further reduced by the highly acid conditions prevailing in the stomach. Any oxidation which these bacteria may accomplish in the alimentary canal will probably occur during passage through the small intestine, but it is extremely doubtful whether in this limited time more than a small percentage of the sterol would be oxidized. All attempts to recover the organisms from faeces have proved unsuccessful. In the case of the guinea-pig, the caecum has recently been identified (Schmidt & Hughes, 1942) as a principal site of the catabolism of cholic acid, the —OH groups of which are oxidized with formation of the corresponding keto-compounds. This oxidation is of a bacterial nature, as shown by the ability of *Alcaligenes faecalis* Castellani & Chalmers, isolated from the caecal contents, to effect the same oxidations *in vitro* (Schmidt, Hughes, Green & Cooper, 1942). The organism, cultured in serum, also oxidizes lithocholic acid and dehydroisoandrosterone to the corresponding keto-compounds (Hughes & Schmidt, 1942). In the author's experiments, neither *A. faecalis* nor the mixed flora of the human caecum has brought about the oxidation of cholesterol. The question, however, whether Δ^4 -cholestenone production *in vivo* is essentially a bacterial oxidation, or whether it results from the enzymic activity of alimentary secretions, still cannot be regarded as settled.

SUMMARY

1. Cholesterol degradation by *Proactinomyces erythropolis* Gray & Thornton *in vitro* was greatly influenced by pH changes in the medium. In buffered media, disappearance of cholesterol was almost complete in 4 months.

2. All *Proactinomyces* spp. tested experimentally oxidized cholesterol to Δ^4 -cholestenone in a varying degree when grown in mineral salt solution with the sterol as sole organic carbon source. It is concluded that oxidation of some of the sterol is effected by the cells of the inoculum, and that actual degradation of the molecule then occurs to provide available carbon for continued bacterial growth. Further oxidation to Δ^4 -cholestenone then occurs, and the process continues until the whole of the cholesterol has disappeared. Certain fractions representing degradation products have been isolated in large-scale experiments, but not as yet identified.

3. Oxidation to Δ^4 -cholestenone also resulted when cholesterol intimately mixed with garden soil was buried in the ground for 1 month.

4. In spite of evidence that *Proactinomyces* spp. are taken into the alimentary tract with certain foodstuffs, it is considered unlikely that these organisms play any appreciable role in the formation of Δ^4 -cholestenone *in vivo*.

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The Utilization of Urea in the Bovine Rumen

4. THE ISOLATION OF THE SYNTHESIZED MATERIAL AND THE CORRELATION BETWEEN PROTEIN SYNTHESIS AND MICROBIAL ACTIVITIES

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Pearson & Smith (1943) showed that when the liquid contents of the bovine rumen were incubated *in vitro* in the presence of certain carbohydrates a decrease in the non-protein nitrogen content of the liquid

occurred. This decrease was assumed to result from protein synthesis, and since the rate of decrease could be controlled by the addition of toxic substances, the protein synthesis was attributed to

biological agencies. An investigation of the microbial population of the rumen was then undertaken, by direct microscopic examination of formalized samples. The micro-organisms observed were shown by Baker (1943) to be distributed amongst a number of representative classes. Using this information Baker demonstrated that, over the period during which the decrease in non-protein nitrogen occurred, microbiological normality was maintained, the flora remaining normal in types though increased in numbers. Pearson & Smith concluded that if the amount of protein apparently synthesized was even an approximate indication of the amount which can be synthesized under favourable conditions in the intact rumen, the non-protein nitrogen and carbohydrate of the diet are together capable of supplying an appreciable proportion of the ruminant's protein requirements. To establish this conclusion it was necessary to find (1) whether protein-containing material is actually formed in sufficient quantities to account for the observed decrease in non-protein nitrogen, (2) whether this material is formed by or incorporated in the micro-organisms of the rumen, and (3) whether the synthesized material can be assimilated during its passage through the gastrointestinal tract.

The present paper embodies the results of experiments designed to meet these requirements by the isolation of the synthesized material in a form in which its microbial components can be identified and its biological value determined. In addition, an attempt has been made to elucidate some essential features of the microbial processes concerned, and to estimate the relative importance, as agents of synthesis, of the several classes of micro-organisms represented in the normal rumen.

METHODS

In vitro incubations and analyses. The methods of obtaining and incubating the samples of liquid rumen contents, and the general methods of analysis, were substantially the same as those described in a previous communication (Pearson & Smith, 1943).

Quantitative and qualitative microbiological examination. The fauna and flora of the rumen consist of: (1) Protozoa (oligotrich ciliates), (2) iodophile bacteria, i.e. bacteria which give a blue colour with iodine, and (3) aniodophile bacteria, i.e. bacteria which do not give a blue reaction with iodine.

The iodophile bacteria comprise: (a) large bacteria of the genera *Amylococcus*, *Amylosarcina*, *Amylobacterium*, *Amylospirillum* and *Oscillospira*, (b) small bacteria with no specifically distinct morphological characteristics (Baker, 1943). The Protozoa and the larger bacteria can be counted but not the other groups.

