toxicological and the nutritional aspects, is in progress.

ACKNOWLEDGMENTS.

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