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The Utilization of Non-protein Nitrogen in the Bovine Rumen

6. THE EFFECT OF METALS ON THE ACTIVITY OF THE RUMEN BACTERIA

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In their investigations on the utilization of urea in the bovine rumen Pearson & Smith (1943*b*) and Smith & Baker (1944) concluded that the bacteria which normally inhabit the rumen assimilate significant amounts of non-protein nitrogen (N.P.N.) and convert it to protein. The conclusion was based on the observed diminution of N.P.N. and the formation of protein in the liquid portion of rumen contents during incubation *in vitro* at 39° after addition of carbohydrate. This *in vitro* technique lends itself to the investigation of both the organic and mineral nutrilites which may be required by rumen bacteria, and in the present communication a description is given of experiments carried out to determine the effect of some of the more common metals on the activity of the rumen bacteria. The subject may have wide practical implications, for it is well known that diseases of stock resulting from mineral deficiencies are widespread and that the requirements of ruminants for some metals seem to be larger than those of other animals, a fact which suggests that the additional requirements may be needed for the intense bacterial activity which is characteristic of the normal rumen. The importance to livestock of metals such as copper and cobalt has been widely discussed in the last few years, and reviews on the subject have been published by Green (1944) and Owen (1947). In Western Australia copper deficiency leads to 'enzootic ataxia' in lambs and to 'falling disease' in cattle, and in Britain to 'sway-

back' in sheep. In many parts of the world diseases of cattle and sheep, such as the 'enzootic marasmus' of Western Australia and the 'vinquish' of Scotland are now known to be caused by cobalt deficiency. Not only deficiency but also slight excess of certain metals in the diet of ruminants may lead to disease. Perhaps the best known example of this is the 'teart' of Somerset, to which only ruminants are subject, and which occurs in animals grazed on pastures having a high content of molybdenum (Ferguson, Lewis & Watson, 1943).

The work described in the present paper was done in two sections. The object in the first section was to determine whether and at what levels copper, cobalt, molybdenum and iron were toxic to rumen bacteria, and in the second section to determine whether certain metals were essential for bacterial growth in rumen contents. Since the growth of rumen bacteria in pure culture is beset with many difficulties (Owen, 1947; Elsdon & Phillipson, 1948) it is not possible to study the mineral requirements by growing the bacteria in a synthetic medium purposely made deficient in a given element. Moreover, there are grave doubts as to whether results obtained with pure cultures would help much in elucidating the processes occurring in the normal rumen where a mixed population of bacteria and protozoa is always found (Baker, 1943) and where symbiotic relationships of one species of micro-organisms to another almost certainly obtain (Owen, 1948).

Even a technique of extraction of chelate complexes with organic solvents as used by Zentmyer (1944) is not at present applicable to rumen work. It is therefore necessary to use a technique such as that of Albert, Rubbo, Goldacre & Balfour (1947), in which synthetic organic compounds are used as chelating substances to form stable complexes with metals and thus render them unavailable to the micro-organisms. These chelating substances bind metals in non-toxic form though they may themselves give rise to complex ions. For example, with 2:2'-dipyridyl, iron forms the complex ferrous tridipyridyl ion, which remains in solution but binds iron so effectively that only one iron atom in 10^{15} remains in the simple ionic form (Albert & Gledhill, 1947). The effect of many of these chelating compounds on the growth of rumen bacteria *in vitro* has been investigated with the results now reported.

In the experiments described in the first section increasing amounts of salts of various metals were added to rumen liquid before incubation in order to demonstrate the toxic limits of the metals used. The experiments described in the second section, in which incubation occurred in the presence of chelating agents, were designed to demonstrate how much of a particular metal was required to permit protein synthesis. It has been possible to estimate the requirements of iron in this way.