Counting procedure. The samples employed were made by diluting 25 ml. of rumen liquid with 75 ml. of approximately 3.5% formaldehyde solution. Each sample was well shaken and 1.0 ml. transferred to a flat-bottomed watch-glass. 1 ml. of Gram's iodine solution was added, the mixture

vigorously agitated with a glass rod and a drop of standard size transferred without delay to a Burkner cell with two counting chambers. Both chambers were filled simultaneously and two ruled areas were counted for each sample. The counts were made under an apochromatic 8 mm. lens with $\times 10$ eyepiece, using a $\frac{1}{2}$ cone. Sixty $\frac{1}{16}$ mm. squares were counted in each chamber, making a total of 120 counts/sample. Micro-organisms projecting over the upper and left-hand borders were reckoned together with the contents of the square. The figures in the present paper represent the total number of a particular type of micro-organism (e.g. *Amylococcus*, *Amylospirillum*, etc.) in 120 squares, divided by 20; they are given to the nearest decimal place. The sum of these figures for the large iodophile bacteria represents the total macroiodophile count. In counting macroiodophiles only forms showing a strong iodine reaction were counted. Individual *Amylococcus* chains of more than three cells were reckoned as one unit. Chains of less than four cells were ignored. Each individual group of *Amylosarcina* or *Amylobacterium* and each *Oscillospira* trichome was reckoned as a unit. Every *Amylospirillum* of more than three turns was regarded as one unit. Forms of less than three turns were ignored. Made in this way the macroiodophile counts afford an index of the density of the members of the macroiodophile population which react with iodine to give a blue colour under the conditions specified. Other factors such as the synthesis and storage of starch by the bacteria may contribute to an increase in density in addition to cell multiplication. This is discussed in the interpretation of the results.

Volume of sediment. During the incubation of rumen contents the microflora tend to associate in zoogloea-like masses. The enumeration of individual micro-organisms in such masses is impracticable. The material can be readily sedimented, and the volume accumulating under different conditions affords an index of the amount of zoogloea formed and therefore of the combined bulk of the microiodophile and aniodophile bacteria. The figures recorded under this heading represent the volume of sediment obtained from 10 ml. of formalized sample when centrifuged for 1 min. at 1000 r.p.m.

Iodine reaction of the sediment. The increase in iodophile bacteria resulted in extensive synthesis of iodophilic polysaccharide. The intensity of the reaction of the sediment with iodine, therefore, afforded an independent macroscopic indication of the microbiological changes. The supernatant liquid was poured off from the sediment and the volume made up to 10 ml. with iodine solution. The mixture was well shaken and allowed to stand for a quarter of an hour. The iodine solution was then decanted and replaced by successive changes of water, centrifuging for 1 min. at 1000 r.p.m. after each change. The colour of the sediment was then examined against a white card.

EXPERIMENTAL AND RESULTS

The isolation of the protein-containing material

One of the first essentials was to find whether the protein-containing material (termed the 'sediment' throughout the remainder of this paper) could be readily isolated from the rumen liquor before and after incubation. For this purpose the following experiment was done.

A sample of rumen liquor was centrifuged for 15 min. at 2000 r.p.m., and the supernatant liquid divided into two

equal portions. (Centrifuging in this way was found in later experiments to give a supernatant liquid in which the amount of synthesis was readily determined.) One of the two portions was incubated with 1% maltose and 0.1% urea, the other with approximately 1% glycerol and 0.1% urea. Both incubations continued at 38° for 6 hr. Small samples were removed before and after incubation for the determination of non-protein nitrogen (N.P.N.) by the tungstate method, and also of pH and total nitrogen. 50 ml. were also removed and treated with 50 ml. of dilute formalin. The sediment in these formolized samples was isolated and examined on both a micro- and macro-scale. On the micro-scale 10 ml. samples were centrifuged at 3600 r.p.m. for 20 min. The supernatant layer was decanted into a Kjeldahl flask; the sediment mixed with 5 ml. dilute formalin, recentrifuged, and the second supernatant layer added to the first. Nitrogen was estimated in the supernatant liquid and also in the total sediment. On a large scale 50 ml. samples of the formolized samples were centrifuged at 3000 r.p.m. for 30 min. in weighed tubes. The supernatant layer was removed and the residue stirred with 20 ml. ethanol and recentrifuged. The residue from the second centrifugation was dried, weighed and its nitrogen content determined.

The more important results, recorded in Table 1, lead to the following conclusions. (1) As in the previous experiments (Pearson & Smith, 1943),

than the bottom fractions which were not only rich in bacteria but also contained much vegetable matter and great numbers of Protozoa. This observation, together with the fact that the synthesized material could now be isolated in a crude form, led to experiments to find whether by centrifuging the rumen liquor for different times and at different speeds, a fraction could be obtained which would not only be relatively simple in its microbial population but would also possess relatively great synthesizing power. It was hoped that detailed biochemical and microbiological examination of such a fraction would give valuable information as to the nature of the agents which promote the synthesis and would yield a sediment rich in synthesized material whose biological value could then be determined. The following experiments were carried out.

