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THE APPLICATION OF MICROBIOLOGICAL METHODS TO THE STUDY OF NUTRITION

Chairman, Professor JOHN CRUICKSHANK

Professor John Cruickshank (Department of Bacteriology, University of Aberdeen): Studies in nutrition have come almost suddenly, it would seem, into the domain of the bacteriologist. Bacteria may be possible sources of vitamins; they may be used in vitamin assays. From the study of man and other animals there has been a rapid slide down the scale of living things to the least differentiated of all, the unicellular organisms. It would seem logical for studies in nutrition to have begun with the morphologically simple and to have proceeded to the study of more complex organisms. Actually it has been the other way round. The intense interest in health of the last century showed itself especially in public-health measures with the intention of preventing or limiting infectious disease. Over a shorter period the effect of nutritional deficiencies, particularly in man, has been intensively studied. Food is now the most common topic of discussion, and governments take heed. To the man in the street, how can bacteria enter into the matter? Is there anything common to the nutrition of these small structures and that of higher animals? Do all forms of life, irrespective of their morphology, depend for food on the same fundamental chemical processes? Different though the food of man appears to be from that of the herbivora and other animals, does it differ merely in appearance, texture, colour, and origin from that of the inhabitants of the microbial world? Fundamentally, are the amino-acids, enzymes and co-enzymes or other catalysts the same for all living creatures? Such are the speculations which arise from the results of recent microbiological studies in nutrition; in particular, from the demonstration that certain groups of bacteria and some moulds demand for adequate nutrition the same range of vitamins as the higher animals, and that they can be successfully used for the assay of these substances.

This irruption of nutritional studies into bacteriology is welcome and stimulating. Hitherto bacterial studies have been dominated by the diseases of men and animals, and investigations of bacteria as a whole have suffered. The most important work in this country on general bacterial nutrition is that of Fildes and his colleagues. They have shown that the different types of nutritional requirements of bacterial species may be regarded as successive levels of an evolutionary scale. At the bottom of the scale are those organisms, the autotrophs, which require only carbon dioxide and inorganic compounds for adequate growth. Such

organisms need no organic compounds and no vitamins. They manufacture these themselves from ammonia, nitrate, or nitrite. The heterotrophic organisms which require for their growth ready-made organic compounds could not have developed until the autotrophs had multiplied and died, thus enriching the environment with organic compounds in the form of bacterial protoplasm, and their metabolic products. The heterotrophs are unable to utilize inorganic nitrogen; they require amino-acids. Many of them, *e.g.*, *Salmonella typhi*, can, however, be trained by successive deprivation in cultures to dispense with amino-acids and to use ammonia instead. The building-up of tryptophane appears to present especial difficulty. Tryptophane is regarded as a necessity for all bacterial protoplasm. A further stage in the evolutionary scale is represented by bacteria which require special nutrient compounds, growth factors. Fildes and other workers described certain growth factors as "bacterial vitamins", but there is in their work no mention of vitamins in the ordinary sense. It is still in doubt whether many such organisms, if submitted to training experiments, could not eventually dispense with them. Still higher in the scale of evolutionary development are the more strictly parasitic bacteria which require for their development the tissues or body fluids of animals. Restriction of synthetic ability leads to strict parasitism, and it is more difficult to secure growth in artificial media or to maintain cultures.

If we turn to the bacteria upon which recent vitamin studies have focussed attention, *Bacterium coli* and *Lactobacillus casei* deserve some consideration. *Bact. coli* is a heterotrophic organism, but some strains are facultative heterotrophs and can readily be trained to use ammonia as a source of nitrogen. In its normal environment in the intestinal canal it is presumably dependent on organic nitrogen (amino-acids). Modern studies in human nutrition and in animal experiments show that vitamins are synthesized by these organisms. This biosynthesis can be inhibited by the relatively insoluble sulphonamides. In the rat the intestinal bacteria are said to synthesize vitamin B_1, riboflavin, nicotinic acid, vitamin B_6, biotin, folic acid, pantothenic acid, inositol and vitamins K and E, quite a battery of vitamins.

L. casei has its normal habitat in milk, cheese and other milk products, in fermenting grain masses, in silage, and, generally speaking, in material in which fermentation with production of much lactic acid occurs. It has come into use in microbiological assay of vitamins and amino-acids, since for growth it requires at least six of the B vitamin series and cannot synthesize them. These vitamins must be supplied together, along with a suitably chosen amino-acid mixture. In its normal habitat presumably all these factors are freely available. It is not a parasitic organism, though members of the same group are always present in the intestines of higher animals and some are present in large numbers in the stools of suckling infants. The lactobacilli in the infant's intestine are present in the vagina of the mother, from whom it is believed the child is infected. Do they play any part in the infant's nutrition? It is of interest to remember that the numerous types of fermented milk product widely used in eastern countries, *e.g.*, kefir, yoghurt, koumiss, are rich cultures of lactobacilli. Metchnikoff was of the opinion that the longevity of the Bulgarians depended on the abundant use of milk soured by

Lactobacillus bulgaricus, another member of the *Lactobacteriaceae*. Are these organisms also a possible source of vitamins for the human subject?

In considering absorption from the intestine it must be remembered that the contents of the intestinal canal are still outside the body, separated from it by the mucous membrane of the intestine. It is unlikely that the bacterial bodies are absorbed as such; some degree of digestion in the lower reaches of the intestinal canal would appear to be necessary. This problem is not yet resolved, but recent studies on the bacterial contents of the rumen of animals with an experimental fistula may yield results. To the bacteriologist the intestinal contents obviously provide a medium for the growth of enormous numbers of living bacteria. The soil, faeces, sewage, and milk are outstanding as culture media for organisms; an enormous number and variety are present. The importance and beneficial action of bacteria in soil and sewage has long been recognized; their importance in the contents of the intestine is only now being considered.

