The Reduction of Sulphate in the Rumen of the Sheep

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The micro-organisms in the rumen of the sheep are capable of reducing nitrate and nitrite to ammonia (Sapiro, Hoflund, Clark & Quin, 1949; Lewis, 1951*a*, *b*). It was thought desirable to investigate whether this mixed population could also carry out the analogous reduction of sulphate to hydrogen sulphide, particularly since sulphate is a significant constituent of grass and hay, the normal foodstuff of ruminants. It has been shown by Thomas, Hendricks & Hill (1950) and by Evans (1931) that the normal concentration of sulphate in grass is of the order of 0·3 % sulphate S in the dry matter, rising under certain circumstances to 1 %. On the basis of a daily intake of 1 kg. dry matter, this is equivalent to 13 and 44 g., respectively, of Na_2SO_4 each day.

Beijerinck (1895) showed that the reduction of sulphate to sulphide in mud was a biological process, and van Delden (1904) isolated an organism, *Vibrio desulphuricans*, which would carry out this process. The organism was a strict anaerobe and it was found that the reduction of sulphate was linked with the oxidation of a variety of organic compounds (see also Baars, 1930). Stephenson & Stickland (1931) isolated from river mud a sulphate-reducing organism which would use gaseous hydrogen as well as organic substances as a hydrogen donor.

The isolation and cultivation of sulphate-reducing bacteria have been described in detail by Butlin, Adams & Thomas (1949). They consider that there is sufficient evidence to assume the existence of several species. Postgate (1951*a*, *b*; 1952) has studied in detail the nutrition and metabolism of *Desulphovibrio desulphuricans*; he used washed suspensions in Warburg manometer vessels for the study of the metabolism of this organism. It was able to utilize sulphate, thiosulphate, sulphite and tetrathionate for growth and its resting metabolism; it was also apparently able to use dithionite and metabisulphite. The author points out that these compounds are not stable. It was considered that the simplest interpretation of the evidence available was that the normal sequence of reactions was as follows:

$$SO_4^{2-} \xrightarrow{H_2} SO_3^{2-} \rightarrow \begin{matrix}\text{(unknown)}\\ \text{(intermediates)}\end{matrix} \rightarrow S^{2-}.$$

Information on sulphate metabolism in the ruminant is limited. Block, Stekol & Loosli (1951) have demonstrated that labelled sulphate fed to the goat is rapidly incorporated into the cystine and methionine of the milk proteins, but no information is available on the mechanism of this conversion. Evidence is already available (Lewis, unpublished) that sulphide is released from L-cysteine by washed suspensions of rumen micro-organisms. It is therefore possible that in the rumen the sulphur-containing amino acids give rise to sulphide and that part at least of the sulphate ingested is converted into sulphide. It would thus seem likely that the bulk of the sulphur supply of the ruminant is at one stage in the form of sulphide. The sulphide produced from sulphate or from sulphur-containing amino acids may be either absorbed from the rumen or the other parts of the alimentary canal into the blood stream, or it may be excreted in the faeces or resynthesized into the sulphur-containing amino acids of the proteins of micro-organisms in the rumen. Thus, information on the production of sulphide in the rumen was thought necessary in order to understand the normal sulphur metabolism of the ruminant and having regard to any possible abnormality or toxicity caused by a high concentration of sulphide.

The present work has been restricted to a study of the reduction of sulphate by rumen micro-organisms. The reduction *in vivo* was investigated by the introduction of sulphate into the rumen through a permanent rumen fistula. The reduction of sulphate *in vitro* was studied with washed suspensions of rumen organisms.

METHODS

Design of animal experiments. The experimental animals were fitted with permanent rumen fistulae: three Scotch blackface wethers at Sheffield and two Clun Forest wethers at Babraham. The Scotch blackface wethers weighed approx. 40 kg. and were fed 230 g. Paul's no. 2 dairy nuts (N, 3·1 %) daily, plus a mixture of chopped hay and straw *ad lib.* The others weighed approx. 60 kg. and were fed 1 kg. of chopped hay daily. During the course of the *in vivo* experiments the sheep were fed at between 9.00 and 9.30 a.m. and the excess of foodstuff was removed 2 hr. later. At this point the initial sample of rumen contents was withdrawn through the fistula. A second sample was withdrawn 1 hr. later and the solution containing a known amount of sodium sulphate was introduced into the rumen.

Succeeding samples were withdrawn at the appropriate times. When the sheep were regularly dosed with sulphate, an aqueous solution of Na_2SO_4 was placed in the rumen through the fistula 2 hr. after feeding commenced. The sheep were not dosed on the days when animal experiments were carried out. The *in vivo* experiments reported were carried out at Babraham and those *in vitro* at Sheffield.

Washed suspensions of rumen bacteria were prepared according to the method of Sijpesteijn & Elsden (1952), save that no Na_2S was added to the washing solution. The sample of rumen contents was withdrawn 5–5·5 hr. after feeding. The activity did not appear to be affected by the omission of the sulphide, provided that the following precautions were adhered to. Oxygen was removed as far as possible from the reagents, including the phosphate buffer (K salts in all cases) by boiling for 2 min., cooling rapidly and gassing with a slow stream of O_2-free N_2 until used. The substrates were not boiled. Unless otherwise stated, all washed suspensions were prepared from rumen contents obtained from sheep that had been previously dosed with Na_2SO_4 for at least 14 days.