A large sample of rumen liquor was strained through muslin and divided into four parts, *A*, *B*, *C* and *D*. *A* was incubated with 1% maltose and 0.1% urea for 5½ hr. at 38°. *B*, *C* and *D* were centrifuged for the times and speeds recorded in Table 2. The top fractions so obtained were then incubated under conditions identical with those for the incubation of *A*. Before and after incubation small samples were removed for the determination of pH, bac-

Table 1. *The amount of synthesis or decomposition in rumen liquor estimated by isolating the dried sediment and by N.P.N. determinations*

(All values are recorded in mg./100 g. rumen liquor; increase is indicated by (+) and decrease by (-).)

Substance added	Large-scale treatment of formolized samples		Small-scale treatment of formolized samples		Change in N.P.N. by sodium tungstate method
	Change in weight of sediment during the incubation	Change in the protein-N of the sediment	N converted to protein, calculated from N.P.N. of supernatant layers	Change in the N of the sediment	
Maltose	+152.2	+6.7	+6.2	+6.4	-8.1
Glycerol	-16.5	-1.4	-0.2	-0.8	+0.7

maltose promoted synthesis whereas glycerol not only failed to do so but tended to cause protein hydrolysis. (2) The sediment containing the synthesized material can be isolated as an amorphous powder. (3) The amount of protein synthesis determined by isolating the sediment was of much the same order whether the isolation was on a micro- or macro-scale. In this particular experiment the synthesis found by isolating the sediment was slightly less than that estimated from the decrease in N.P.N. by the tungstate method.

The effect of different rates and times of centrifuging

Pearson & Smith (1943) have shown in one experiment that, when rumen liquor was centrifuged for 5 min. at 1000 r.p.m., the top layers of the supernatant liquid which contained great numbers of many different types of bacteria gave more synthesis

terial counts, total nitrogen and N.P.N. by the tungstate method. For the quantitative isolation of the 'sediment' 100 ml. samples were treated with 10 ml. formalin. These formolized samples were centrifuged for 30 min. at 3000 r.p.m. and the supernatant layers removed. The residues were stirred well with dilute formalin, the mixtures recentrifuged and the second batch of supernatant layers added to the first. The sediment was then washed twice with ethanol and once with ether, the mixtures being centrifuged after each washing. The ethanol-ether washings (*E*) were united and their nitrogen content determined. The sediment was dried overnight in a vacuum desiccator with P_2O_5 and finally in an oven at 100° for 1½ hr. It was then weighed. The aqueous supernatant liquors were heated for 10 min. at 100° with sufficient trichloroacetic acid to give a concentration of 10%. A precipitate (*F*) was formed which was filtered off and washed. This precipitate would contain any protein which was soluble in water or which was too finely dispersed to be centrifuged down with the sediment. The nitrogen contents of both the precipitate and the filtrate were determined. In this way the total nitrogen of the liquors before and after incubation was divided into 'sediment' nitrogen (*S*), nitrogen removed

Table 2. *Biochemical and microbiological results for incubations of rumen liquor before and after centrifuging*

Centrifugation of rumen liquor ...	(Nitrogen results are recorded in mg./100 g. rumen liquor.)											
	A			B			C			D		
	Uncentrifuged rumen liquor			1000 r.p.m. for 3 min.			2000 r.p.m. for 15 min.			3000 r.p.m. for 25 min.		
	Initial	Final	Change	Initial	Final	Change	Initial	Final	Change	Initial	Final	Change
pH	7.6	6.0	- 1.6	7.6	6.1	- 1.5	7.6	6.2	- 1.4	7.6	6.5	- 1.1
N removed by ethanol- ether (<i>E</i>)	3.2	5.3	+ 2.1	0.8	2.1	+ 1.3	0.4	1.7	+ 1.3	0.2	1.2	+ 1.0
Protein N not in 'sediment' but precipitated by trichloroacetic acid (<i>F</i>)	18.2	12.5	- 5.7	18.6	16.1	- 2.5	17.5	15.6	- 1.9	15.4	13.9	- 1.5
True N.P.N. not precipitated by trichloroacetic acid	55.3	40.5	- 14.8	54.5	41.1	- 13.4	57.9	43.1	- 14.8	59.5	47.3	- 12.2
Total N.P.N. as determined by tungstate method	60.1	43.5	- 16.6	55.2	35.5	- 19.7	56.7	38.1	- 18.6	55.9	43.5	- 12.4
Sediment nitrogen (<i>S</i>)	101.6	118.6	+ 17.0	11.3	29.0	+ 17.7	7.2	24.8	+ 17.6	3.2	16.0	+ 12.8
True synthesis of protein, i.e. change in <i>S</i> corrected for change in <i>F</i>	—	—	11.3	—	—	15.2	—	—	15.7	—	—	11.3
Weight of sediment, mg./100 g. liquor	1388	1615	+ 227	127	358	+ 231	77	366	+ 289	33	256	+ 222
% N in the dry sediment	7.3	7.3	—	8.9	8.1	- 0.8	9.3	6.8	- 2.5	9.7	6.3	- 3.4
Microbiological counts*												
Protozoa	17	12	- 5	0.6	0	- 0.6	0	0	0	0	0	0
<i>Amylococcus</i> chains	21	44	+ 23	9	27	+ 18	2	13	+ 11	0.6	5	+ 4.4
<i>Amylospirillum</i>	2	4	+ 2	2	3	+ 1	0	0	0	0	0	0
<i>Amylobacterium</i>	0.4	0.9	+ 0.5	0.3	0.8	+ 0.5	0	0	0	0	0	0
Total macroiodophile bacteria	23	49	+ 26	11	31	+ 20	2	13	+ 11	0.6	5	+ 4.4
Volume of zoogloea (ml.)	3.0	4.5	+ 1.5	0.1	0.5	+ 0.4	0.1	0.7	+ 0.6	0.1	0.5	+ 0.4
Iodine reaction	Some	Some		None	Strong		None	Strong		None	Strong	