EXPERIMENTAL

In both sections of the work the *in vitro* incubation technique of Pearson & Smith (1943*a, b*) was used. The procedure in a typical experiment was to remove through a permanent rumen fistula in a bullock or cow about 3 l. of semi-solid rumen ingesta. This material was strained through muslin to remove coarse vegetable matter, and then, for most experiments, centrifuged at 2000 rev./min. (approximately $800 \times g$) for times varying up to 5 min. to remove finer vegetable debris, protozoa and the larger zoogeal masses. The strained and centrifuged rumen liquid was divided into the required number of portions, and 1% maltose and 0.05% urea added to each. The urea was added to ensure that lack of N.P.N. would not be a limiting factor, for on some diets the N.P.N. level in rumen liquid can be very low. One of the portions acted as a control, while to the other portions known concentrations of metals or chelating agents were added. Where the salt of a metal such as Cu was added to some portions a Na or K salt was usually added to one other portion to ensure that any effect attributed to the metal was not caused by an increase in salt concentration *per se*. Samples from each of the incubation flasks were taken both before and after incubation for 4 hr. at 39°, and their N.P.N. content determined by the sodium tungstate method described by Pearson & Smith (1943*a*). It has been shown by Smith & Baker (1944) that a decrease in N.P.N. occurring during incubation is correlated with an increase in bacterial protein, and that an increase in N.P.N. is associated with a decrease in bacterial protein. In the present work a measure of bacterial activity was obtained from the changes which occurred in N.P.N. expressed in mg. N/100 g. rumen liquid.

RESULTS AND DISCUSSION

The tolerance of metals by rumen bacteria

Copper. It was realized that when a Cu salt was added to rumen contents some of the Cu would probably be adsorbed by the vegetable particles suspended in the rumen liquid, and that this would affect the amount of metal which the bacteria would appear to tolerate. Three separate tests were therefore made. In one of the tests (no. 3) the rumen liquid was strained through muslin and not centrifuged; in the other two (nos. 1 and 2) the protozoa and vegetable particles were partially or almost entirely removed by centrifuging the rumen liquid at 2000 r.p.m. for either 2 or 4 min. It will be seen from Table 1 that when 10 p.p.m. of Cu were added to the rumen liquid, which was relatively free from protozoa and vegetable particles (no. 1), the amount of N.P.N. converted to protein in 4 hr. was only 0.9 mg. N/100 g. rumen liquid compared with 12.0 mg. in the control which contained 50 p.p.m. of added Na but no added Cu. Higher concentrations of Cu up to 50 p.p.m. caused a similar decrease in the amount of protein synthesis, the values obtained varying from 0.6 to 1.9 mg. N/100 g. rumen liquid. (A value of 0.6 cannot be regarded as differing significantly from one of 1.9 when the difficulties involved in making the determinations are considered.) In the rumen liquid which had been centrifuged for only 2 min. and also in the uncentrifuged sample, bacterial growth appeared to be practically unaffected by the addition of 10 p.p.m. of Cu. It is probable, therefore, that in these samples the vegetable particles adsorbed some of the Cu and thus rendered that portion of it harmless to the bacteria.

With the sample centrifuged for 2 min. the addition of 100 p.p.m. of Cu resulted in a slight hydrolysis of the protein, whereas 1000 p.p.m. caused complete fixation. With the sample which was not centrifuged, increasing the amount of Cu reduced the amount of synthesis and ultimately hydrolysis tended to predominate and increase. These results are similar to those obtained by Pearson & Smith (1943*b*) for the effect of NaF in similar experiments with rumen contents. From the earlier work with fluoride and the present work with Cu it is clear that as the concentration of a toxic element increases, protein synthesis is reduced and hydrolysis gradually predominates until ultimately, when the concentration of the element is sufficiently high, enzyme action is completely inhibited and no change occurs. Since Na_2SO_4 at a concentration equivalent to 500 p.p.m. of Na had no marked influence, it can be concluded that the inhibitory effect when CuSO_4 was present was due solely to the Cu.

