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THE NUTRITIONAL ROLE OF THE MICROFLORA IN THE ALIMENTARY TRACT

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The Microbiological Aspect of Rumen Digestion

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In some quarters it used to be customary to refer to the illustrious name of Pasteur when opening a discourse on any subject of microbiology. While it is granted that such homage may have been overdone sometimes, it does seem relevant to the subject to mention here that 60 years ago Pasteur (1885) remarked to an audience of the French Academy that, if appropriate experiments could be carried out, it would in his opinion be found that no animal could develop normally in the absence of an intestinal microflora. The date of Pasteur's remark and the still sketchy state of knowledge in 1944 of the pattern of cellulose digestion by ruminants illustrate the complexity of this problem. Let it not be supposed that a great deal of interest has not been taken in this subject during the interval of 60 years.

I have recently once again read through the relevant literature and can state that many attempts have been made to interpret the function of the intestinal microflora in terms of cellulose digestion.

I shall not give here a record of the information available in the literature except in so far as it is needed to outline the present admittedly blurred picture of the subject. This picture, if I correctly interpret it, presupposes the existence within the intestinal tract of the ruminant of a great variety of micro-organisms, including protozoa. These forms are supposed to subsist on the foods ingested by the host and, by so doing, to convert the cellulose of the plant tissues into soluble and gaseous products, notably organic acids and methane. From some of these degradation products the host is assumed to derive nutritional benefits. This conception of a symbiotic relationship between host and microflora was proposed many years ago by Tappeiner (1882) when he found that the disappearance of cellulose in the alimentary tract of ruminants could not be ascribed to enzymes secreted by the animals. It has found favour right up to the present day though not without opposition, mainly because, as some early writers remarked, it failed to account satisfactorily for the high nutritive value of cellulose in the feeding of ruminants (Henneberg and Strohmann, 1885). In amplification of Tappeiner's theory these writers, therefore, suggested that, apart from gases and organic acids, the intestinal microflora might, from ingested cellulose, set free monoses

by the absorption of which the host would derive added nutritional benefit from the cellulose.

That monoses and possibly disaccharides are in fact liberated from cellulose when it is broken down by certain bacteria was shown by Pringsheim (1912), and it is possible, therefore, that the suggestion of Henneberg and Strohmann (1885) can be substantiated, but this has still to be done experimentally, either directly, if possible, or indirectly in some way if, as is likely, the direct method is impracticable.

The picture which I have sketched of a symbiotic digestion of cellulose, lays great stress on the benefits accruing to the host but little if any on the nutritive value of cellulose for the intestinal micro-organisms. It is not without interest to invert the picture for a moment, and to examine the process of cellulose digestion in the rumen from the point of view of the intestinal micro-organisms. It is revealing to say the least that, though several attempts have been made, the most natural technique to employ, that of direct microscopic observation, has been adopted only by two workers, somewhat superficially by Henneberg (1921–22) and systematically by Baker (1933, 1934, 1943).

When ingested plant tissues enter the rumen of an ox or a sheep, they are submerged in a dense suspension of micro-organisms contained partly in the liquid portion of the rumen content and partly on tissues already present there. Instantly the fresh tissues become infected with this microflora and, before long, a variety of cells can be observed under the microscope to have invaded the tissues and to be growing on them in the manner characteristic of micro-organisms decomposing cellulose. Their development results in time in a more or less continuous covering of the tissues with densely packed cells. Such spreading of cells could not occur without vigorous reproduction of the micro-organisms of the rumen. In other words, a process of synthesis of organic matter must take place there in addition to the breakdown on which so much emphasis has already been laid.

A measure of the quantitative significance of the growth and synthetic processes resulting from cellulose digestion has only been obtained quite recently and as yet only in the vaguest outline but, even so, it appears justifiable to assert that it must be of considerable magnitude. The method which I adopted in arriving at an estimate presupposed that an ox ingests some 2 kg. of cellulose daily and passes through its rumen a total of 100 l. of liquid during 24 hours. Further, my calculations assumed that, for the numbers of the micro-organisms to remain constant within a period of 24 hours, the rate of growth of the rumen micro-organisms must be such that one new generation of cells is produced every 24 hours, a very conservative estimate considering that the generation time of most microbial cultures during their logarithmic phase of growth is of the order of $1\frac{1}{2}$ to 2 hours.