* Total number per 60 × $\frac{1}{16}$ mm. squares/20 (see text).

from the wet sediment by ethanol-ether (*E*), protein nitrogen not contained in the sediment but precipitated by trichloroacetic acid (*F*), and true N.P.N. unprecipitated by trichloroacetic acid.

The results (Table 2) lead to the following conclusions:

(1) In all five incubations some of the protein nitrogen (*F*), not initially in the sediment but precipitated by trichloroacetic acid from the supernatant liquid, was either decomposed during incubation or was transferred to the sediment. This was particularly noticeable in *A* but much less so in *B*, *C* and *D*. To estimate the true amount of protein synthesis, the increase in sediment nitrogen (*S*) must be corrected for this change in *F*.

(2) The true protein synthesis attained a maximum of 15–16 mg. N/100 g. liquor in fractions *B* and *C*, where the liquor had been centrifuged for 3 min. at 1000 r.p.m. and for 15 min. at 2000 r.p.m. In the uncentrifuged material (*A*) and in the material centrifuged for 25 min. at 2000 r.p.m. (*B*) it was

much less. It has been shown for uncentrifuged liquor (Pearson & Smith, 1943) that protein hydrolysis and synthesis appear to proceed concurrently and that either one or the other may predominate according to the conditions. It is probable, therefore, that as much synthesis actually occurred in *A* as in *B* and *C*, but that a greater amount of hydrolysis also occurred. That the synthesis in *D* was less than in *B* and *C* may have been due to the fact that the more intensive centrifuging had greatly reduced the number of micro-organisms initially present so that there were fewer to multiply.

(3) In the four incubations the actual weight by which the sediment increased was 0.22–0.29 g./100 g. rumen liquor. In *A*, the initial sediment was so great that this was equivalent to a change of only 16%, but in *B*, *C* and *D*, where the initial sediment was very much smaller, due to the fact that much of the suspended matter had been removed in the preliminary centrifuging, the weight of sediment was increased almost 3, 5 and 8 times respectively.

D gave a sediment of which at least 80 % was synthesized during incubation.

(4) Synthesis in itself is obviously not dependent on the presence of Protozoa but is accompanied by a great increase in the numbers of micro-organisms, particularly iodophile types which synthesize polysaccharide and give an intense blue iodine reaction to the medium. In *D*, and to a less extent in *B* and *C*, the nitrogen percentage of the final sediment was less than in the initial. This suggests that the synthesis of protein is accompanied by an even greater synthesis of non-protein substances which, from other evidence, consist mainly of polysaccharide.

(5) As in the previous experiment the amount of synthesis calculated from the changes in N.P.N. by the tungstate method was generally higher than the synthesis shown to have occurred by isolating the 'sediment'.

Incubations without added carbohydrate and in the presence of glycerol, glucose or maltose

The technique just described for iodophile counts and also for arriving at the true synthesis by isolating the sediment and applying a correction for the difference in protein nitrogen precipitated by trichloroacetic acid was now applied to compare incubations in which no carbohydrate was added with those in which glycerol, glucose or maltose was present. Since in the previous experiment maximum synthesis was observed in a fraction which had been obtained by centrifuging at 2000 r.p.m. for 15 min., this speed and time were adopted in the present experiment. A large volume of centrifuged liquor was divided into four parts which were all incubated with 0.1 % urea. One part contained no added carbohydrate; each of the other three contained

1 % glycerol, 1 % glucose or 1 % maltose. Since the initial and final values for the chemical data were of a similar order to those recorded for incubations *B* and *C* in the previous experiment (Table 2), only the actual changes observed during incubation and microbiological data are recorded in Table 3. They lead to the following conclusions: (1) During incubation in the absence of added carbohydrate or with glycerol, hydrolysis tended to predominate. This was shown by a small increase in N.P.N. and by a small decrease in the weight of the sediment and in the nitrogen which it contained. With glucose and maltose, on the other hand, marked synthesis was observed. (2) Protein synthesis appeared to be accompanied by synthesis of polysaccharide. The fact that the nitrogen percentage in the final sediment was so much less than it was in the initial again suggests that the increase in polysaccharide may have been considerably greater than the increase in protein. (3) The iodophile counts, volume of zoogloea and iodine reaction greatly increased when synthesis predominated but decreased or showed little change when hydrolysis predominated. (4) The tungstate method of N.P.N. again gave results for protein synthesis which were higher than those found by isolating the sediment and correcting for protein nitrogen not contained in the sediment but precipitated by trichloroacetic acid (*F*).