It is clear from the results in Table 1 that a concentration of 10 p.p.m. of Cu was the maximum

Table 1. *Effect of copper on the activity of rumen bacteria as judged by the amount of protein synthesis occurring in 4 hr. at 39°*

Exp. no.	Treatment of the rumen liquid before incubation	Substances added	Concentration of metal (p.p.m.)	Synthesis (+) or hydrolysis (-) (mg. N/100 g. rumen liquid)
1	Strained through muslin and centrifuged for 4 min. at 2000 rev./min. (relatively free from vegetable particles and protozoa)	Na as Na ₂ SO ₄	50	+ 12.0
		Cu as CuSO ₄	10	+ 0.9
			12	+ 0.6
			14	+ 1.6
			25	+ 0.8
			50	+ 1.9
2	Strained through muslin and centrifuged for 2 min. at 2000 rev./min. (many of the vegetable particles and protozoa removed)	Na as Na ₂ SO ₄	10	+ 11.7
		Cu as CuSO ₄	500	+ 10.6
			10	+ 10.2
			100	- 1.6
			1000	- 0.1
3	Strained through muslin but not centrifuged (rich in vegetable particles and protozoa)	No addition	0	+ 7.0
		Na as Na ₂ SO ₄	50	+ 6.4
		Cu as CuSO ₄	10	+ 7.1
			12	+ 2.8
			14	+ 2.3
			17	+ 1.8
			20	+ 0.4
			25	- 3.1
			33	- 1.4
			50	- 5.3

which could safely be present in the rumen liquid even when vegetable particles were present, since with 12.0 p.p.m. the amount of synthesis was greatly reduced.

Cobalt, molybdenum and iron. For the tests with these three metals rumen liquid strained through muslin and centrifuged for 2 min. at 2000 r.p.m. was used. The results for Co in Table 2 show that 10 p.p.m. brought about a reduction in the amount of protein synthesis, but that some synthesis occurred even with 100 p.p.m. With 1000 p.p.m., however, no measurable change in N partition occurred. The results for Mo (Table 2) were similar in that 10 p.p.m. reduced the amount of synthesis as compared with the control, but at least 2000 p.p.m. were required to reduce it to a negligible amount. With Fe, 10 and 100 p.p.m. did not appear to have any effect, but 1000 p.p.m. reduced the synthesis which occurred in 4 hr. to a negligibly small amount. At higher concentrations of Fe, up to 2000 p.p.m., protein hydrolysis predominated.

Of the metals tested, Cu was clearly the most toxic, and the rate of increase of toxicity with concentration was much greater with Cu than with the others. One instance has been published by Becker, Neal, Arnold & Rusoff (1938) in which feeding 2 g. Cu/1000 lb. body weight/day to cattle caused toxic effects. Assuming that the capacity of the rumen of a 1000 lb. animal is 20 gal., 2 g. Cu if given all at one time could give a concentration of 22 p.p.m. in the rumen contents. It may be, therefore, that the toxicity of the Cu was due at least in part to its effect on the rumen microflora. On the other hand,

Liebscher (1937) observed no toxic effects when 2.13 g. Cu were fed.

Table 2. *Effect of cobalt, molybdenum and iron on the activity of rumen bacteria as judged by the amount of protein synthesis occurring in 4 hr. at 39°*

Substance added	Concentration of metal (p.p.m.)	Synthesis (+) or hydrolysis (-) (mg. N/100 g. rumen liquid)
No addition	0	+ 11.9
Na as Na ₂ SO ₄	1000	+ 12.6
Co as cobaltous acetate	10	+ 8.8
	100	+ 3.4
	1000	+ 0.6
No addition	0	+ 11.6
K as KOH	1670	+ 11.3
Mo as K ₂ MoO ₄	10	+ 8.9
	100	+ 8.6
	1000	+ 5.3
	2000	+ 1.0
	4000	0.0
No addition	0	+ 10.1
Fe as FeCl ₃	10	+ 11.5
	100	+ 11.6
	1000	- 1.0
No addition	0	+ 6.4
Fe as FeCl ₃	100	+ 6.0
	1000	+ 0.8
	2000	- 1.6

On feeding 0.5 g. Co/1000 lb. body weight/day, an amount which would give a concentration of only 5 or 6 p.p.m. in the rumen liquid, Becker *et al.* (1938) and Keener, Percival, Morrow & Ellis (1947) ob-

served no toxic effects, and certainly from the results recorded here the possibility of depressing normal bacterial action in the rumen by therapeutic treatment with even quite large doses of Co seems very remote.