Manometric methods. The reactions were carried out at 37° in Warburg manometers with double side-bulb cups. The main compartment contained 2 ml. of the washed cell suspension and the first side bulb contained the potential H-donor and the sulphate. The contents of this side bulb were tipped in when thermal equilibrium had been attained. The second side bulb contained 0·4 ml. 4 N-H_2SO_4 which was tipped in at the end of the incubation period to stop the reaction and to release any H_2S which had been bound by the medium. Failure to do this led to low recoveries of sulphide. The centre wells contained 0·2 ml. 20% (w/v) NaOH and a plug of acid-washed glass wool to trap any sulphide produced and to absorb any CO_2 formed. Glass wool was used in preference to filter paper, since the latter disintegrated in the presence of alkali.

Total nitrogen. This was determined by the Kjeldahl method as modified by Chibnall, Rees & Williams (1943) using a Markham (1942) steam-distillation apparatus and the boric acid reagent of Conway & O'Malley (1942).

Sulphide estimation. Sulphide was determined by a colorimetric method developed by Wilkinson & Fry (1950). It is based on the reaction of H_2S with I_2 in acid solution:

$$H_2S + I_2 \rightarrow 2HI + S.$$

A solution of I_2 in CCl_4 is purple in colour (maximum absorption at 520 mμ.) and Fig. 1 shows that Beer's law is obeyed over the range of 0–0·0007 M. When a solution of I_2 in CCl_4 is shaken with an acidified aqueous solution of H_2S, the I_2 reacts with the sulphide and the depth of colour in the CCl_4 is reduced. Thus, from a determination of the decrease in optical density after treatment with a solution containing H_2S, it is possible to obtain an estimate of the amount of sulphide present. This method has been used for the determination of sulphide produced in biological systems.

The sulphide produced in the manometer vessels was absorbed by alkali in the centre well. At the end of the period of incubation, the acid was added from the side bulb and the manometer shaken for a further period of 30 min. The plug of glass wool was carefully transferred with forceps, afterwards washed, into a separating funnel. The total volume at this stage was 3 ml. To the separating funnel were added 2 ml. of 0·005 M-I_2 in redistilled CCl_4, 3 ml. CCl_4 and 5 ml. 2 N-HCl. The I_2 solution had been previously standardized by shaking with a known excess of aqueous

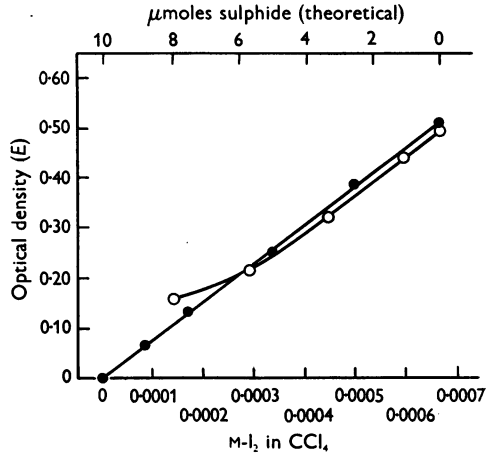

Fig. 1. Optical density of iodine in carbon tetrachloride. ●, dilution of 0·00503 M-I_2 in CCl_4; ○, addition of known amounts of sulphide to 2 ml. 0·00503 M-I_2 in CCl_4. Volume made up to 15 ml. with CCl_4. For conditions see text.

$Na_2S_2O_3$ and back-titrating with a standard solution of I_2 prepared from potassium bi-iodate. After being shaken vigorously for 2 min., the CCl_4 layer was run into a 15 ml. measuring flask and the contents of the separating funnel were washed twice by shaking with 3 ml. volumes of CCl_4. Care was taken to avoid evaporation by keeping all vessels stoppered. The volume was made up to 15 ml. with redistilled CCl_4 and the optical density measured in an EEL (Evans Electroselenium Ltd., Harlow, Essex) colorimeter with a green filter.

The sulphide concentration in rumen contents was estimated using Conway units (Conway, 1950). In the outer compartment of the dish were placed 2 ml. of rumen contents which had been filtered through muslin, and in the inner compartment 1 ml. 5% (w/v) NaOH; the dishes were then covered and 0·4 ml. 4 N-H_2SO_4 was added to the outer compartment as described by Conway (1950). The vessels were allowed to stand overnight at room temperature and the alkali was transferred, with washing, to a separating funnel. The procedure thereafter was the same as described above.

Fig. 1 shows the relationship between the optical density of the I_2 solution and μmoles sulphide added. Within the range of 0–6 μmoles sulphide added to 2 ml. 0·005 M-I_2 in CCl_4, this was linear. With larger amounts of sulphide, however, it appeared that the reaction was no longer quantitative. For the purpose of this experiment, a solution of Na_2S was used which had been standardized directly against an I_2 solution prepared from potassium bi-iodate. The difference between the standard I_2 curve and that following the addition of sulphide may be due to mechanical losses of I_2 in the manipulations. The recoveries of sulphide added to washed suspensions of rumen contents in Warburg manometer vessels and Conway dishes were also determined. A representative selection of the results is presented in Table 1. A standard curve was prepared for each series of estimations by dilution of 0·005 M-I_2 in CCl_4 solution. The figures of apparent recovery of sulphide from 2 ml. of distilled water probably refer to losses of the I_2 in CCl_4

Table 1. *Recovery of sulphide added to rumen contents*

(The observed values are corrected for the mean of duplicate estimations of sulphide in rumen contents.)