On this basis I have attempted to evaluate the weight of micro-organisms produced daily in unit samples of rumen liquor taken at various periods during the 24 hours. These samples were first subjected to gentle treatment in the centrifuge for the purpose of eliminating tissue debris and protozoa, and the microflora present in them, therefore, represented part only of the total produced, since that part which adhered to the debris must have been removed. No account was taken of

those organisms which during the 24 hours were digested by the protozoa present in the rumen, and the substantial protozoan population itself also was excluded from the estimate.

In 16 separate determinations the weight of the microflora of 100 ml. of centrifuged rumen liquor was found to amount to an average of 404 mg. of dry microbial substance, or 404 g. in 100 l. of rumen liquor, the amount assumed to pass daily through the rumen.

A provisional chemical analysis of this material showed that it contained some 45 per cent. of protein, about 20 per cent. of carbohydrates, and about 2 per cent. of ether soluble substance, giving a daily minimum production of 180 g. of microbial protein, 80 g. of carbohydrates and 8 g of ether soluble substance, containing a total of about 139 g. of carbon. If derived from the 2 kg. of ingested cellulose this would represent some 15 per cent. of its total carbon. As already mentioned, this is undoubtedly an appreciable underestimate, and careful evaluation of the synthetic microbial processes of the rumen may well raise to a much higher figure the percentage of the ingested carbon utilized for the maintenance of the rumen population at their normal numbers. Even when this amount is taken at its lowest it is interesting to compare it with the percentage of carbon appearing in the rumen in the form of organic acids, produced by the microflora from cellulose.

Tappeiner (1884) claims to have found in his experiments 2·35 g. of such organic acids, estimated as acetic acid, per l. of liquor or, on our basis of calculation, 235 g. of acetic acid derived from 2 kg. of cellulose. This would represent a recovery in the form of acetic acid of 10·6 per cent. of the ingested carbon.

Barcroft, McAnnally and Phillipson (1944) have recently shown that the organic acids produced in the rumen are rapidly utilized by the host. Nutritively these acids must be inferior to the microbial substance synthesized in the rumen, if it can be assumed that the latter is digested by the ox. Is there any evidence in support of this assumption? In the literature, I am afraid not, at any rate no direct evidence, for the bacterial dry matter has only recently become available in bulk, but indirect evidence, supplied primarily by Baker (1943), shows that the typical rumen microflora and the microfauna as well undergo marked changes during their passage through the alimentary canal. The protozoa and the larger forms of the bacterial population of the rumen are usually absent from the faeces. The smaller forms, in so far as they can be grown on the standard bacteriological media, have been shown by Ankersmit (1905-06) to be eliminated during the passage of the rumen contents through the small intestine. In no cases has Baker (1943) been able to discover in the faecal microflora those forms which contain in their cells the iodine staining polysaccharide which both Henneberg (1921-22) and he emphasize as typical for a very large section of the rumen microflora. It is probable, therefore, that a percentage, perhaps the bulk of the rumen microflora, can be digested by the host.

If this is in fact the case, it is of interest to consider once more the chemical composition of this microflora. I mentioned that it contains approximately 45 per cent. of protein and 20 per cent. of carbohydrates. The former may, on examination, be found to have the high nutritive value ascribed to yeast protein by Macrae, El-Sadr and Sellers (1942).

The view that protein might be synthesized in the rumen as a result of microbial activity had previously been advanced, though without any reference to the significance of such synthesis from a nutritional point of view. It was dismissed as unlikely by Krebs (1937) because of the adverse conditions prevailing in the rumen. Recently Pearson and Smith (1943) have shown that a synthesis is in fact possible and that it goes hand in hand with an increase in weight of the microflora of the rumen liquor.

According to a private communication from Baker the carbohydrates of the rumen microflora consist essentially of a starch like polysaccharide which reacts with iodine and yields glucose on hydrolysis. As Baker (1943) points out it must be available nutritionally since it is not found in the microflora of the lower intestine. Its presence within the rumen microflora is perhaps the clearest available evidence in support of the contention of Henneberg and Strohmann (1885) that glucose is produced as an intermediate decomposition product of cellulose.