The present results for Mo suggest that the 'teart' of Somerset is not caused directly by interference with bacterial activity in the rumen. The disease occurs on pastures in which there is up to 100 p.p.m. of Mo in the dry matter, and this would be expected to give a concentration of less than 10 p.p.m. in the rumen contents.

The experiments with Fe showed that the rumen bacteria are not likely to suffer from Fe poisoning.

It is known that pure cultures of some organisms are inhibited by small amounts of Fe in the medium (1-3 p.p.m.) but this inhibition is strongly antagonized by citrate, so that the low toxicity of Fe in rumen liquid may be attributed in part to the organic acids which it is known to contain.

Experiments on the mineral requirements of rumen bacteria

The effect of some chelating agents. As in the first section the bacterial activity, obtained by incubating strained and centrifuged rumen liquid containing 1 % maltose and 0.05 % urea *in vitro* in the presence of the substances being tested, was estimated by the

Table 3. *Effect of various chelating agents (10^{-3} M) on bacterial activity in rumen liquid as judged by the amount of protein synthesis occurring on incubation for 4 hr. at 39°*

Reagent	Protein synthesis (+) or hydrolysis (-) mg. N/100 g. rumen liquid		Depression of synthesis expressed as % of synthesis in the control	Metals with which some of the agents were found by Albert & Gledhill (1947) to chelate at pH 6.5-7.3 and 37°
	Control (no chelating agent present)	Chelating agent added		
8-Hydroxyquinoline	+10.4	+ 1.4	87	Cd, Co, Cu, Fe ⁺⁺ , Fe ⁺⁺⁺ , Mn, Pb, Zn
Thioglycollic acid	+ 7.1	+ 7.5	—	Co
Cupron (benzoinoxime)		+ 6.8	—	Cu
Titan-yellow*		+ 7.8	—	Cu
Potassium ethyl xanthate		+ 4.9	31	Cd, Co, Cu, Pb
2:2'-Dipyridyl		- 0.8	>100	Fe ⁺⁺
Sodium diethyldithiocarbamate	+13.0	+ 7.8	40	Cd, Co, Cu, Fe ⁺⁺ , Fe ⁺⁺⁺ , Mn, Pb, Zn
Dithizone (diphenylthiocarbazone)		+11.2	Slight	Cd, Co, Cu, Pb, Zn
1-Nitroso-2-naphthol		+ 3.2	75	Co, Cu, Fe ⁺⁺ , Fe ⁺⁺⁺ , Mn, Pb, Zn
Cupferron (ammonium nitrosophenylhydroxylamine)		+11.1	Slight	Cu, Fe ⁺⁺ , Fe ⁺⁺⁺
Rubeanic acid (dithioxamide)		+ 4.1	68	Cd, Co, Cu, Pb, Zn
Dipicrylamine	+5.4	- 2.4	>100	—
Salicylaldoxime		+ 3.3	39	Cu, Fe ⁺⁺⁺ , Pb
o-Phenanthroline		- 1.2	>100	Fe ⁺⁺
Alizarin-S (sodium 1:2-dihydroxy- anthraquinone-3-sulphonate)		+ 4.1	Slight	—
Quinalizarin (tetrahydroxyanthra- quinone)		- 1.1	80	Cd, Co, Cu, Fe ⁺⁺ , Fe ⁺⁺⁺ , Mn, Pb, Zn
Nitroso-R-salt (sodium 1-nitroso-2- naphthol-3:6-disulphonate)	+5.1	+ 5.8	—	Co, Cu, Fe ⁺⁺ , Fe ⁺⁺⁺
2-Nitroso-1-naphthol		+ 3.5	31	—
Curcumin†		+ 5.6	—	—
Diphenylcarbazone		+ 4.8	—	Cu, Zn
Ammonium thiocyanate	+7.5	+ 8.7	—	—
Potassium ferrocyanide		- 8.5	—	—
Diacetyl monoxime		+ 6.9	—	—
Resorcinol		+ 7.8	—	—
Phenylhydrazine		+ 0.6	92	—
Triethanolamine		+ 6.6	—	—
Phenylthiohydantoic acid	+4.5	+ 2.1	53	Cd, Co, Pb, Zn
Chromotropic acid (sodium 1:8-di- hydroxynaphthalene-3:6-disulphonate)		+ 5.2	—	Cu, Mn, Pb
Thiourea	+8.1	+ 8.0	—	Cu
Benzidene sulphate		+ 6.8	Slight	—
Sodium 2-chloro-5-nitrotoluene-8- sulphonate		+ 7.3	—	—

* Sodium 4:4'-di-(6-methyl-2-benzthiazolyl)-diazaminobenzene-2:2'-disulphonate.