I should like to return for a moment to the biological assay of vitamins and amino-acids by the use of bacteria. Fildes and his collaborators declare that "unprecedented purity and cleanliness are of primary importance, that no nutrient should be considered indispensable until growth has consistently failed without it. Synthetic products only are permissible." When extracts or filtrates are used the results of such assays must be given with great reserve. Many bacterial cultures of the volume of 10 l. may yield only 1·5 mg. of dry bacteria, and the amount of any one nutritive component may be infinitesimally small, almost beyond our conception. The variability of any strain of bacterium in repeated culture is a common experience; they are subject to changes in morphology, in division and in antigenic constitution. When there is opportunity for many thousands of millions of generations to occur, as in bacterial cultures, the possibility of mutations arising must be considered. The use of bacteria in the assay of infinitesimally small quantities demands an irreproachable technique.

Many other considerations of speculative character arise in my mind. Is the time approaching when with pure products of amino-acids and growth factors available it will be possible in synthetic media to grow any species of bacterium or other living cell, no matter how strictly parasitic it has become? Will this knowledge be applicable to the most parasitic of all, the viruses, which hitherto have not been cultivated outside living tissues?

The Microbiological Assay of Amino-acids

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Microbiological methods of assay have come into prominence in recent years for the quantitative estimation of a number of the components of the vitamin B complex. The initiative and drive for this type of work have come almost entirely from the U.S.A. and rapid advances have been made. The organisms that have been chiefly used are certain strains of bacteria which produce lactic acid (lactobacilli). From the practical

point of view these lactic bacteria have proved to be suitable and convenient organisms for this purpose, because by a happy chance, in these particular strains, lactic acid production is proportional, over a certain range, to the concentration of vitamin in the basal medium, and simple titration with standard alkali is sufficient to establish a standard curve.

The microbiological assay of amino-acids with lactic bacteria is the logical outcome of the earlier work on the assay of vitamins. The principle upon which the microbiological assay of vitamins is based demands a basal medium which contains all the specific constituents, except the one to be estimated, in ample supply. Graded doses of the particular vitamin to be estimated are added to the basal medium, and growth or lactic acid production is found to be proportional, over a certain range, to the concentration of the essential nutrient added. A parallel series of tubes with the substances which are to be analysed for that vitamin are set up at the same time and a comparison is made of the growth in the two series.

The lactic bacteria, like other living organisms, require for normal metabolism and growth a utilizable source of energy, nitrogen, mineral salts and a wide assortment of vitamins. Glucose is now generally used as a source of energy, and a casein hydrolysate or peptone as source of nitrogen. Casein hydrolysate consists of a mixture of amino-acids and, when reinforced with tryptophane and cystine, is an excellent source of nitrogen for the lactic bacteria. The question therefore arises: Can a casein hydrolysate be replaced by a mixture of the individual amino-acids composing it and still give as good growth of the lactic bacteria? This question has been answered in the affirmative. It was shown by Hutchings and Peterson (1943) that *Lactobacillus helveticus* (*casei* ϵ) will grow, growth being measured by lactic acid production, as well on a medium composed of 20 amino-acids as in the presence of a casein hydrolysate.

So far as the lactic bacteria are concerned the amino-acids can be divided into two classes: essential and stimulatory. If the basal medium is composed solely of essential amino-acids, growth, but not maximum

TABLE 1

EFFECT OF PRESENCE OF STIMULATORY AMINO-ACIDS ON ACID PRODUCTION OF *Lactobacillus helveticus* IN A MEDIUM CONTAINING 10 AMINO-ACIDS

Amino-acid added to basal medium	Acid formed as measured by ml. 0·1 N-NaOH needed per 10 ml. medium
None	3·2
Threonine	6·3
Threonine and methionine	7·4
Up to 20 amino-acids	10·8
Casein hydrolysate	10·9

growth, will take place. It is only on the addition of the stimulatory acids that maximum growth is secured. This has been well illustrated by Hutchings and Peterson (1943) for *Lactobacillus helveticus*, which was found to grow on a medium composed of glucose, sodium acetate, adenine, mineral salts, 6 vitamins, riboflavin, nicotinic acid, pantothenic acid,

pyridoxin, biotin and folic acid, together with the following 10 amino-acids in place of a casein hydrolysate: glutamic acid, aspartic acid, leucine, serine, valine, tryptophane, cystine, arginine, phenylalanine and tyrosine. Maximum acid production, however, was not attained on this mixture (see Table 1) but, when the number of acids was increased to 20, acid production attained the same level as with a casein hydrolysate.

Just as in the absence of certain essential vitamins the lactic bacteria will not multiply, so, in the same way, in the absence of any one essential amino-acid, growth, *i.e.*, multiplication, will not take place. In the microbiological assay of amino-acids with the lactic bacteria the casein hydrolysate or peptone of the basal medium is replaced by its constituent amino-acids, with the omission only of the particular amino-acid that is to be assayed. Graded doses of the acid are then added, and subsequent procedure is the same as in the assay of a vitamin.

A practical point that should be mentioned here is that the optimum *p*H for growth of the majority of lactic bacteria lies between 6 and 7; in practice it is usual to employ a *p*H of 6·8, but growth will proceed until a *p*H of 4 or even less is attained. Since lactic acid is formed during growth, buffer must be added to the medium to prevent any sudden change or shift in *p*H to levels which would seriously interfere with, or even entirely inhibit, normal growth. It has been shown (Snell, Strong and Peterson, 1937) that sodium acetate is especially suitable for this purpose, and it is now invariably added to the medium.

There is a further point that has a fundamental bearing upon the microbiological assay of amino-acids which can be conveniently discussed at this stage, namely, the phenomenon of antagonism. This phenomenon was very completely investigated by Gladstone (1939) for the pathogen *Bacillus anthracis*. Strains of *B. anthracis* were grown on a pure synthetic medium composed of the following 17 amino-acids: aspartic acid, valine, leucine, alanine, glutamic acid, *iso*leucine, phenylalanine, lysine, glycine, proline, hydroxyproline, tyrosine, arginine, histidine, tryptophane, methionine and cystine, together with the usual mineral salts and glucose. All the strains that were tested grew well on this medium, although some grew more quickly than others.