Sulphide added (μmoles)	Fluid	Recovered from Conway dishes			Recovered from Warburg manometers		
		μmoles	μmoles (corr.)	%	μmoles	μmoles (corr.)	%
0	2 ml. water	0·20	—	—	0·18	—	—
0		0·18	—	—	0·17	—	—
0	2 ml. RC*	0·74	—	—	0·66	—	—
0		0·69	—	—	0·73	—	—
1·13		1·83	1·11	98	1·89	1·19	105
1·13		1·88	1·16	103	1·74	1·04	92
3·39		4·11	3·39	100	3·95	3·25	96
3·39		4·15	3·43	101	3·86	3·16	93
5·65		5·82	5·10	90	5·95	5·25	93
5·65		5·97	5·25	93	5·78	5·08	90
7·91		7·19	6·47	82	7·20	6·50	82
7·91		7·11	6·39	81	7·38	6·68	84

* RC = Rumen contents after filtering through muslin.

solution. The quantity recovered from rumen contents appears to fall off with larger amounts of added sulphide. The method has been used only within the range of 1–6 μmoles sulphide, where the recoveries were always within the range 90–105 %.

Sulphate estimation. Sulphate was determined by a modification of the standard, gravimetric $BaSO_4$ procedure (Vogel, 1951). A volume of 200 ml. rumen contents was withdrawn and filtered through muslin. Duplicate 50 ml. samples of this were used for analysis. A suitable volume of 2 N-HCl was added to give a final concentration of 0·01 N added HCl and after being stirred, the whole was centrifuged at 3600 rev./min. for 30 min. The residue was washed with 50 ml. 0·01 N-HCl and spun again. The combined supernatants were heated to 100° and 25 ml. 5 % (w/v) $BaCl_2.2H_2O$ added. After being kept in a boiling-water bath for 20 min., the precipitate was filtered through a weighed, sintered-glass crucible (no. 4), washed, dried and weighed again. Recoveries of 200–1200 μmoles Na_2SO_4 added in aqueous solution (weighed out as $Na_2SO_4.10H_2O$) to the 50 ml. samples of filtered rumen contents, were always within the range of 95–101 % after subtracting the blank concentration in rumen contents which was always less than 1·5 μmoles sulphate/ml. This was composed of sulphate and some undefined materials precipitated by the $BaCl_2$.

RESULTS

Reduction in the rumen

In order to determine whether sulphate is reduced in the rumen of the sheep, the concentrations of sulphate and sulphide were determined following the introduction of sulphate into the rumen. If the sheep had not been previously dosed with sulphate, the sulphate added disappeared at a steady rate and some sulphide was always formed, but both the amount of sulphide formed and the rates at which it was produced and disappeared were very variable. The results of a typical experiment are presented in Fig. 2, including those of a control experiment in which no sulphate was introduced into the rumen.

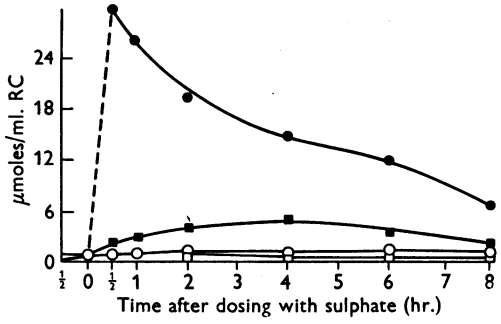

Fig. 2. Sulphate and sulphide in the rumen contents (RC) following dosing with sulphate. Dose, 80 g. $Na_2SO_4.10H_2O$ (41·4 μmoles/ml. RC, assuming the rumen has a capacity of 6 l.). Control, no dose administered. □, control sulphide; ○, control sulphate; ■, experimental sulphide; ●, experimental sulphate.

The concentration of sulphide in the rumen in the absence of added sulphate is consistently low and usually less than 0·8 μmole/ml. rumen contents. It can be seen that there is a rapid disappearance of added sulphate; within 4 hr. the concentration is less than half that found 0·5 hr. after dosing. Within 24 hr. the concentration had fallen to the blank level of 0·5 μmole/ml.

Experiments were also carried out after the sheep had been dosed daily with 40 g. $Na_2SO_4.10H_2O$ in 200 ml. water. The daily dose was administered 2 hr. after feeding, and 24 hr. later the concentration of both sulphate and sulphide in the rumen had returned to the blank level. The results of one such experiment are given in Fig. 3, after the sheep had been dosed for 14 consecutive days. The usual dose was not administered on the day that the experiment was carried out. In this experiment the disappearance of sulphate was more rapid and the concentration of sulphide obtained was greater. The

concentrations of sulphate in the rumen contents 4 hr. after the test substance had been administered were 14·7 μmoles (no pre-dosing) and 6·0 μmoles (after 14 doses)/ml. and the sulphide concentrations at the same time were 4·8 and 8·5 μmoles/ml., respectively.