So far no reference has been made to the possibility of a synthesis of the vitamin B complex in the rumen as a result of the reproduction of the microflora living there. That such synthesis must be of a considerable order can be judged from the observations that the washed and dried bacterial content of the rumen was recently found by me to contain 142 μg. riboflavin per g. A microbiological test of the riboflavin content of the food ingested by the ox from which the bacterial material was taken, yielded values of 1·5 to 2 μg. per g. for the dried grass and oats, and 6 μg. for the dried grass. In connexion with this subject of riboflavin synthesis in the rumen, it should be added that, while the dried bacteria taken direct from the rumen in my experiments yielded 142 μg. riboflavin per g., I found that the riboflavin content fell to 81 μg. per g. of dried bacteria when rumen liquor was incubated *in vitro* for some hours in the presence of glucose or maltose, and the bacterial content was collected, washed and dried.

This observation indicates that incubation *in vitro*, even when of short duration, interferes seriously with the rumen microflora as it functions in its natural habitat.

From what has been said it appears reasonable to conclude that, if the synthetic activities of the rumen microflora are taken into account as part of cellulose digestion by ruminants, it is possible to account for a considerably greater part of the carbon in the ingested food than is possible when such calculations are based solely on the output of organic acids liberated in the rumen by a microbiological breakdown of cellulose.

It is worth while to emphasize also the nutritional significance of any protein and B vitamins produced during the multiplication of the native microflora of the rumen.

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Discussion

Mr. F. Baker (County Technical College, Guildford, Surrey), opener: The methods I have applied to the systematic study of the microbial populations of the rumen and caecum of herbivora may be dealt with under four headings: The collection and preservation of material; preliminary microscopical examination; the use of counting methods; the application of histochemical and histophysical procedures to the examination of cellulosic substrates.

Collection and Preservation of Material

Samples are collected *post mortem*, or from an animal with a fistula, or from cultures at specified periods during incubation of rumen or caecal contents. They are fixed by addition of formaldehyde. By proceeding in this way the examination can be made at leisure and material is available for subsequent reference. A close check can thus be kept upon the results obtained, and observations upon any particular sample indefinitely extended. Also, the preserved material is available for biochemical investigation; for instance, its content of protein or polysaccharide may be determined.

Where, as with rumen contents, the samples are markedly heterogeneous, they are strained through muslin and the suspended components of the strained liquid separated with the centrifuge. In this way rumen liquid can be made to yield as successive fractions the bulk of the coarser vegetable fragments, the rumen ciliates, the larger, and finally the smaller, bacterial species. The volume or weight of these fractions may be determined and under certain circumstances affords valuable information.

Preliminary Microscopical Examination

An essential part of the microscopic technique is the examination of wet iodine preparations. In these the presence of an entire class of micro-organisms, the iodophile species, which have never been isolated in pure culture, can be disclosed. These micro-organisms may accumulate in sufficient numbers to give a reaction visible to the naked eye, showing how much can escape notice through over reliance on plating out and through failure to utilize the simplest resources of the microscope. Wet preparations of bacteria which do not react with iodine can be obtained by irrigation with erythrosin or Bengal rose and yield far more accurate information than can be obtained from the examination of dried films. For protozoa dry preparations are entirely useless.

The Use of Counting Methods

A counting technique was long ago applied by Ferber (1928) to the rumen ciliates. I have since extended it to the larger iodophile bacteria of the rumen and caecum. Counts are made in iodine solution by use of a double cell with Burker ruling. A set of 60 squares is counted in each of the 2 cells under a 4 mm. dry apochromatic objective. Average values afford an index to the relative population densities of particular species in a given sample. Incubations of rumen contents *in vitro* demonstrate that the final value is increased or diminished by the presence of particular substances. Thus the metabolic requirements of micro-organisms which do not appear on plates can be systematically explored. Also, as Dr. Smith and I have shown in collaboration, a very definite relation can be established between the population density attained and the amounts of protein and polysaccharide synthesized (Smith and Baker, 1944).