Gladstone then omitted single amino-acids from the medium and found that growth was considerably affected by the omission of the following:

Leucine: no growth even in 9 days;
Valine: no growth even in 9 days;
*Iso*leucine: initial delay in growth, full growth after 40 hours;
Glycine: initial delay in growth, full growth after 40 hours;
Cystine: initial delay in growth, full growth after 72 hours.

On this evidence it is reasonable to assume that leucine and valine are essential amino-acids for *B. anthracis*, while *iso*leucine, glycine and cystine can be synthesized after an initial lag period. Further, since there is no growth at all in the absence of valine and leucine, and delayed growth in the absence of *iso*leucine, it is reasonable to assume also that there will be no growth at all when all three are omitted from the medium. When this assumption was experimentally tested, however, the surprising result was obtained that, after an initial lag period of 48 hours,

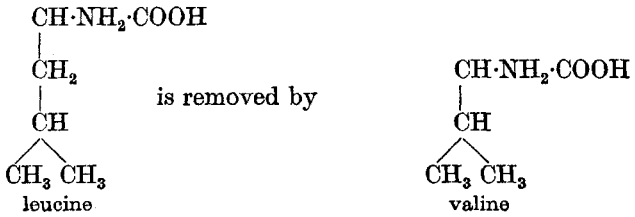
there was only slightly less growth than the maximum obtained in the presence of all 17 amino-acids. One further fact: the addition of any one of these three amino-acids, leucine, valine or *iso*leucine, to a mixture from which they were all absent, completely abolished growth. A concentration as low as M/187,500 valine, M/42,500 leucine or M/312,500 *iso*leucine was sufficient to prevent all growth. On the other hand, if all three amino-acids were added to the medium in the above concentrations maximum growth was attained in 22 hours. It is clear from these figures that *iso*leucine is the most toxic of these three amino-acids in its growth-inhibitory action, while valine occupies an intermediate position.

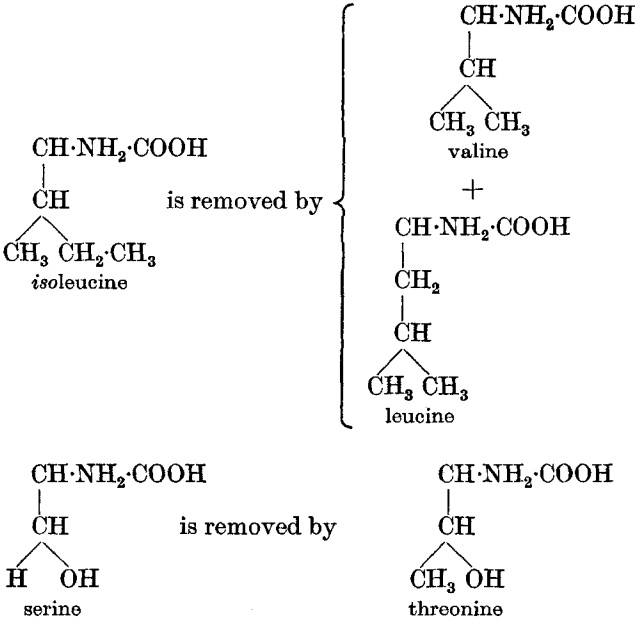
When all three amino-acids, leucine, valine, and *iso*leucine, are present in the medium, not only is growth inhibition abolished but growth is actually speedier and more luxuriant than in their absence. They must, therefore, be capable not only of neutralizing one another's toxic influence but, having done so, of actually stimulating growth. When leucine, valine, and *iso*leucine are absent, serine and threonine show toxicity when added singly to the medium. When added together they neutralize one another's toxic effects.

The immediate question to be answered, therefore, is whether all three acids, leucine, valine, and *iso*leucine, are equally involved in this phenomenon of antagonism. We have already seen that in the presence of leucine with valine, and in the absence of *iso*leucine, there is eventual growth, despite an initial lag period. Thus, the toxic influence of leucine or valine, when present singly, is neutralized when they are present together. On the other hand, the toxic influence of *iso*leucine is neutralized only when valine and leucine are present together.

This phenomenon of antagonism has been encountered with other micro-organisms. In the absence of aspartic acid or asparagine, β-alanine will inhibit the growth of the yeast *Saccharomyces cerevisiae*, whereas when they are added together not only does growth take place, but it is actually stimulated. Antagonism of amino-acids has been encountered also among the lactic bacteria.

Gladstone has advanced some theoretical views on the possible mechanism of this phenomenon of antagonism. Firstly, it will be recalled that amino-acids which are able to neutralize toxic effects are themselves toxic when added singly. Secondly, the particular amino-acids concerned or associated in this way have a general similarity of chemical structure. Thus, the toxicity of:



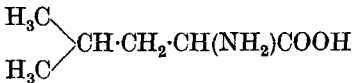


and vice versa.

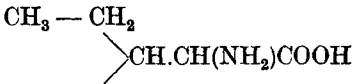
This similarity of structure led Gladstone to suggest the view that there is some common key reaction which is necessary if normal growth is to take place, and that this reaction can be inhibited if there is an excess of one or other of the amino-acids taking part. The most obvious function of amino-acids in cell metabolism is the building up of bacterial protoplasm. Growth, however, is not dependent upon the presence of these amino-acids, but in their absence is delayed. This does not mean that these amino-acids are not essential for growth. When a particular amino-acid accelerates growth which would nevertheless take place in its absence, all the evidence suggests that such an amino-acid is necessary for growth, but that when it is not added it is synthesized by the organism. It may be assumed, therefore, that all these amino-acids are required for growth, but can be synthesized by the organism. It is possible that the presence of one amino-acid in excess may block this master or key reaction, or the enzymes responsible for the reaction, either by causing synthesis of another amino-acid of similar chemical composition, or by preventing an amino-acid which has been synthesized from being built into bacterial protoplasm.

We can summarize these findings of Gladstone by saying that it is not possible to determine optimum concentrations for single amino-acids, and that the necessity for any single amino-acid is in many cases not absolute but relative. Thus an amino-acid A may be required to neutralize the toxic influence of an amino-acid B but, in the absence of B, A no longer functions as an essential amino-acid. This is the case, for instance, with serine and threonine.