In this experiment maltose and glucose gave rise to very similar amounts of protein synthesis, whereas in earlier work (Pearson & Smith, 1943) maltose was superior to glucose. The explanation for this difference may lie partly in the nature of the rumen sample used for incubation and partly in the fact that in the work now being discussed both sugars were present to the extent of 1 % and incubation

Table 3. *Protein synthesis and iodophile counts for incubation in absence of added carbohydrate and in presence of glycerol, glucose or maltose*

(Nitrogen results are recorded in mg. N/100 g. rumen liquor.)

	No added carbohydrate		Glycerol		Glucose		Maltose	
Change in pH*	+ 0.1		- 0.1		- 1.2		- 1.2	
Change in protein N not in the sediment but precipitated by trichloroacetic acid (<i>F</i>)	- 1.9		+ 0.4		- 5.9		- 6.7	
Change in N.P.N. not precipitated by trichloroacetic acid together with the small amount of N removed by ethanol-ether from the sediment	+ 1.7		+ 1.3		- 12.5		- 11.7	
Change in N.P.N. (tungstate method)	+ 0.3		- 0.7		- 15.1		- 14.6	
Change in sediment N (<i>S</i>)	- 1.2		- 1.7		+ 18.6		+ 18.2	
True synthesis of protein (<i>S</i> corrected for <i>F</i>)	- 3.1		- 1.3		+ 12.7		+ 11.5	
Change in weight of sediment, mg./100 g. rumen liquor†	- 15		- 16		+ 346		+ 339	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
N in dry sediment (%)	10.3	10.6	10.7	10.8	10.5	6.9	10.3	6.8
Counts of total macroiodophile bacteria	2	0	3	0	2	53	2	50
Volume of zoogloea (ml.)	0.1	0.1	0.1	0.1	0.1	1.0	0.1	1.0
Iodine reaction	None	None	None	None	None	Strong	None	Strong

* The initial pH was 6.9.

† The initial sediments were respectively 146, 148, 149 and 146 mg./100 g. liquor.

proceeded for 2-3 hr., whereas in the earlier work their initial concentration was only 0.3% and incubation continued for 5½ hr.

The correlation between synthesis and iodophile counts

The technique just described for making iodophile counts and estimating true synthesis by isolating the synthesized material and correcting for *F* was applied in three types of experiment to determine whether synthesis was always accompanied by an increase in the microbial population of the medium. One example of each type will be cited.

(a) *Changes in rumen liquor during incubation.* In the previous experiments samples were taken for examination and analysis before and after incubation for 5½ or 6 hr. In the present experiment

Fig. 1. Synthesis and macroiodophile counts estimated at intervals during an incubation of rumen liquor in the presence of added urea and maltose. The relative volume of sediment gives a measure of zoogloea formation.

samples were taken at the beginning and then after ½, 1, 2, 4 and 6 hr. so that a study could be made of the changes occurring as incubation proceeded. The results are illustrated in Fig. 1. The weight of sediment increased from 176 mg./100 g. rumen liquor initially to 495 mg. after 2 hr., while protein synthesis in the same period was equivalent to 16.3 mg. N or 102 mg. protein/100 g. rumen liquor. Thereafter the total weight of sediment decreased slightly while the amount of protein in the sediment remained almost constant, with a slight tendency to decrease towards the end of the 6 hr. The intensity of the iodine reaction (not shown in the figure) and the volume of sediment, which is a measure of the zoogloea formation, followed a course similar to that of the sediment weight. Up to 2 hr., a period

in which the pH was within the limits normally encountered in fresh rumen contents, synthesis was accompanied by roughly corresponding increases in the iodophile population of the medium.

Since the total weight of sediment increased by 319 mg./100 g. rumen liquor in the first 2 hr., while the corresponding increase in protein was only 102 mg., it is evident that some substance was being elaborated much more rapidly than protein. Later, during the remaining 4 hr. when the sediment was tending to diminish, this substance tended to decrease slightly while the protein remained almost constant. The intensity of the iodine reaction at intervals during incubation suggested that the substance, which was synthesized and also hydrolyzed more rapidly than protein, was probably a starch-like polysaccharide which stained blue with iodine and which by direct microscopic examination could be seen to exist within the cells of the iodophile bacteria. This suggestion was confirmed by a further experiment, with an entirely different sample of rumen liquor in which the polysaccharide content of the sediment was estimated by the gravimetric cuprous oxide method after hydrolysis with HCl. The results recorded in Table 4 show that during