† 1:7-Bis-(4-hydroxy-3-methoxy-3-methoxyphenyl)-hepta-1:6-diene-3:5-dione.

change which occurred in N.P.N. concentration. The chelating agents were added in amounts sufficient to give a final concentration of 0.001M. The results of the tests made with thirty-one chelating agents are given in Table 3. Nineteen of these reagents were among those which had been shown by Albert & Gledhill (1947) to chelate with metals at pH 6.5-7.3 and 37°, conditions similar to those existing in the rumen incubation flasks in the present experiments. The remaining twelve were all chelating agents frequently used in the estimation of metals. Of the thirty-one reagents only eleven caused more than a 35% depression of synthesis as compared with the appropriate control. Probably most of these eleven caused the depression by chelating with metals particularly Fe, but at least one of them, phenylhydrazine, is well known as an enzyme inhibitor apart from its chelating properties. Further tests were made with some of the eleven inhibiting reagents to determine whether they exerted their effect by reducing the concentration of certain metals to less than the minimum essential for bacterial growth.

8-Hydroxyquinoline (oxine). The preliminary test (Table 3) indicated that synthesis was almost entirely depressed by the presence of 0.001M oxine. Further tests were made with concentrations ranging from 0.00006M to 0.004M. The results which are recorded under Exp. no. 1 in Table 4 showed that increasing the concentration of oxine caused the amount of synthesis to decrease until with 0.004M

hydrolysis predominated. Attempts were made to reverse the inhibition of oxine by adding a solution containing Co, Cu, Fe, Mn, and Zn in the proportions found in pasture (Mitchell, 1944). Sufficient of the metals was added to chelate with all the added oxine. The results recorded for Exp. 3 in Table 4 show that the mixture of metals only partially overcame the depression of synthesis caused by the oxine.

Further attempts to reverse the effect of oxine were made with CuSO_4 , FeSO_4 , CoCl_2 and a solution of the ash of rumen liquid. The metals were added in twice the amount believed to be necessary to combine with all the oxine. The results in Table 4 show that there was a very slight reversal with Cu and Fe but none with Co or with the ash solution. It was concluded from these experiments that either the oxine was not reacting solely with the metals, or the solutions used to reverse its effects did not have an optimal ionic balance.

Oxine as a chelating agent has the disadvantage that it is not sufficiently specific for bringing about a deficiency of any one metal since it forms complexes with so many (Albert & Gledhill, 1947). It was therefore decided to concentrate on the two reagents, 2:2'-dipyridyl and *o*-phenanthroline, which caused inhibition of synthesis in the preliminary tests (Table 3) and which were found to be specific for Fe under the conditions used by Albert & Gledhill (1947).

2:2'-Dipyridyl and o-phenanthroline. The results of the first experiment with these reagents are shown in

Table 4. *Effect of 8-hydroxyquinoline (oxine), when added to rumen liquid alone and also with various metals, on the amount of protein synthesized by rumen bacteria in vitro*

Exp. no.	Substance added to rumen liquid	Protein synthesis (+) or hydrolysis (-) (mg. N/100 g. rumen liquid)
1	None	+ 11.2
	8-Hydroxyquinoline { 0.00006M	+ 10.0
	{ 0.00025M	+ 7.3
	{ 0.001M	+ 4.4
	{ 0.004M	- 1.1
2	None	+ 6.8
	Metals only*	+ 7.8
	8-Hydroxyquinoline { 0.002M alone*	- 0.9
	{ 0.001M + metals*	+ 2.9
3	None	+ 10.4
	8-Hydroxyquinoline { 0.001M	+ 1.4
	{ 0.001M + CuSO_4 0.0005M	+ 3.1
4	None	+ 5.2
	8-Hydroxyquinoline { 0.001M	- 0.9
	{ 0.001M + CoCl_2 0.001M	- 0.2
	{ 0.001M + FeSO_4 0.001M	+ 1.2
5	None	+ 4.9
	8-Hydroxyquinoline { 0.001M	- 0.2
	{ 0.001M + solution of ash of rumen liquid	- 0.2

* The metals consisted of Co, Cu, Fe, Mn and Zn in the proportions found in pasture (Mitchell, 1944).