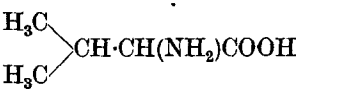
The estimation of any amino-acid by chemical methods is a difficult and tedious operation and requires much skill and practice. Moreover, the aliphatic amino-acids, leucine, *iso*leucine and valine, when present together in a protein hydrolysate, are particularly difficult to estimate with any degree of accuracy by chemical methods. These three compounds are closely related chemically, as may be seen from their constitutions:



Leucine (α-amino-*iso*caproic acid)



*Iso*leucine (α-amino-β-methyl-*n*-butyric acid)



Valine (α-amino-*iso*valeric acid)

and it becomes a matter of very real practical difficulty to devise suitable quantitative methods of separating them for chemical estimation. On the other hand, these three acids are amongst the simplest to estimate microbiologically, and differentiation is specific and exact.

For the actual estimation of amino-acids with the lactic bacteria certain practical aspects must be kept in mind. As far as possible the less exacting strains of lactic bacteria should be used for this task. It is now possible to estimate 16 of the known amino-acids by using three lactic organisms, *Lactobacillus arabinosus* 17/5, *Leuconostoc mesenteroides* P60 and *Streptococcus faecalis* R. I strongly urge that *Lactobacillus helveticus* should not be used for this type of work. In any case there is no advantage to be gained by its use instead of the organisms mentioned above, which will estimate all the acids that can be estimated with *L. helveticus*.

Some examples of the practical details of the assay of amino-acids may be considered. For instance, the assay of tryptophane differs in certain respects from the assay of the remaining amino-acids and will be described separately. The organism recommended for its assay is *Lactobacillus arabinosus*. This organism is now used for the microbiological assay of nicotinic acid. It is less fastidious in its nutritional requirements than some other species of lactobacilli, because it does not require the addition of folic acid to the medium. The medium employed for the assay of nicotinic acid has the composition given in Table 2.

With slight modification this same medium can be employed for the assay of tryptophane. Tryptophane is unique among the amino-acids because it is completely destroyed by prolonged hydrolysis with strong acids under the conditions used for the hydrolysis of casein. Thus it becomes possible to use the same medium as is used for the assay of nicotinic acid for the assay of tryptophane, by omitting the latter and

TABLE 2

COMPOSITION OF BASAL MEDIUM FOR THE ESTIMATION OF NICOTINIC ACID WITH *Lactobacillus arabinosus*

Substance	Amount
Vitamin-free acid-hydrolysed casein	1·0 per cent.
dl-Tryptophane	0·01 per cent.
l-Cystine	0·02 per cent.
Glucose, anhydrous	2·0 per cent.
Sodium acetate, anhydrous	2·0 per cent.
Xylose	0·1 per cent.
Sodium chloride	0·5 per cent.
Ca *d*-pantothenate*	0·1 p.p.m.
Vitamin B_1	0·1 p.p.m.
Riboflavin	0·2 p.p.m.
Vitamin B_6	0·1 p.p.m.
p-Aminobenzoic acid	0·1 p.p.m.
Biotin	0·0004 p.p.m.
Adenine	10·0 p.p.m.
Guanine	10·0 p.p.m.
Uracil	10·0 p.p.m.
Xanthine	10·0 p.p.m.
Ammonium sulphate	0·3 per cent.
Inorganic salt solution A†	0·5 ml. per 100 ml.
Inorganic salt solution B†	0·5 ml. per 100 ml.

* If this is unobtainable, twice the amount of Ca *dl*-pantothenate may be used.
† Barton-Wright (1946).

adding excess (0·4 p.p.m.) of nicotinic acid. Again, by replacing casein hydrolysate in the above medium with the amino-acids listed in Table 3 and adding excess of vitamins, it is possible to estimate leucine, valine, *iso*leucine and cystine.

TABLE 3

AMINO-ACID COMPOSITION OF MEDIUM FOR THE ESTIMATION OF LEUCINE, VALINE, *Iso*LEUCINE AND CYSTINE WITH *Lactobacillus arabinosus*

Amino-acids	Amount per tube (10 ml.)
	mg.
l (+)-Glutamic acid	4
dl-Aspartic acid	8
dl-Lysine	4
dl-Threonine	2
dl-Valine*	2
dl-*Iso*leucine*	2
dl-α-Alanine	2
l(−)-Cystine*	1
l(−)-Leucine*	1
dl-Methionine	1
dl-Phenylalanine	1
dl-Serine	1
l(−)-Arginine HCl	0·5
l(+)-Histidine HCl	0·5
dl-Tyrosine	0·8
dl-Tryptophane	0·8

* This amino-acid is omitted from the medium when it is the object of the assay.

Certain precautions must be observed with these microbiological assays if failure and invalid results are to be obviated. In the first place, any possibility of toxicity of individual acids must be avoided by increasing the number of amino-acids to at least 16 and preferably to 20. Secondly, the purity of the individual amino-acids used in a medium must be unassailable. Minute traces of contaminants are sufficient to lead to high blanks and invalid assays. For example, in the assay of methionine, high blanks are frequently encountered because natural leucine, unless carefully purified, carries traces of methionine. A safe rule to follow in carrying out these microbiological assays is always to use the synthetic amino-acid wherever possible. One case in which this rule cannot be applied is the assay of *iso*leucine. Synthetic *dl*-leucine should not be added to the medium because it is usually contaminated, often heavily, with *iso*leucine. The synthetic amino-acids are racemates, *i.e.*, are composed of equal amounts of the *l*- and *d*-enantiomorphs. It is usually assumed that the *d*-enantiomorph has no biological activity, but this is an assumption that should be tested. It is not always true, *e.g.*, although glutamic acid is an essential amino-acid for *L. arabinosus* and *L. helveticus*, both organisms are stimulated to a degree greater than 50 per cent. by *dl*-glutamic acid as compared with *l*-glutamic acid.