The Application of Histochemical and Histophysical Procedures to the Examination of Cellulosic Substrates

Plant residues are brought down in the centrifuge, washed repeatedly, and mounted in an aqueous medium of high refractive index. I employ a saturated solution of mercuric iodide in potassium iodide, to which crystalline iodine is added to a concentration of 0·5 per cent. Preparations are examined under the polarizing microscope with an intense source of light. Cellulose is birefringent, and decomposition is accompanied by loss of double refraction. In these conditions, therefore, the cavities excised enzymically are outlined as black regions or pits on the luminous surfaces of the walls. The micro-organisms responsible, which are species of iodophile bacteria, can be seen in the interior of the cavities merely by uncrossing the nicols. The presence of a vast microbial population is thus disclosed, the role of whose members as agents of decomposition and *ipso facto* as agents of synthesis would otherwise escape attention. None of these organisms makes any appearance on plates or in mineral salt media to which cellulose has been added. Observations made in polarized light can be extended by the use of histochemical reagents. Chlor-zinc-iodine and the tetrazonium dyes such as Congo red and azo-blue can most usefully be employed. Colour reactions cease in the affected regions. Cellulosic substrates treated with these reagents show dichroism under a single nicol so that cell wall structures can be distinguished with ease and certainty from other stained material. By these several procedures the disintegration of plant material can be followed from start to finish and the stages recorded as photomicrographs.

Advantages of Direct Microscopical Observation

Three points in regard to the use of methods of direct microscopical observation deserve special emphasis.

First, methods of pure culture present an inadequate picture of the microbial populations of the gut. Their inherent deficiencies can only be made good by direct microscopical observation of the micro-organisms present in relation to their natural habitat.

Second, experiments conducted *in vitro* afford insight into changes occurring *in vivo* only when the survival of the normal microbial population has been demonstrated. Direct microscopical observation is essential to the control of cultures obtained by incubation *in vitro*.

Third, by incorporating counting procedures into the microscopic technique the significance of the biochemical data in regard to synthesis and decomposition can be elucidated in terms of the changes observed in the density of the known components of the normal microbial population of the gut.

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Sir Jack Drummond (Ministry of Food, Portman Court, Portman Square, London, W.1): Can Mr. Baker say whether the interesting group of iodophile bacteria he has described, which appears to be of great importance, is related to the organisms which Hutchinson and Clayton (1918-19) found in their early work on the breakdown of cellulose and which, to the best of my recollection, could not be obtained in pure culture?

REFERENCE

Hutchinson, H. B. and Clayton, J. (1918–19). *J. agric. Sci.* **9**, 143.

Mr. A. L. Bacharach (Glaxo Laboratories, Ltd., Greenford, Middlesex): Have these organisms been demonstrated in animals other than the bovine?

Mr. R. Benesch (L.C.C. Central Pathological Laboratory, Epsom, Surrey): Is anything known about the changes brought about in the rumen by the microflora of silage?

Dr. J. Stewart (Moredun Institute, Gilmerton, Midlothian): Is there any proof that the type of micro-organism described by Mr. Baker is mainly concerned with the breakdown of cellulose and not with the other more easily digestible carbohydrates?

Wing Commander T. F. Macrae, R.A.F.V.R. (R.A.F. Institute of Pathology and Tropical Medicine): We have heard only of the beneficial effects of the micro-organisms of the rumen in increasing the nutritive value of the ingested food. There is, however, a detrimental effect, the formation of large quantities of methane which results in a considerable loss of energy to the animal. What is the source of the nitrogen contained in the synthesized protein of the rumen bacteria; does it originate from material which would otherwise be available to the animal?

Mr. W. S. Ferguson (Jealott's Hill Research Station, Bracknell, Berks.): Could Dr. Thaysen or Mr. Baker give any information on the effect of the ration of the host on the growth of the mixed flora of the rumen? I believe that work at Onderstepoort in South Africa has shown that the type of ration given influenced greatly the make up of the micro-organisms in the rumen.

Dr. R. Sutherland (Health Office, Brighouse, Yorks.): Is there any evidence that the the microflora of the human alimentary tract plays a part in any way comparable in human nutrition?

Dr. A. C. Thaysen gave the following replies:

To Sir Jack Drummond: I do not think that the organism observed by Hutchinson and Clayton (1918-19) has any connexion with the iodophile bacteria. It could not be grown at the time on any medium then known but was eventually obtained in pure culture.

To Mr. Benesch: I do not know of any work on the effects of the microflora of silage on that of the rumen.