Table 5. The inhibitory effect of the reagents at a concentration of 0.001M was confirmed, and it was found that when just enough Fe to react with the added chelating agents was present protein synthesis was restored. Since these two agents have similar chelating properties and behave so similarly in rumen liquid, only one of them (*o*-phenanthroline) was used in further investigations. In later experiments, as shown in Table 5, it was found that as low a concentration as 0.0001M *o*-phenanthroline was sufficient to inhibit synthesis.

bacteria with the help of *o*-phenanthroline was pursued. Experiments were made in which the concentration of 'available' Fe in the rumen liquid was adjusted by the addition of *o*-phenanthroline and FeSO₄ in varying amounts. The 'available' Fe refers to the Fe in the rumen liquid not in combination with *o*-phenanthroline. By the method of Pyenson & Tracy (1945) it was found that the amount of Fe occurring naturally in the rumen liquid was between 1 and 2 p.p.m. It was calculated, therefore, that approximately 0.0001M *o*-phenanthroline would be

Table 5. *Effect on protein synthesis of adding 2:2'-dipyridyl, or o-phenanthroline alone and also in combination with iron or other metals*

Exp. no.	Substance added	Protein synthesis (+) or hydrolysis (-) (mg. N/100 g. rumen liquid)
1	None	+11.4
	<i>o</i> -Phenanthroline (0.001M)	- 0.9
	<i>o</i> -Phenanthroline (0.001M) + FeSO ₄ (0.00033M)	+11.2
	2:2'-Dipyridyl (0.001M)	- 0.3
	2:2'-Dipyridyl (0.001M) + FeSO ₄ (0.00033M)	+10.1
2	None	+ 6.7
	<i>o</i> -Phenanthroline (0.0001M)	0.0
	<i>o</i> -Phenanthroline (0.0001M) + CoCl ₂ (0.0001M)	+ 5.1
	<i>o</i> -Phenanthroline (0.0001M) + FeSO ₄ (0.0001M)	+ 9.7
	<i>o</i> -Phenanthroline (0.0001M) + CuSO ₄ (0.0001M)	+ 0.4
	<i>o</i> -Phenanthroline (0.0001M) + ZnSO ₄ (0.0001M)	+ 4.6
	<i>o</i> -Phenanthroline (0.0001M) + MnSO ₄ (0.0001M)	+ 2.0

To ascertain whether *o*-phenanthroline is entirely specific for Fe the effect of adding some other metals to rumen liquid containing sufficient *o*-phenanthroline to give complete inhibition was investigated. The results given in Table 5 showed that only Fe completely restored synthesis. In fact with this particular sample of rumen liquid extra Fe appeared to stimulate synthesis. Of the other metals tested Co and Zn produced significant reversals of inhibition which suggested either that these metals could be utilized by the bacteria in place of Fe or that they competed with Fe for *o*-phenanthroline and so released some Fe. The latter explanation seemed the more likely. An experiment was made, therefore, in which *o*-phenanthroline was added to a solution of Fe alone, and to solutions containing mixtures of Fe and Co or Zn. Only enough *o*-phenanthroline was added to react with the Fe in each solution. In the solutions containing mixtures of Fe and Zn or Co it was shown that some Fe was not chelated. This is in agreement with the findings of Fortune & Mellon (1938), who showed that in analytical work the presence of Zn and Co affected the formation of the ferrous *o*-phenanthroline complex. It was concluded, therefore, that these metals partially reversed the inhibition caused by *o*-phenanthroline by freeing some of the Fe from the chelate complex.