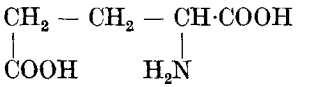
Glutamic acid forms a high percentage of many proteins, such as casein and gluten, and all chemical methods for its estimation that have been devised up to the present are particularly tedious to carry out. It is, therefore, most desirable for practical reasons that suitable microbiological methods of assay should be available. Unfortunately, the microbiological assay of glutamic acid is beset with a number of difficulties which have still to be surmounted.

A few of the difficulties may be discussed here. In the various assays of amino-acids that have already been mentioned, growth, or acid production, shows an increase directly proportional to the concentration of the amino-acid added. This result is to be expected if the amino-acids are used directly by the organisms for synthesis of bacterial protoplasm and this protoplasm has a constant composition. When we turn to glutamic acid, however, we find that the curve is sigmoid in shape. There is little growth above that of the blanks in the lower concentrations, and then suddenly a sharp break appears in the curve and growth increases rapidly with increasing concentration until a maximum is attained. This behaviour towards glutamic acid would appear to be general among lactic organisms. It is certainly true for *L. arabinosus*, *L. helveticus*, *Leuconostoc mesenteroides* and *Streptococcus faecalis* R. Such behaviour strongly suggests that part at least of the glutamic acid present in the medium must first of all be converted into some other substance before it can be utilized by the organism, and that this conversion takes place most readily when the glutamic acid is in high concentration or when vigorous growth has been initiated.

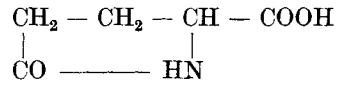
It has been established that glutamine ($CONH_2 \cdot CH_2 \cdot CH_2 \cdot CHNH_2 \cdot COOH$) is a growth stimulant for many organisms, and Lyman, Kuiken, Blotter and Hale (1945) tested this obvious possibility for *L. arabinosus*. They found that the initial lag period in response to glutamic acid was abolished by the addition of 0·25 mg. glutamine per tube of medium. The concentration of glutamine added may appear high, but most of it

is converted into pyrrolidone-carboxylic acid when the medium is autoclaved, and sufficient is left only to initiate growth. In the circumstances, therefore, the temperature and time of autoclaving must be carefully controlled. Hac and Snell (1945) partly overcame this difficulty of an initial lag period in the assay of glutamic acid with *L. arabinosus* by adding ammonium sulphate to the medium, increasing the concentration of inoculum, and lowering the *p*H of the medium. With these modifications the curve obtained tended to approach that obtained with glutamine.

Yet another method of attack upon this problem of the assay of glutamic acid has been used by Lewis and Olcott (1945). Instead of a medium composed of a long series of amino-acids from which glutamic acid had been omitted, they used a casein hydrolysate in which the glutamic acid had been converted into pyrrolidone-carboxylic acid by successive autoclavings at *p*H 2·9, followed by extraction of the pyrrolidone-carboxylic acid with ethyl acetate.



Glutamic acid



Pyrrolidone-carboxylic acid

The organism used for assay was *L. arabinosus*, and standard curves were obtained in which the initial lag period was greatly reduced. In my own personal view this is the most promising line of attack on this particular problem. One disadvantage of the method is the very tedious procedure involved in converting the whole of the glutamic acid in the casein hydrolysate into pyrrolidone-carboxylic acid and its subsequent extraction with ethyl acetate. There is no doubt that media for the microbiological assay of amino-acids are expensive and costly to prepare. If, therefore, some means could be devised for the separation of individual amino-acids from a casein hydrolysate, the cost of an assay would be greatly reduced. Some preliminary experiments have been made in this direction by the method of electrodialysis (Foster and Schmidt, 1921; Kuhn and Desnuelle, 1937; Macpherson, 1946). It is possible to obtain a quantitative separation of the basic amino-acids, histidine, lysine and arginine, on the one hand and the acidic amino-acids, glutamic acid and aspartic acid, on the other, by this process. It now remains to be seen whether a casein hydrolysate freed from the basic and acidic amino-acids and re-fortified with the missing acids can be used in the basal media in place of a mixture of individual amino-acids.

There is an additional objection to the use of either *L. arabinosus* or *L. helveticus* for the assay of glutamic acid. We have already seen that both these organisms are stimulated by the *d*-enantiomorph. On the other hand, neither *Leuconostoc mesenteroides* P60 nor *Streptococcus faecalis* R is stimulated by *d*-glutamic acid, and both give only a 50 per cent. response with the *dl*-acid. Thus, when the microbiological assay of glutamic acid has been perfected, it will probably be these two organisms which will be used for this purpose.

To revert again to more general considerations, I hope that I have been able to demonstrate, in this necessarily brief and cursory review of a vast subject, that a powerful and flexible instrument is being forged

for the assay of amino-acids. The advantages of these methods are manifest and many. The initial apparatus is inexpensive and to be found in any well equipped bacteriological laboratory. The assays are relatively rapidly performed, and a number of samples can be dealt with at one time. The accuracy of the methods compares favourably with that of the best chemical methods. The laborious separations often necessary in protein analysis are done away with, and the methods are extremely sensitive because they are essentially micromethods. The analysis of the amino-acid content of rare proteins, such as viruses, should now become a practical possibility.

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The Genetical Control of Nutritional Requirements in Micro-organisms and its Application to Microbiological Assays

Dr. G. Pontecorvo (Department of Genetics, University of Glasgow)

Modern biochemical research strongly suggests that the synthesis of complex constituents like polysaccharides, polypeptides and nucleic acids follows paths which, as a first approximation, can be considered the same in all living organisms. The difference between an autotrophic organism, like a green alga or a sulphur bacterium, and one with nutritional requirements as complex as those of a mammal, is a difference in the ability to perform the earlier steps in these syntheses. The autotroph can perform the synthesis from inorganic compounds; the mammal can start only from substances which are further up in the chain, such as amino-acids, simple carbohydrates, and vitamins. The evolutionary implications of the loss of the ability to synthesize have been considered by Knight (1936) and Lwoff (1938).