Table 4. *The increase of protein and polysaccharide during incubation for 6 hr.*

Duration of incubation (hr.)	Sediment (mg./100 g. rumen liquor)	N content of the sediment (%)	Protein content of the sediment ($N \times 6.25$) (mg. protein/100 g. rumen liquor)	Poly-saccharide content of the sediment (mg./100 g. rumen liquor)
0	49.4	8.1	25.0	6.4
1	69.3	6.7	28.8	18.4
2	98.0	6.3	38.8	28.0
4	162.4	6.5	65.6	62.0
6	196.2	6.1	75.0	78.6

the first hour the protein of the sediment increased by only 3.8 mg./100 ml. rumen liquor, whereas the polysaccharide increased by 12.0 mg. After 2 hr. the polysaccharide formation was almost twice as great as the protein formation, while the nitrogen content of the sediment, which was originally 8.1%, had decreased to 6.3%. By direct microscopical examination it can be observed that initially some of the iodophile bacteria are almost entirely depleted of the starch-like polysaccharide. It may be, therefore, that in the early stages of incubation in the presence of readily available sugar or starch the process of building up reserves of this polysaccharide greatly predominates over actual cell division. Later, when multiplication reaches a maximum and begins to decline, as it did after 2 hr. in the previous experiment (Fig. 1), the polysaccharide reserves tend to

diminish appreciably before any hydrolysis of protein can be observed.

(b) *The effect of different amounts of maltose.*

A sample of rumen liquor which had been centrifuged for 10 min. at 2000 r.p.m. was divided into five portions. One portion was incubated without maltose and the others with amounts of maltose varying from 0.21 to 0.84 %. All five portions contained 0.1 % urea. Samples were analyzed at the beginning and after 4 hr. The results (Table 5) again

hydrolysis (Pearson & Smith, 1943). The following experiment was undertaken to determine whether the decreased synthesis caused in this way with centrifuged liquor was accompanied by a corresponding decrease in the weight of the sediment and in bacterial count. A sample of centrifuged liquor was divided into four portions, all of which were incubated for 3 hr. with 1 % maltose and 0.1 % urea. The first sample was incubated without NaF; the others contained 0.005, 0.1 and 0.5 % NaF respec-

Table 5. *Synthesis and iodophile counts for incubations in the presence of different concentrations of maltose*

Mean initial values	pH	True protein synthesis (mg. N/100 g. liquor)	Change in weight of sediment over the initial weight (mg./100 g. liquor)	N in the sediment (%)	Iodophile counts			Iodine reaction	Volume of zoogloea (ml.)
					<i>Amylo-coccus</i>	<i>Amylo-spirillum</i>	Total		
	7.0	—	—	10.1	1	1	2	Slight	0.1
Concentration of maltose (%)									
None	7.6	0.3	-14	10.9	0	0	0	Slight	0.1
0.21	7.2	3.7	55	10.3	4	5	9	Strong	0.2
0.42	6.9	6.4	91	9.7	10	25	35	Strong	0.5
0.63	6.3	9.7	207	8.5	19	30	49	Very strong	0.5
0.84	5.8	11.5	358	7.4	24	35	59	Very strong	0.9

Table 6. *Synthesis and iodophile counts in the presence of NaF*

Mean initial values	pH	Change in sediment N (mg./100 g. liquor)	Change in weight of sediment, compared with the initial value (mg./100 g. liquor)	Iodophile counts			Volume of zoogloea (ml.)	Iodine reaction
				<i>Amylo-coccus</i>	<i>Amylo-spirillum</i>	Total		
	6.1	—	—	2	1	3	0.5	Very weak
NaF (%)								
None	5.5	13.7	311	17	3	20	1.0	Strong
0.005	5.6	12.7	303	15	4	19	1.0	Strong
0.1	5.7	2.8	106	8	2	10	0.5	Weaker
0.5	6.1	-5.7	-44	3	1	4	0.5	Very weak

show that protein synthesis is accompanied by a relatively large increase in the weight of sediment, in the volume of zoogloea and also in the iodophile count and in the intensity of the iodine reaction. Synthesis of both protein and polysaccharide increased with corresponding increases in the initial concentration of maltose. In the absence of maltose no protein synthesis occurred and the weight of sediment and iodophile count showed a small decrease.

(c) *The effect of sodium fluoride.* In uncentrifuged rumen liquor increasing concentrations of NaF decrease the protein synthesis and bring about protein

tively. The results are recorded in Table 6. With no NaF and with 0.005 % NaF protein synthesis was accompanied by an increase in sediment weight and iodophile count and by an intensification of the iodine reaction. When the protein hydrolysis predominated, as with 0.5 % NaF, the weight of sediment decreased slightly and there was no significant change in the iodophile count or in the intensity of the iodine reaction. With 0.1 % NaF intermediate values were obtained. These observations all indicate a general correlation between synthesis of protein and of polysaccharide on the one hand and multiplication of the iodophile population on the other.