In view of these findings the original plan of attempting to assess the Fe requirement of the

sufficient to reduce the available Fe to zero. This was the minimum concentration of *o*-phenanthroline already found by experiment to be necessary to inhibit synthesis. Two experiments were made in which the available Fe in several samples of the same rumen liquid was adjusted, first by adding amounts of *o*-phenanthroline between 0.00002 and 0.0001M, and then by the addition of Fe as FeSO₄ in amounts up to approximately 4 p.p.m. The results given in Table 6 show that in both experiments protein synthesis was much reduced when the available Fe was made less than 1 p.p.m. by the addition of *o*-phenanthroline. On adding Fe to the rumen liquid in which all the naturally occurring Fe had been coupled with *o*-phenanthroline the addition of as little as 1 p.p.m. was all that was required to restore synthesis.

The iron content of rumen bacteria. Because the amount of Fe present in the rumen liquid of animals not having access to pasture lay between 1 and 2 p.p.m., and because slight stimulation of protein synthesis occurred on increasing the Fe content, it was thought that the naturally occurring Fe might be suboptimal on some diets. In one of the experiments (Exp. B, Table 6) an attempt was therefore made to estimate the Fe occurring naturally in the rumen organisms and in the 'cell-free' filtrate. The organisms were separated by centrifugation and washed twice with water. The Fe contained in the cells amounted to 0.43 p.p.m. in terms of the whole

Table 6. *Effect on protein synthesis of varying the concentration of available iron*

Incubation no.	o-Phenanthroline added	Exp. A				Exp. B			
		Fe bound by o-phenanthroline (p.p.m.) (a)	Added Fe (p.p.m.) (b)	Naturally occurring Fe 1.93 p.p.m. (c)		Naturally occurring Fe 1.38 p.p.m. (d)			
				Available Fe (p.p.m.) (b+c-a)	Protein synthesis (+) or hydrolysis (-) (mg. N/100 g. rumen liquid)	Available Fe (p.p.m.) (b+c-d)	Protein synthesis (+) or hydrolysis (-) (mg. N/100 g. rumen liquid)		
1	M/50,000	0.37	None	1.56	+4.5	1.01	+4.6		
2	M/40,000	0.47	None	1.46	+4.9	0.91	+1.0		
3	M/30,000	0.62	None	1.31	+5.0	0.76	+1.5		
4	M/20,000	0.93	None	1.00	+4.4	0.45	-0.1		
5	M/10,000	1.86	None	0.07	+0.8	None	-0.5		
6	M/10,000	1.86	0.47	0.54	+1.6	None	-0.7		
7	M/10,000	1.86	0.93	1.00	+3.9	0.45	+1.0		
8	M/10,000	1.86	1.41	1.48	+5.7	0.93	+2.5		
9	M/10,000	1.86	1.86	1.93	+5.9	1.38	+4.6		
10	M/10,000	1.86	3.72	3.79	+6.8	3.24	+3.6		
11	None	None	3.72	5.65	+6.6	5.10	+6.2		
12	None	None	None	—	—	1.38	+5.0		

rumen liquid, whereas the amount in the cell-free rumen liquid was 0.9 p.p.m. The two fractions therefore accounted for 1.33 p.p.m. of Fe, which agreed closely with the Fe content of 1.38 p.p.m. recorded in Table 6 for the whole rumen liquid. In another experiment the amount of Fe in the micro-organisms centrifuged from a sample of rumen liquid before incubation was 0.36 p.p.m. in terms of the whole rumen liquid, whereas after incubation it had increased to 0.65 p.p.m., thus demonstrating a concentration of Fe by the organisms. The fact that Co is concentrated by rumen bacteria from their environment has been shown recently by Tosic & Mitchell (1948).

SUMMARY

Using the diminution of non-protein nitrogen in bovine rumen liquid when incubated *in vitro* as an index of bacterial growth, the following observations were made.

1. In rumen liquid which was strained through muslin and centrifuged at 2000 r.p.m. (800 × g) for 2 min., the bacteria were able to tolerate 100 p.p.m. iron, 10 p.p.m. copper, somewhat less than 10 p.p.m. cobalt and between 100 and 1000 p.p.m. molybdenum.