In order to determine whether there was a significant increase in the rate of sulphate disappearance from the rumen when the sheep had been dosed daily with sulphate, a series of experiments was carried out before and after dosing the sheep. The results are expressed as the time taken to decrease the concentration of sulphate in the rumen by a half. The curve of sulphate concentration is extrapolated to the time of dosing, and the time after dosing at which the concentration is halved is recorded. These semiquantitative data for a series of experiments are presented in Table 2; the differences are consistent and probably significant. The results of these *in vivo* studies show that sulphate is reduced to sulphide in the rumen of sheep and that the rate of sulphate disappearance and sulphide production is increased by the repeated dosage of the sheep with sulphate. No information is available about the quantity of sulphate absorbed from the rumen nor the amount that passes along the alimentary tract.

Washed-suspension experiments

The reduction of sulphate with gaseous hydrogen as hydrogen donor. The ability of washed suspensions of rumen micro-organisms to reduce sulphate with gaseous hydrogen as the hydrogen donor was tested. When sulphate was incubated with the washed cells in an atmosphere of hydrogen, there was a steady uptake of hydrogen greater than that observed in the control manometer which contained no sulphate (Fig. 4). An additional control was arranged in which sulphate was incubated with the suspension under an atmosphere of nitrogen. The first control was used for the correction of the observed hydrogen uptake and the second for the correction of the figures of sulphide formation. Hydrogen sulphide was estimated in the alkali withdrawn from the centre wells and the results are presented in Table 3. The hydrogen uptake corresponded to 111 and 114 % (not corrected) and 89 and 92 % (corrected) of the theoretical value for reduction to sulphide: the figures for sulphide production were 100 and 104 %, and 74 and 78 %, respectively. Despite the apparently good agreement with the uncorrected values, it is thought that the figures after the subtraction of the blanks are probably more reliable, since almost half of the sulphide in the blank can be accounted for in terms of that initially present in the washed suspension. It was found that the sulphide content of the suspension was not markedly affected by the washing procedure. The fact that the amount of hydrogen used, even correcting for the control, is

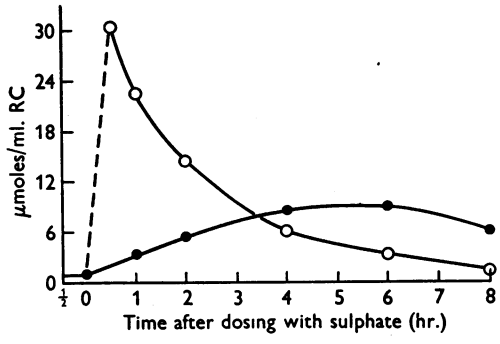

Fig. 3. Sulphate and sulphide in the rumen following repeated dosing of a sheep with sodium sulphate. Sheep given daily dose of 40 g. $Na_2SO_4.10H_2O$ for 15 days, and experimental dose of 80 g. $Na_2SO_4.10H_2O$ at time 0. ○, sulphate; ●, sulphide.

Table 2. *Disappearance of sulphate from the rumen of the sheep*

(The animals dosed with sulphate were given a solution of 40 g. $Na_2SO_4.10H_2O$ daily, and the experimental dose consisted of 80 g. $Na_2SO_4.10H_2O$ in 250 ml. water given 3 hr. after feeding.)

	Time for reduction of sulphate to half of the initial concentration (hr.)	
	Sheep not dosed	Sheep dosed on 14 successive days
Sheep 8	4·2	1·4
Sheep 8	2·6	1·2
Sheep 11	4·3	1·7
Sheep 12	2·5	1·3
Sheep 8	3·2	2·5

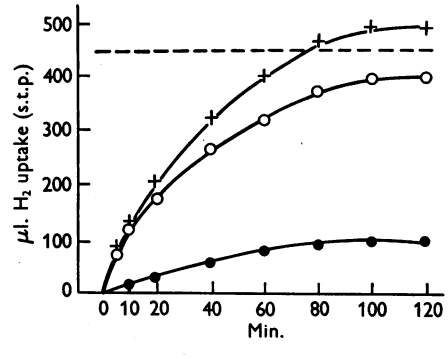

Fig. 4. Hydrogen uptake by washed micro-organisms in the presence of sulphate. Theoretical uptake is for reduction to sulphide. 2 ml. washed-cell suspension in 0·1 M potassium phosphate buffer (pH 6·5), containing 16·4 mg. total N/100 ml.; 0·1 ml. 0·05 M-Na_2SO_4 in side bulb; 0·2 ml. 20 % (w/v) NaOH in centre well. ●, control; +, observed; ○, corrected.

greater than the sulphide produced suggests that hydrogen is being used in some other reaction.

Reduction of other inorganic sulphur compounds. The ability of the washed suspensions in an atmosphere of hydrogen to reduce sulphite, dithionite, and thiosulphate, in the presence of hydrogen, was next examined. There was a rapid uptake of hydrogen in the presence of sulphate, thiosulphate and sulphite, but not when dithionite was added (Fig. 5).

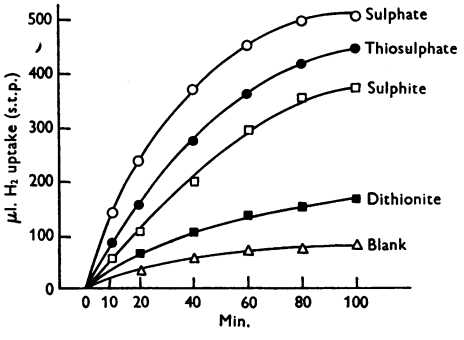

Fig. 5. Hydrogen uptake in the presence of sulphate, sulphite, dithionite and thiosulphate. 2 ml. Suspension in 0·1 M phosphate buffer (pH 6·5) containing 19·4 mg. total N/100 ml.; 0·2 ml. 0·025 M substrate in side bulb; 0·2 ml. 20% (w/v) NaOH in centre well. △, control; ■, sodium dithionite; □, sodium sulphite; ●, sodium thiosulphate; ○, sodium sulphate.