REFERENCE

Hutchinson, H. B. and Clayton, J. (1918–19). *J. agric. Sci.* **9**, 143.

Mr. F. Baker gave the following replies:

To Sir Jack Drummond: The general physical features of decomposition of cellulose in the rumen are similar to those occurring in the soil but the microbial agents concerned are of entirely different types. The organism studied by Hutchinson and Clayton (1918-19), the so called *Spirochaeta cytophaga*, is strongly aerobic and can readily be grown in mixed culture *in vitro*; it does not produce the polysaccharide reacting with iodine. The organisms operating in the rumen have been described by Henneberg (1921-22) and myself. Few if any of the many types have been grown *in vitro* and all are facultative or obligate anaerobes; they synthesize bacterial starch and stain blue with iodine.

To Mr. Bacharach: Very many types of iodophile micro-organisms are present in the caecum of the horse, guineapig and rabbit (Baker, 1933; Baker and Martin, 1937, 1937-38, 1938-39). I have also observed them in the caeca of hens (unpublished work). The iodophile micro-organisms of ruminants and non-ruminants include a wide range of readily identifiable morphological types. Each host animal tends to maintain a characteristic microflora. The specificity persists in the case of guineapigs and rabbits even when animals are kept in the same cage and given identical diets (Baker, 1944).

To Dr. Stewart: My observations on incubation of ox rumen contents were made in collaboration with Dr. Smith. Incubation lasts up to 6 hours and starch or soluble carbohydrate is added to the incubated material. During this period no appreciable decomposition of cellulose occurs. In the living animal, however, there is a complex bacterial population of high density, many of whose members are iodophile. Some of these types are capable of utilizing only starch or soluble carbohydrate; others are responsible for the breakdown of cellulose and hemicelluloses. The activity of the latter can be demonstrated in polarized light by the methods I have described today. The distinctive feature of the iodophile microflora as a mixed microbial population is its ability to synthesize a polysaccharide reacting with iodine from a wide variety of carbon compounds (Baker, 1943; Smith and Baker, 1944).

To Mr. Ferguson: According to the diet conditions, the growth rate of particular members of a complex microbial population of the caecum or of the rumen is accelerated or retarded. That different hosts maintain in the caecum or rumen characteristic microbial populations is, however, shown for the rabbit and guineapig by the experiments I mentioned in my reply to Mr. Bacharach. Incubation also illustrates this point.

For instance, the iodophile populations of the rumen of sheep and oxen alike show a marked increase in density in a 6 hour incubation period. The predominant micro-organisms from the ox are, however, bacteria which synthesize starch (Baker, 1942), while, in the sheep, they are pseudo-yeasts and other organisms which synthesize glycogen (Quin, 1943). Among the yeasts is the organism referred to by Quin as *Schizosaccharomyces ovis*. It seems reasonable to state, therefore, that diet may induce marked quantitative changes in the characteristics of the bacterial population but that the general qualitative characteristics persist.

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The Formation of Protein

Dr. J. A. B. Smith (The Hannah Dairy Research Institute, Kirkhill, Ayr)

Introduction

During the last 40 years the claim has frequently been made that for some animals a portion of the dietary protein required to maintain normal health and growth can be replaced by substances containing only non-protein nitrogen (N.P.N.). The work supporting or refuting the claim has been reviewed fairly recently by Krebs (1937) and by Owen (1941). In discussing the earlier researches carried out mainly on the continent Krebs cites 126 references. He shows that the earlier results and conclusions were conflicting and that the conflict of opinion arose mainly from the fact that the earlier experiments were often so badly planned and so inadequately controlled that the findings did not lend themselves to decisive interpretation.

Until the manufacture of great quantities of non-protein nitrogenous compounds such as urea from atmospheric nitrogen became possible, and until it appeared that war might again curtail the supplies of available feeding stuffs, the question was regarded as of little importance in Britain. During the past few years, however, the subject has been studied in much detail both here and in America with the result that the problem which was hitherto so confused has now been greatly clarified.

The object of this paper is to summarize the recent work. The results of the investigations fall naturally into 2 sections, evidence obtained for the conversion of N.P.N. to protein from practical feeding trials, and evidence which supports the principal theory put forward to explain the ability of the ruminant to utilize N.P.N. in this way.