Among micro-organisms one finds at one end autotrophs, like the unicellular algae and the sulphur and iron bacteria requiring only inorganic nutrients, at the other end many forms, especially pathogens, as exacting in their nutritional requirements as a mammal. Between these two extremes, forms are found that give a practically continuous series. Even more, among forms requiring a particular nutrilite, say vitamin B_1, some require the whole molecule, others grow as well on its two moieties, and others need only one of these.

All this is, of course, very well known. What is not equally well known is that a large amount of variation in nutritional requirements

occurs also between individuals of a species, the term species being used here in a purely systematic sense. For example, *Penicillium notatum* (one of the *fungi imperfecti*), *Neurospora crassa* (one of the ascomycetes), *Bacterium aerogenes*, and *Bacterium coli* have extremely simple requirements. They are able to grow on a medium supplying a simple source of carbon, and mineral salts; biotin is, in addition, necessary for *Neurospora*. Yet, when a culture from a single-celled strain of any one of these organisms is plated, a very small proportion of single-colony isolates shows one of a number of new nutritional requirements, in addition to the minimal ones of the original strain. One of these "mutant" isolates may require nicotinic acid, another thiazole, a third lysine, a fourth choline, a fifth monomethylamino-ethanol, and so on. Each type of mutant arises repeatedly but with exceedingly small frequency; 10^{-8} per bacterium per "generation", or per nucleus per division in the case of fungi, may be taken as a rough estimate of this frequency.

Biochemical study of these mutants shows that one or other chain of essential biosyntheses has been interrupted at a specific link, with the result that growth now becomes dependent on the availability in the medium of one of the substances which come in the chain *after* the broken link (Beadle, 1945,1). In some cases the intermediate immediately *preceding* the broken link may be secreted into the medium, thus showing that we are actually dealing with the failure of a synthesis to proceed (Tatum, Bonner and Beadle, 1944). Incidentally, the enormous possibilities in the field of industrial fermentations opened up by this discovery have not yet been fully grasped.

This individual variation in nutritional requirements has been investigated genetically. In the first place this has been done with microorganisms having sexual reproduction, which makes the use of standard genetical techniques possible. Most of the work has been done with the ascomycete *Neurospora*, by Beadle, Tatum and their co-workers at Stanford University (Beadle, 1945,2), with yeast by Winge and coworkers at the Carlsberg Laboratory, and by Lindegren and co-workers at Washington University, St. Louis (Lindegren, 1945). The results of this work can be summarized in the following way.

A mutant strain differs from the original strain in its inability to perform a specific, simple step in a chain of biochemical reactions; this failure is often, perhaps always, due to the lack or inactivity of a specific enzyme; the ability or inability to perform the reaction, *i.e.*, the presence or absence of the active enzyme, is controlled by a single gene, or, to put it in other words, is inherited as a single unit. This gene may exist in one or more active forms (allelomorphs) determining the normal production of the enzyme, and in one or more mutant forms determining complete or partial absence of enzyme activity. These results open up the fundamental problem of the relationships within the cell of genes in the nucleus and enzymes in the cytoplasm. This is a crucial problem in cell physiology which cannot be discussed here.

The discovery that specific biochemical steps are controlled by specific genes makes it possible to control the biochemical steps by playing on the genes the usual tricks of genetics. For instance, we can increase enormously the rate at which a gene mutates towards one of its inefficient allelomorphs by treatment with X-rays, ultraviolet light, neutrons,

mustard gas, and by other means. We can, in other words, increase the frequency with which a mutant strain arises from, say, 10^{-8} to 10^{-3}. This means that the production of a whole collection of mutants differing from the parent strains in all possible nutritional requirements becomes a practical and relatively simple process.

The remarkable thing is that after all this was established for micro-organisms with a sexual stage, exactly the same results were obtained with micro-organisms without a sexual stage, *i.e.*, with bacteria (Anderson, 1944; Roepke, Libby and Small, 1944; Gray and Tatum, 1944; Tatum, 1945; Demerec, 1946; Devi, unpublished) and with *fungi imperfecti* like *Penicillium notatum* (Pontecorvo, unpublished) and *Aspergillus terreus* (Raper, Coghill and Hollaender, 1945). We cannot speak here of genes as units of Mendelian inheritance, though we are certainly dealing with some sort of particulate basis for inheritance. What this identity of results means is of course a very important point in modern genetics, but for the purpose of the present discussion it is sufficient to note that in bacteria, as well as in *fungi imperfecti*, hereditary blocks of specific biochemical steps can occur just as in organisms with sexual reproduction, and that these blocks occur spontaneously with a very low frequency and, after treatment, with a much higher frequency.

How easy the technique is for the production and identification of mutants with specific growth requirements can be gathered from Figure 1.

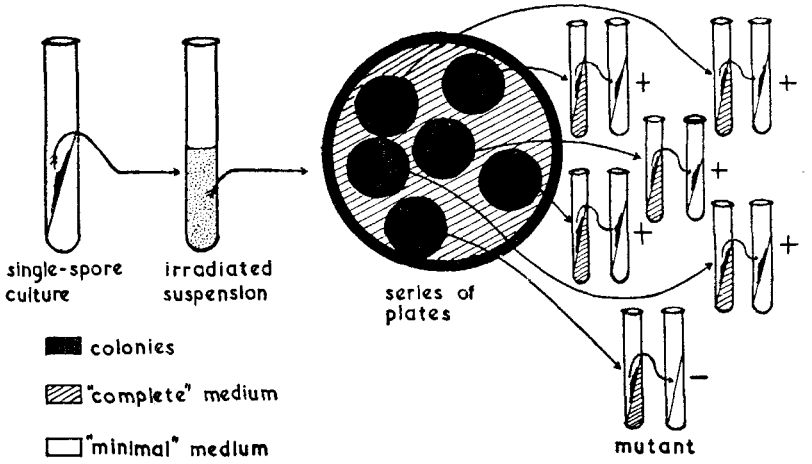

FIGURE 1.

PRODUCTION AND IDENTIFICATION OF MUTANTS WITH SPECIFIC NUTRITIONAL REQUIREMENTS.

The "complete" medium supplies amino-acids, water-soluble vitamins and purine and pyrimidine bases. The "minimal" medium supplies only glucose, nitrates and inorganic salts. A mutant is identified by its inability to grow on "minimal" medium.