2. Growth of the bacteria was definitely inhibited by 1000 p.p.m. iron, 25 p.p.m. copper, 1000 p.p.m. cobalt and 2000 p.p.m. molybdenum.

3. Of a series of organic reagents used in analysis of

solutions for metallic ions several were found which strongly inhibited bacterial growth. The inhibitions of growth produced by 8-hydroxyquinoline, 2,2'-dipyridyl and o-phenanthroline were further studied. The inhibition produced by 8-hydroxyquinoline did not seem to be due to a specific interference, for it could be reversed only in part by metallic ions or mixtures of metallic ions. The inhibitions due to the other two compounds were however completely reversed by adding iron and partly reversed by adding cobalt or zinc. It is suggested that the reversals of these inhibitions by cobalt and zinc were due to their reacting with some of the chelating agents and thus making some iron available.

4. With increasing concentrations of o-phenanthroline inhibition of bacterial growth increased. When just sufficient o-phenanthroline was added to cause complete inhibition of growth successively increasing amounts of added iron were found to cause increasing amounts of protein synthesis. By this means it was shown that 1–2 p.p.m. of iron in rumen liquid produced good growth.

5. The amount of iron associated with the micro-organisms was found to increase on incubation *in vitro*.

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The Turnover of Radioactive Phosphate Injected into the Subarachnoid Space of the Brain of the Rat

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In the literature little information is given on the rate of phosphorylations in the brain, and even the few studies which have been made in this field are without real value because of unsatisfactory experimental conditions and methods. Perhaps the main reason for this neglect is that many acidic substances hardly penetrate into the brain at all from the remainder of the organism. The 'blood-brain barrier' acts as an extremely effective obstacle to any exchange of such substances between the central nervous system and the rest of the organism, and thus makes the former with respect to permeability a virtually 'closed' system. Hevesy (1939*a, b*) and Hevesy & Hahn (1940) have shown that even a fairly long time after subcutaneous or intravenous injection of ^{32}P only a very small fraction of the substance could be found in the brain and a number of workers have, erroneously, interpreted these results as implying that the brain must have a very low turnover rate (Borell & Örstöm, 1947; Reiss, Badrick & Halkerston, 1949). This, however, by no means follows from the experimental findings.

Bakay & Lindberg (1949), in their investigations on the role of the cerebrospinal fluid in the metabolism of the brain, showed that ^{32}P injected intracisternally spread very rapidly into different parts of the rabbit's brain, and that after a relatively short time a considerable part of the newly introduced phosphate could be found in organic linkage. But these experiments did not yield a detailed picture of the metabolic conditions. The object of the present study was to investigate the extent and the rate of incorporation of the newly introduced phosphate into the various acid-soluble phosphorus fractions and also the turnover rate of these fractions inside the barrier.

EXPERIMENTAL

Operative procedure. White rats (100–150 g.) were injected with about 0.1 ml. of a solution containing approx. 0.1 mc. ^{32}P and a negligible amount of phosphate. The injections were made without anaesthetic as follows: the injection needle was placed in the sutura sagittalis on a level with the connecting line between the two eyes and forming an angle of about 45° with the frontal plane (see Fig. 1) and

Fig. 1. Intracranial injection on rats.

was introduced by rotation under moderate pressure. The injected solution thus enters the space between the bulbi olfactorii, whence it can spread easily in the subarachnoid space without injuring important motor or vegetative centres. Following the injection the condition of the animals was observed. Usually no change was noted. In the cases in which convulsions, paralysis, or other symptoms of relatively severe brain injury were observed, however, the animals were either rejected or used for longer experimental periods.

Preparation of samples. The animals were killed by drowning in acetone cooled to -60 to -70° with CO_2 , in which they died instantly. They were taken out about 15 min. after death and kept in solid CO_2 until required.

The brains were removed under continuous cooling with solid CO_2 with the aid of a hammer and chisel (see LePage, 1946). A small portion of the medulla oblongata (1–5 mg.) was placed directly in the electron counter to see whether the labelled phosphate had entered and spread in the brain.