In previous experiments (Pearson & Smith, 1943), 0.1% NaF caused very marked hydrolysis to predominate, whereas in the present experiment a small amount of synthesis was observed. This difference is almost certainly due to the fact that in the previous work uncentrifuged liquor was used. The large numbers of Protozoa and possibly also the quantities of vegetable matter present in uncentrifuged liquor result in a greatly increased protein hydrolysis in the presence of NaF.

Isolation of the sediment in amounts suitable for detailed examination

In order to prepare large samples of the sediment before and after incubation the following series of experiments were carried out.

Rumen liquor (2.5 l.) was centrifuged at 2000 r.p.m. for 10 min. and 1% maltose and 0.1% urea added to the supernatant liquid. One litre (A) of the liquid was then removed from the mixture for the preparation of the initial sediment and the remainder incubated at 38° for 4 hr. At the end of this period a second litre (B) was removed for the preparation of the final sediment. Formaldehyde (50 ml. of 36% (w/v)) was added to A and B to preserve them. In the present experiment some 10 l. of supernatant liquid were treated in this way. The formalized samples, 5 l. of A and 5 l. of B, were centrifuged for 30–40 min. at 3000 r.p.m. The sediments so obtained were transferred to volumetric flasks with water, made up to a known volume and the mixture well shaken. From both initial (A) and final (B) samples half the mixture was withdrawn by pipette and centrifuged. The sediment was washed twice with water and dried in a vacuum desiccator (method I). The other half was centrifuged, washed four times with ethanol, once with ether and then air-dried (method II). Method I gave a very dark green brittle glass-like substance which was readily reduced by pestle and mortar to a green-grey powder. In method II, the ethanol and ether removed the green colouring matter and left a white amorphous powder which was readily dried in air. The samples before incubation each amounted to approximately 1 g., containing 10.8% ash and just over 50% crude protein. After incubation the corresponding yield was 4 g., containing 5.3% ash and 33% crude protein. From the various results which have just been described, this relative decrease in the content of ash and protein can be ascribed mainly to an increase in polysaccharide. In another experiment, after incubation, a 7 g. sample of sediment was prepared by method II and analyzed. Polysaccharide was determined by hydrolyzing a portion of the material with dilute HCl and estimating the reducing sugar by the usual gravimetric cuprous oxide method. Lipoid matter was determined by saponification with ethanol-KOH, followed by ether extraction. Glucosazone (m.p. 203° decomp.) was readily prepared from the sediment after hydrolysis with HCl. No reducing sugar was present before hydrolysis.

The results of the analysis of the synthesized material are shown in Table 7, where they are compared with typical results recorded for linseed cake. The similarity in the general composition of the two products is most striking.

Table 7. *The analysis of the synthesized material from the rumen liquor compared with typical analytical figures for linseed cake*

	Rumen material (%)	Linseed cake (%)
Moisture	0.5	0.5
Ash	6.2	5.8
Crude protein (N × 6.25)	36.3	33.0
Total carbohydrate	46.6	50.0
Lipoids	9.5	10.7
	99.1	100.0

The values for the sediment are those found for a typical sample after washing it with ethanol and ether. The values for the linseed cake were obtained from values recorded in standard tables and corrected to 0.5% moisture.

Microscopical examination of dried sediment

Preparations of the dried sediment before and after incubation were examined microscopically to determine their microbiological characteristics, and to form a rough estimate of the kind and amount of plant residue which they contained.

Examination of the initial sediment prepared from unincubated material stained with iodine showed the presence of macroiodophile species in small numbers only, *Amylococcus* being the prevalent type. Most of the cocci were in pairs, chains being infrequent. Many of them did not react at all strongly with iodine. Individual fragments of the abundant zoogloea gave a pale yellow to brown reaction. These particular fragments appeared to be composed in large part of minute rods, cocci and vibrios which gave no blue colour with iodine. Similar micro-organisms were dispersed throughout the preparation.

Examination of the sediment from incubated material showed that pronounced changes had taken place. Macroiodophiles were present in very large numbers, the majority giving a dense, almost black, reaction. Some forms which did not give a blue colour with iodine were also observed. *Amylococcus* was again the prevalent type. Chains of 4–8 cells were frequently observed. *Amylospirillum*, *Amylosarcina*, and *Amylobacterium* were also identified. The iodine reaction of the zoogloea was much intensified. The micro-organisms present were similar to those in the unincubated sediment, but a great increase in the number of forms which reacted with iodine was apparent. Amongst such microiodophile species, coiled and interlacing chains of vibrios were conspicuous.

In order to detect fragments of vegetable material, the sediment was examined by polarizing microscope and in ultra-violet light. The latter method, which discloses the intrinsic fluorescence of encrusting substances, was found more convenient than the former, which makes use of the birefringence of cellulose

structures. Preparations were made up in dilute ammonium hydroxide, which intensifies fluorescence. Examination of both incubated and unincubated material showed that plant residues were present only in very small amounts. Most of the fragments were identified as broken or detached epidermal hairs. In addition, isolated annular and small spiral vessels and pollen grains were observed. The proportion of plant residue to bacterial matter was greater in the unincubated than in the incubated material. In both sediments a few of the smaller kinds of ciliates could be detected.