At the end of the 100 min. incubation period, the acid was tipped in from the side arm. The sulphide production was estimated; the results, together with theoretical values for reduction to sulphide, are presented in Table 4. The appropriate corrections have been made; the control values were of the same order as those in Table 3. There was clearly a reduction of sulphate, sulphite, and thiosulphate, to sulphide, with a hydrogen uptake and sulphide production within the range of 70–100% of the theoretical. In the presence of dithionite it is doubtful whether there was a significantly greater hydrogen uptake or sulphide production than in the control. Postgate (1951*b*) has shown that *D. desulphuricans* is able to utilize sulphate, thiosulphate, sulphite and tetrathionate for its growth and resting metabolism. However, though it was also apparently able to use dithionite and metabisulphite, the author considers the results to be of doubtful significance because of the spontaneous decomposition of these compounds.

The effect of pH *on the reduction of sulphate by washed suspensions in the presence of hydrogen.* The preparation of the washed-cell suspension differed in that the rumen micro-organisms were both washed with and suspended in a saline prepared as follows: a solution of 0·9% (w/v) NaCl was boiled immediately before use and cooled rapidly under the tap; to this were added 5% aqueous thioglycollic

Table 3. *Hydrogen uptake and sulphide formation by washed suspension in the presence of sulphate*

(2 ml. washed-cell suspension in 0·1 M phosphate buffer (pH 6·5) containing 19·8 mg. total N/100 ml.; 0·1 ml. 0·05 M-Na_2SO_4 (5 μmoles) in first side bulb: 0·4 ml. 4 N-H_2SO_4 in second side bulb: 0·2 ml. 20% (w/v) NaOH in centre well. Theoretical values are for reduction to sulphide.)

		H_2 uptake			Sulphide formation		
Substrate	Gas phase	Obs. (μmoles)	Corr. (μmoles)	% Theory corr.	Obs. (μmoles)	Corr. (μmoles)	% Theory corr.
Na_2SO_4	H_2	22·2	17·8	89	5·0	3·7	74
Na_2SO_4	H_2	22·8	18·4	92	5·2	3·9	78
Na_2SO_4	N_2	0·3*	—	—	1·2	—	—
Na_2SO_4	N_2	0·4*	—	—	1·4	—	—
None	H_2	4·6	—	—	0·6	—	—
None	H_2	4·3	—	—	0·9	—	—

* An apparent uptake of gas that may have been due to traces of O_2 in the N_2 used.

Table 4. *Reduction of inorganic sulphur compounds to sulphide*

(2 ml. suspension in 0·1 M phosphate buffer (pH 6·5) containing 19·4 mg. total N/100 ml.; 0·2 ml. 0·025 M sodium salt of substrate in first side bulb (5 μmoles); 0·2 ml. 20% (w/v) NaOH in centre well; 0·4 ml. 4 N-H_2SO_4 in second side bulb. Theoretical results are for reduction to sulphide and figures have been corrected for appropriate blanks.)

	H_2 uptake			Sulphide production		
	Theory (μmoles)	Found (μmoles)	% Theory	Theory (μmoles)	Found (μmoles)	% Theory
Control (N_2/SO_4^{2-})	—	0	—	—	0·9	—
Control (H_2 only)	—	3·8	—	—	0·4	—
Sulphate	20	18·9	95	5	3·6	72
Sulphite	15	13·0	87	5	4·1	82
Thiosulphate	20	16·2	81	10	7·5	75
Dithionite	25	3·8	15	10	2·3	23

acid to a final concentration of 0·02% (w/v) and sufficient 0·1N-NaOH to bring the pH to 6·5. The main compartment of the manometer vessels contained 1 ml. of the suspension and 1 ml. 0·2M potassium phosphate buffer, of the appropriate pH, which had also been boiled and cooled rapidly and which contained 0·02% (w/v) thioglycollic acid. The buffers covered the range of pH 5·8–8·0. A control manometer containing no substrate was set up at each pH. The sulphate (0·2 ml. 0·05N) was tipped in from a side bulb after a period of equilibration. Initial rates of hydrogen uptake, from 5 to 10 min. after tipping in the substrate, were determined and corrected for the gas uptake in the controls; the blanks were of the order of 15–20% of the values in the presence of the substrate. The results (Fig. 6) are expressed as percentages of the maximum initial rate of hydrogen uptake, and show that the optimum pH for sulphate reduction by the washed rumen micro-organisms is around 6·5.