It shows an adaptation by the writer of the original technique of Beadle and Tatum (1941) for the production of mutants in *Penicillium notatum*. For other organisms, *e.g.*, bacteria, only minor additional modifications are necessary. A simple method for the preliminary ascertainment of

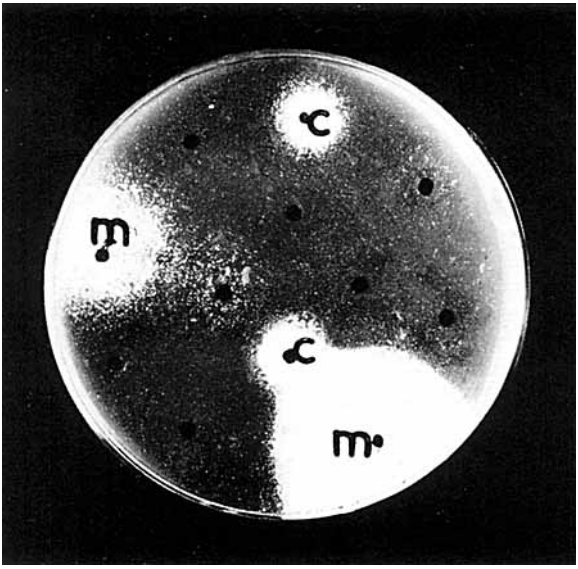

FIGURE 2.

PRELIMINARY INVESTIGATION OF THE NUTRITIONAL REQUIREMENTS OF A MUTANT.

A thick spore suspension is plated on "minimal" medium and a small crystal of each of a number of substances (amino-acids, vitamins, etc.) is placed in duplicate in marked positions on the medium. Growth takes place only around the position of the substance or substances required by the mutant. The photograph shows a mutant of *Penicillium notatum* responding to methionine (*m*) and cystine (*c*) but not to several other amino-acids. With minor adaptations this method can reveal also interaction or competition between two or more nutrilites.

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the specific growth requirement of any one mutant is illustrated in Figure 2 where a mutant of *P. notatum* is shown responding to methionine and cystine. The same method has been successfully used with bacteria (*Bact. aerogenes*) by Miss Devi at the Hannah Dairy Research Institute.

The range of mutants so far obtained by such techniques in moulds, *Neurospora* (Beadle and Tatum, 1946), *P. notatum* (Pontecorvo, unpublished), *Aspergillus terreus* (Raper *et al.*, 1945), *Ophiostoma multiannulatum* (Fries, 1945), and in bacteria, *Bact. coli* (Anderson, 1944; Gray and Tatum, 1944; Tatum, 1945; Roepke *et al.*, 1944; Demerec, 1946), *Acetobacter melanogenum* (Gray and Tatum, 1944) and *Bact. aerogenes* (Devi, unpublished), covers almost all essential amino-acids, most vitamins of the B group, purines and pyrimidines. It is obvious that an artificially produced mutant, requiring a certain nutrilite as a consequence of induced mutation, can be used for the assay of that nutrilite in just the same way as a natural strain requiring the same nutrilite, but the requirement alone does not make a strain suitable for a standardized assay.

In the first place there are practical questions such as the time required for full growth, the ease of making an estimate of the amount of growth, and the cost of the basal medium; for instance, it is well known that lactobacilli are in favour because the amount of growth of a culture can be estimated by simple titration of the acid produced. Secondly, there is the question of specificity; the organism must, ideally, respond to one, and only one, substance. Thirdly, there is the question of the variability of the results, which of course determines the reliability and precision of the assay.

If all these points are taken into consideration, there would certainly be a possibility of greatly improving the microbiological assays already used, and of developing new ones for additional substances if the problem of producing suitable strains were tackled, in general, by the genetical technique. Since, in fact, we have no control of the order in which mutants arise following irradiation, it may take as long to obtain one mutant, requiring, say, vitamin B_1, as to obtain a whole series of different ones.

In my opinion a reasonable way of tackling this problem would be the following. In the first place, the analyst should state which essential practical features an organism must have to be suitable. In the second place he should list all the substances for which an assay is wanted, especially those for which no satisfactory method of assay is at present available. The geneticist should choose among the bacteria or other micro-organisms fulfilling the above desiderata one which has nutritional requirements as simple as possible and also a low spontaneous mutability. The genetical work should then aim at the production of as many mutants as possible for *each* of the nutritional requirements desired. Among all the mutants requiring the same substance the one giving the highest specificity of response, presumably because in it the block affects the last step in the synthesis of the substance, should be chosen for the assay. If none of the mutants is specific enough, more should be produced, and in many cases the right one would sooner or later be obtained.

The result of work organized along the lines suggested would be that the mutants of a single organism, about which a good deal of information

would gradually have accumulated, would be used for all assays. The advantages in standardization and in avoiding pitfalls should be considerable.

A start along these lines has been made recently in collaboration between the Hannah Dairy Research Institute and my laboratory; success, however, can be obtained only if the closest contact is maintained between those who produce the mutants and those who use them.

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The Role of Micro-organisms in the Nutrition of Farm Animals

Dr. E. C. Owen (Hannah Dairy Research Institute, Kirkhill, Ayr)

In this country by farm animals we mean cows, sheep, horses, pigs and poultry, and perhaps cats, dogs, rats, mice and rabbits. In other countries than Britain animals not already listed are, however, extensively domesticated. For example, the goat and the buffalo supply much of the world's milk, and the indigenous tylopods of South America, the llamas, alpacas and vicuñas, were domesticated by the old Indian civilizations there. The elephant is used as a beast of burden in the tropics, while in desert areas the camel provides transport and milk. In the tundras of north Eurasia a ruminant, the reindeer, living on tree lichen and reindeer moss, provides man with transport, meat, milk and clothing. Foxes are farmed extensively on Cape Breton Island in Canada, and ostriches in South Africa. The domestic production of rabbits and pigeons also is widely distributed.