These results entirely confirmed those obtained by the microscopic examination of formalized samples. They demonstrated also that the micro-organisms, of which the sediment is almost entirely composed, had undergone very little structural alteration in consequence of the procedures to which they had been subjected. Samples of these sediments are now being prepared from rumen liquor without addition of formalin so that their vitamin content and biological value may be determined.

DISCUSSION

Earlier experiments by Pearson & Smith (1943) showed that when rumen liquor is incubated under favourable conditions at 38°, the N.P.N. content of the mixture decreases while the total nitrogen remains constant. Pearson & Smith assumed therefore that protein was synthesized. The present experiments have confirmed this assumption and have shown that the decrease in N.P.N. is accompanied by the accumulation of a bulky sediment which gives an intense blue colour with iodine. This sediment is readily isolated and has been found to consist almost entirely of bacteria. It contains a starch-like polysaccharide and at least the main portion of the synthesized protein. In all experiments where protein synthesis occurred there was also a corresponding increase in the numbers of iodophile bacteria and a synthesis of polysaccharide. When the conditions were unfavourable to bacterial multiplication, i.e. in the absence of added carbohydrate or in the presence of NaF, neither protein nor polysaccharide synthesis was observed.

By centrifuging the rumen liquor before incubation, protein synthesis was shown to occur in the absence of Protozoa. The chief contributors to the protein synthesis are obviously the iodophile bacteria. They comprise a variety of macroiodophile and microiodophile species (Baker, 1943). Figures for the macroiodophiles indicate that *Amylococcus* was the predominating species in most of the incubations, though on occasion *Amylospirillum* and *Amylosarcina* reached comparable densities. Removal of the greater portion of the macroiodophiles by centrifuging showed, however, that the observed increases

in the numbers of macroiodophiles in centrifuged liquor cannot account for more than a small fraction of the observed synthesis. From direct microscopical examination and other considerations there can be no doubt that the bulk of the synthesized polysaccharide and protein is incorporated in the small rods, cocci and vibrios of the microiodophile population.

Not all the organisms present in the sediment react with iodine. Among the non-reacting types are some, such as spirochaetes, which appear to synthesize starch-like polysaccharide. Direct observation shows that these do not accumulate in sufficient numbers to have any quantitative significance. Other non-reacting types, however, frequently attain a higher density. Except that they do not react with iodine, most of these are indistinguishable microscopically from the microiodophile population. Every transition can be observed between strongly reacting, weakly reacting and non-reacting forms. Moreover, observations on readily identifiable macroiodophile species have repeatedly shown that the amount of reacting polysaccharide present may vary within wide limits; forms replete with polysaccharide and forms devoid of it are encountered. By analogy, therefore, it is probable that most of the smaller non-reacting types do not belong to distinct aniodophile species, but that they represent dead or depleted members of the microiodophile population. Counts recorded in the present experiments for macroiodophiles show that the numbers of actually reacting types (i.e. those which stain blue with iodine), accumulating during incubation were greater than might reasonably be assumed to result from cell multiplication alone. It must be supposed therefore that many of the non-reacting forms present in the initial samples were still viable and that, in a proportion of these, re-storage of polysaccharide occurred in addition to cell division. This agrees with the chemical results, which showed that the synthesis of polysaccharide was greater than that of protein during the first 2 hr. of incubation, whereas towards the end of some incubations the polysaccharide-protein ratio and the iodine reaction both tended to decrease. The simplest conclusion is therefore that the bulk of the synthesized material is incorporated in the microiodophile population, but that the initial rate of synthesis and final rate of hydrolysis is greater for polysaccharide than for protein.

Since the greater part of the synthesis occurs within the first 2 hr. of incubation, while microbiological normality is maintained in the samples (Baker, 1943), it is reasonable to assume that in the intact rumen, the iodophile bacteria, which abound there, readily convert significant amounts of N.P.N. to protein, and of sugars (probably derived mainly from the starch in the diet) to polysaccharide, and

that by synthesizing protein they supply part of the ruminant's protein requirements. Experiments are still required to determine the biological value of the synthesized material and to assess the nutritive requirements of the bacterial agents of synthesis. Investigation along these lines is now in progress.

SUMMARY

1. Rumen liquor has been incubated *in vitro* with and without added carbohydrates. The synthesis which results under favourable conditions has been estimated by isolating the synthesized material as an amorphous powder which consists mainly of bacteria.

2. Synthesis of protein is accompanied by synthesis of a starch-like polysaccharide and by

an increase in the iodophile population of the medium.

3. The greater portion of the synthesis is attributed to the masses of microiodophile bacteria which abound in the rumen. Protozoa do not appear to contribute to the synthesis.

4. Typical analytical figures for the sediment containing the synthesized material are 0.5 % moisture, 36.3 % protein, 46.6 % polysaccharide, 9.5 % lipoid matter and 6.2 % ash. These values are very similar to the corresponding values for a typical feeding stuff such as linseed cake.

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