Hydrogen donors other than gaseous hydrogen. The compounds tested are either known to occur in the rumen or are thought to be present under certain conditions. For convenience the experiments were carried out in Warburg manometer vessels in an atmosphere of nitrogen. In addition to the control in which sulphate was incubated with the suspension in the absence of hydrogen under nitrogen, a second manometer was set up in which the hydrogen donor was hydrogen gas. This latter served as a reference standard whereby the efficacy of the hydrogen donors under examination could be judged. The test substances were incubated in the presence of sulphate and the washed suspension for 2 hr. and the ability of the compounds to act as hydrogen donors was assessed in terms of the amounts of sulphide produced. It was considered that a significant reduction of sulphate was effected when the sulphide produced in the presence of the potential hydrogen donor was at least double that in the control. In no case did the reaction go to completion in the 2 hr. incubation period. There was a significant reduction of sulphate in the presence of hydrogen, glucose, fructose, DL-lactate, pyruvate, formate, succinate, ethanol, citrate and L(−)-malate but not in the presence of the fatty acids, D-xylose, mannitol or glycerol (Table 5).

Fractions of rumen micro-organisms. The experiments *in vivo* show that dosing the sheep with sulphate increases the rate of sulphide production from sulphate. In view of this, a series of experiments was designed to determine whether dosing the sheep with sulphate affects the rate of sulphate reduction by fractions of washed-cell suspensions of rumen micro-organisms. Each of the fractions was suspended in 0·1M phosphate buffer (pH 6·5) in a volume equivalent to one-third of that initially withdrawn from the rumen. At the time of the first experiment (expt. 1) the sheep had not been dosed with sulphate for 28 days: after this, a dose of 40 g. $Na_2SO_4.10H_2O$ in 200 ml. water was introduced daily into the rumen of the sheep except on the days when a sample was withdrawn for investigation. Succeeding experiments were carried out on the third day (expt. 2), the 6th day (expt. 3) and the 18th day (expt. 4) after the commencement of the daily dosing of the sheep. The separation of the fractions was effected on a M.S.E. 'Angle' centrifuge (Measuring and Scientific Equipment Ltd., London, S.W. 1). The sample of rumen contents was filtered through muslin and spun at 700 rev./min. for 2 min.

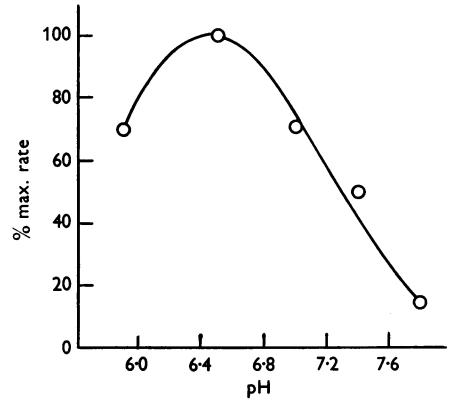

Fig. 6. Effect of pH on rate of hydrogen uptake by washed cell suspensions in presence of sulphate. Rates expressed as % of the maximum initial rate of hydrogen uptake. Suspension containing 25·4 mg. total N/100 ml. For details see text.

Table 5. *Production of sulphide from sulphate by washed suspensions of rumen bacteria in presence of potential hydrogen donors*

(2 ml. washed-cell suspension in 0·1M phosphate buffer (pH 6·5); 0·4 ml. 0·1M H-donor; 0·2 ml. 0·1M-Na_2SO_4 (20 μmoles) in first side bulb and 0·4 ml. 4N-H_2SO_4 in second side bulb; 0·2 ml. 20% (w/v) NaOH in centre well. Incubation for 2 hr. in N_2 atmosphere.)

H-donor	μmoles sulphide	H-donor	μmoles sulphide
Expt. A		Expt. C	
Control	2·1	Control	1·2
Hydrogen	15·4	Hydrogen	15·3
Glucose	16·1	Acetate	1·6
Fructose	13·9	Propionate	1·2
DL-Lactate	12·9	Butyrate	1·8
Pyruvate	11·2	Valerate	2·1
Expt. B		Expt. D	
Control	3·7	Control	1·9
Hydrogen	18·9	Hydrogen	16·3
Formate	16·2	Citrate	8·2
Succinate	11·1	D-Mannitol	3·2
Ethanol	10·0	Glycerol	3·7
D-Xylose	4·2	L(−)-Malate	7·0

Table 6. *Rate of sulphide production and hydrogen uptake by fractions of rumen micro-organisms (see text) in the presence of sulphate at different stages of a course of daily dosing with sulphate*

(2 ml. suspensions: total N approx. 8 mg. (residue 1), 6 mg. (residue 2) and 4 mg. (residue 3). 0·3 ml. 0·02 M-Na_2SO_4 in first side bulb; 0·4 ml. 4 N-H_2SO_4 in second side bulb; 0·2 ml. 20% (w/v) NaOH in centre well. Incubation for 30 min. under H_2 atmosphere.)

	Expt. 1 (before dosing)		Expt. 2 (3rd day of dosing)		Expt. 3 (6th day of dosing)		Expt. 4 (18th day of dosing)	
	Obs.	Corr.	Obs.	Corr.	Obs.	Corr.	Obs.	Corr.
				μmoles H_2S/mg. total N/hr.				
Residue 1	0·2	0·1	0·7	0·4	0·6	0·5	0·8	0·6
Residue 2	0·4	0·2	1·1	0·7	1·5	1·1	1·8	1·5
Residue 3	0·9	0·5	2·0	1·5	3·3	2·5	3·0	2·4
				μmoles H_2/mg. total N/hr.				
Residue 1	0·5	0·2	1·8	1·2	1·2	0·8	3·4	2·5
Residue 2	1·0	0·4	4·7	2·8	4·9	3·8	6·4	4·8
Residue 3	2·6	1·2	9·1	7·1	10·3	8·0	11·9	10·0

(residue 1); the supernatant was spun at 1900 rev./min. for 5 min. (residue 2); and in the same way at 3550 rev./min. for 25 min. (residue 3). The residues were then washed with and suspended in the 0·1 M phosphate buffer (pH 6·5) prepared as before but containing no thioglycollic acid.

The manometer vessels contained 2 ml. of these suspensions, together with 6 μmoles Na_2SO_4 in an atmosphere of hydrogen, with alkali in the centre well. The incubation was stopped after 30 min., the hydrogen uptake was recorded and the sulphide absorbed in the alkali was estimated. Control manometers were arranged in each instance in the absence of sulphate (for the correction of the hydrogen uptake) and in the presence of sulphate in nitrogen atmosphere (for correction of sulphide production). The results (Table 6) are expressed in the form of metabolic quotients, as μmoles H_2 or H_2S/mg. total N/hr. The calculations were made on the basis of the gross uptake of hydrogen or production of sulphide in the 30 min. incubation period. Corrections have also been made for the blank reactions. This form of expression was considered desirable since it compensated for differences in the total nitrogen of the suspensions, but it must be pointed out that the hydrogen-uptake curves were not strictly linear throughout the 30 min. incubation. In no instance did the reaction go to completion. The maximum value of $Q_{H_2}^{H_2}$ recorded was an uptake 266 μl. H_2/mg. total N/hr., which may be compared with a $Q_{H_2}^{H_2}$ (sulphate) of 200 on a dry-weight basis found by Postgate (1951*b*).

In all cases there was a more rapid reduction of sulphate by the fraction containing the smaller organisms (residue 3). The rate was almost invariably more than twice as great as with residue 2. With some exceptions the sulphide production was greater than expected in view of the hydrogen uptake, suggesting that in these instances other hydrogen donors were present to some extent in the suspension. The relationship between hydrogen uptake and sulphide production does not always agree well with the expected 4 moles H_2 taken up/mole sulphide formed. It was also clearly shown that dosing of the sheep with sulphate encouraged the rate of sulphide production.

DISCUSSION

It has been shown that sulphate is reduced to sulphide by the rumen micro-organisms both *in vivo* and *in vitro* using washed suspensions of rumen micro-organisms. There is no reason to believe that the presence of sulphate in any way stimulates the release of sulphide from such compounds as the sulphur amino acids. The parallel findings *in vivo* and *in vitro* present a further confirmation of the reliability of the washed-suspension method, although the objections listed by Sijpesteijn & Elsden (1952) remain. The pH optimum of the reaction, about 6·5, is about the normal pH of rumen contents (Phillipson, 1942). A similar pH optimum was shown by Lewis (1951*b*) for the reduction of nitrate, nitrite and hydroxylamine, and by Sijpesteijn & Elsden (1952) for the decarboxylation of succinic acid. Postgate (1949) stated that the optimum pH for sulphate reduction by *D. desulphuricans* with hydrogen gas as hydrogen donor is 6·3, confirming the results of Stephenson & Stickland (1931). Butlin *et al.* (1949) recommend that these bacteria be grown at pH 7·2. Postgate (1951*a*) showed that *D. desulphuricans* grew well from pH 6·3 to 8·6, growth being more rapid at the higher pH, a feature attributed to the concentration of bicarbonate in the medium.

The hydrogen donors found to be active for sulphate reduction by the washed suspensions are similar to those found to be effective in nitrate reduction (Lewis, 1951*b*). There is no evidence available to indicate whether or not these are the

compounds effective for sulphate reduction in the rumen. The evidence for their occurrence in the rumen is very limited; only the fatty acids have been shown to be present in significant quantities and these are not active hydrogen donors. In both the nitrate and the sulphate reduction, hydrogen gas was amongst the most active hydrogen donors; in a careful analysis of rumen gases, Lugg (1938) was not able to demonstrate the presence of hydrogen.

The reduction is presumed to be carried out by sulphate-reducing bacteria in the rumen, but the organism or organisms responsible have not been isolated. Several of the properties of the washed suspension of rumen micro-organisms are similar to those of *D. desulphuricans*. A comparison may be made of the inorganic sulphur compounds reduced (cf. dithionite), the effective hydrogen donors (Baars, 1930), the strict anaerobiosis required, and the pH optimum. The presence of sulphate-reducing organisms is strongly suggested by the results of dosing the sheep with sulphate; the more rapid reduction *in vivo* is interpreted as being due to the rapid proliferation of the organisms in a sort of 'enrichment' medium. A parallel enhanced rate of reduction was demonstrated *in vitro*. It was also shown that the fractions containing the smaller organisms have the greater ability to reduce sulphate. None of these results contradicts the idea that the reduction is brought about by organisms resembling *Desulphovibrio*.

Preliminary efforts to isolate sulphate-reducing organisms from the rumen have not been successful. It is, however, desirable that this be carried out. An attempt was made to assess the number of such organisms present by the technique of including ferrous sulphate in a solid agar medium, so that in the vicinity of a sulphate-reducing organism the sulphide gives rise to a black spot. The results obtained were, however, uncertain and in some cases no colonies were observed even after 14 days incubation. Tentative figures obtained were of the order of 10^3 organisms/ml. of rumen contents in the sheep not dosed with sulphate, and 10^5 organisms/ml. following the dosing of the sheep for 14–28 days. It is realized that the results were variable, that the figures found were low and also that the technique does not exclude the possibility of sulphide production from sulphur-containing amino acids. The results suggested an increase in numbers following dosage. It must be pointed out that there have been certain inconsistencies in the results of the experiments on sulphate reduction with the techniques used. During some *in vivo* experiments the quantity of sulphide produced was very low, the washed suspensions sometimes did not appear to reduce sulphate, particularly if the sheep had not been previously dosed with sulphate, and often there were no demonstrable colonies in the solid agar medium when the method described above was used (see Butlin *et al.* 1949).

Since hydrogen sulphide is toxic, the possibility that diets rich in sulphate might exert toxic effects had to be considered. However, when a dose of 150 g. $Na_2SO_4.10H_2O$ was given, no toxic effects were observed even though the concentration of sulphide in the rumen reached 14·7 μmoles/ml. rumen liquor. Assuming the normal and maximum concentrations of sulphate in the dry matter of the diet to be 0·3 and 1 % sulphate S, respectively, and a daily intake of 1 kg. dry matter, this is equivalent to an intake of 30 and 100 g., respectively, of $Na_2SO_4.10H_2O$. So at this range no gross abnormality or toxicity can be expected.

However, the sulphide produced in the rumen probably contributes to the normal sulphur metabolism of the ruminant. Block *et al.* (1951) showed that, when sulphate containing ^{35}S was fed to a ewe, 4 hr. after dosing the bulk of the isotope was in the trichloroacetic acid-insoluble fraction of the rumen contents. This suggests a rapid incorporation of sulphate S into the rumen protein and in view of the present results, hydrogen sulphide may be an intermediate in this process. Several workers have shown that sulphide can be utilized by animal tissues in the production of sulphur-containing amino acids (Dziewatkowski, 1946; Melchior & Tarver, 1947; Smythe, 1943). It has also been shown in the goat that labelled sulphur, fed as sulphate, rapidly enters the milk proteins; the labelling was found equally in the cystine and methionine (Block *et al.* 1951). Some radioactivity was also found in the trichloroacetic acid filtrate of the milk. Thus it was suggested that some sulphate was absorbed from the rumen, some converted into protein of rumen contents and also part finally found in the amino acids of milk proteins. The significance of the present work may lie in the demonstration of the formation of sulphide from sulphate, which may then be utilized in the rumen for the production of the sulphur-containing amino acids of the protein of the bacteria.

SUMMARY

1. Sulphate is reduced to sulphide in the rumen of the sheep and also *in vitro* using a washed suspension of rumen contents under various conditions.

2. It was shown *in vitro* using hydrogen gas as hydrogen donor, that the optimum pH of the reduction is around 6·5.

3. Sulphite and thiosulphate are also reduced to sulphide.

4. In addition to hydrogen gas, the following hydrogen donors were also effective: glucose, formate, fructose, lactate, pyruvate, succinate, ethanol, citrate and malate.

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A Simple Technique for the Estimation of Radioactive Components of Plasma after the Administration of Radioactive Iodide

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Radioactive components of plasma released from the thyroid gland after the administration of ^{131}I have recently been studied in rats and in cases of thyroid disease by using in conjunction the methods of paper chromatography and radioautography (for a review, see Gross & Pitt-Rivers, 1952). The use of these techniques is time-consuming and not suitable for plasma containing small amounts of radioactivity. Furthermore, we have found losses of radioactive iodide to occur on paper chromatograms, whilst a similar observation has been made in the case of thyroxine by Taurog, Chaikoff & Tong (1950); the technique therefore is not suitable for quantitative work. This communication describes the use of methanol and silver phosphate for the fractionation of plasma components containing radioactive iodine. It has been found that silver phosphate will remove iodide and thyroxine from solution under specified conditions, whilst methanol liberates thyroxine from its state of physiological combination with plasma proteins; at the same time any thyroid protein present is precipitated with the proteins. Suitable application of these two reagents enables comparative estimations of the above components to be made with rapidity and accuracy. The determinations may easily be carried out on small serial samples of plasma, and enable graphs to be constructed of the relative changes occurring in the various fractions after doses of radioactive iodide have been delivered to the gland. The results obtained in several patients and in rats are described and correlated with chromatographic and radioautographic studies.

EXPERIMENTAL

Precipitation of plasma proteins. (*a*) Methanol. Plasma (0·5 ml.) was mixed thoroughly with methanol (1·16 ml.) and centrifuged.

(*b*) $(NH_4)_2SO_4$. Plasma (0·25 ml.) was diluted with 27·8% (w/v) $(NH_4)_2SO_4$ soln. (4·75 ml.) and a sample (0·2 ml.) taken immediately for counting. The suspension of globulins was then removed by centrifugation and a similar sample of the supernatant taken for counting. This procedure was adopted in order to eliminate the absorption error in counting, which error was due to the high salt content.

Treatment with Ag_3PO_4. (*a*) Plasma (0·5 ml.) was diluted with 0·067 M phosphate buffer, pH 7·4 (2·0 ml.) and the solution vigorously shaken for at least 5 min. with Ag_3PO_4 (about 100 mg.).