Very few of the animals mentioned have been subjected to intensive nutritional investigation and I shall confine my remarks mainly to the cow, sheep, horse, pig and poultry. The rat has already been extensively considered by Kon (1945). I have endeavoured to avoid repetition of material already published in the Society's *Proceedings*.

It is a commonplace of zoologists that the more vegetarian an animal is, the longer, more capacious and more complicated is its gut. That showpiece of the zoologist, the frog, is a perfect example. As a tadpole it is mainly vegetarian and has a long, coiled intestine. As a frog it

eats insects and worms and has a very simple alimentary tract. Thus, animals' tracts range from a relatively simple gut, as found in the carnivores, *e.g.*, the dog, through a gut of somewhat longer relative length, as found in the pig, to very complicated structures reaching their highest development in the ruminants. The pure carnivore relies almost entirely on its own digestive enzymes to bring its food into a state ready for absorption. Other animals with more complicated tracts have their digestion assisted by fermentations due to micro-organisms which they harbour. This assistance of digestion by fermentation reaches its highest development in the ruminants.

The symbiotic organisms responsible for the fermentation consist of bacteria, yeasts and sometimes other fungi, together with a variety of species of protozoa. None of these organisms are pathogenic, so that they must be regarded as symbionts. It is best to use the term symbiosis to signify an intimate association of two species without implication as to the benefits conferred by one upon the other, provided that the association is not harmful to either partner. If, with the development of scientific research, it is found that the species harboured confers benefits upon the host, then the relation between the two species becomes one of mutualism. A classical example of mutualism is the association of legumes and the bacteria in the nodules of their roots.

Symbiotic organisms may benefit their hosts by enabling them to digest cellulose, by enabling them to utilize ammonium nitrogen for protein synthesis, or by elaborating vitamins for them. Of these three processes the digestion of cellulose is probably by far the most important.

Known examples of mutualistic symbiosis in animals are shown in Tables 1, 2, and 3.

TABLE 1

MICRO-ORGANISMS CONCERNED AS SYMBIONTS IN THE DIGESTION OF CELLULOSE IN VARIOUS PARTS OF THE ALIMENTARY TRACT OF CERTAIN ANIMALS*

Species of animal	Organ of body harbouring symbionts	Micro-organisms
Mammals		
Ruminants[2] (cattle, sheep, goats) ..	Rumen, reticulum (caecum, colon)	Bacteria, yeasts + protozoa
Rabbit[2]	Caecum	Bacteria
Horse[2]	Caecum and colon	Bacteria
Birds		
Fowl (*Gallus*)[11]	Caeca	Bacteria
Insects		
Wood-wasps (*Sirex*)[14]	—	Fungus
White ants (termites)[6]	Hind-gut	? Protozoa ? Bacteria ? Fungi

* The numerals refer to the authorities set out in the special bibliography for Tables 1 to 3, p. 189.

Note.—Question marks in Tables indicate that the evidence is inconclusive.

TABLE 2

MICRO-ORGANISMS CONCERNED AS SYMBIONTS IN THE SYNTHESIS OF PROTEIN FROM AMMONIA IN THE ALIMENTARY TRACT OF CERTAIN ANIMALS WITH AN INDICATION OF THE NATURE OF THE SUBSTANTIATING EVIDENCE*

Species of animal	Organ inhabited	Nature of evidence	Micro-organism
Cow	Rumen	Nitrogen balance[13]; milk production[13]	Iodophile bacteria[15]
Calf, Lamb	Rumen	Growth on subnormal protein intake[12]	Iodophile bacteria ? Yeasts
Sheep	Rumen	Nitrogen balance[12]	? Yeasts Iodophile bacteria
Pig[5]	—	Cannot use ammonia as a source of protein	—
Fowl[3]	—		—
Rat[9]	—		—

* The numerals refer to the authorities set out in the special bibliography for Tables 1 to 3, p. 189.

TABLE 3

NATURE OF THE EVIDENCE FOR THE SYNTHESIS OF VITAMINS BY MICRO-ORGANISMS LIVING SYMBIOTICALLY IN THE ORGANS OF ANIMALS*

Vitamin	Species of animal	Organ inhabited	Nature of evidence	Organism responsible
Vitamin B complex	Cattle[8]	Rumen	Analysis of rumen contents. Determination of excess of output over intake	? Bacteria
	Rat[8]	Caecum	Experiments on refection	? Bacteria, ? Yeasts
	Fowl[1]	Site not specified ? caecum	Effect of sulphonamides by analogy with rat	? Yeasts ? Bacteria
	Beetles infesting stored grain, *e.g. Lasioderma* and *Sitodrepa*[4]	Mycetomes	Vitamin B requirement of insects reared from sterilized eggs	Bacteria
Vitamin K ..	Cow[8]	Rumen	—	? Bacteria
	Rat[8]	Caecum	Effects of caecectomy and sulphonamide treatment	Bacteria
Vitamin E[7] ..	Rat	—	Effect of sulphonamides	?
β-Carotene[10] ..	An insect, *Cicadella viridis*	Mycetomes	Effect of egg sterilization	Bacteria

* The numerals refer to the authorities set out in the special bibliography for Tables 1 to 3, p. 189.

Note.—Question marks in Tables indicate that the evidence is inconclusive.

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A further function of symbiotic micro-organisms in the rumen of sheep and cattle must be added to those listed in Tables 1, 2 and 3. It is the power the rumen bacteria have of producing lower fatty acids by fermentation of carbohydrates, including cellulose. This process occurs also to a small extent in the stomach of the horse, owing to inefficient mixing of food (Mangold, 1929). The production of lower fatty acids by rumen micro-organisms is no new discovery (Mangold, 1929), but only as a result of recent work at Cambridge (McAnally and Phillipson, 1944; Elsden, 1945) has the quantitative importance of the production of fatty acids in the rumen been fully realized. Further, Phillipson and McAnally (1942) have made the important discovery that organic acids produced in the rumen are directly absorbed through its walls into the portal circulation. The free fatty acids are more rapidly absorbed than their ions (Danielli, Hitchcock, Marshall and Phillipson, 1945), thus aiding the saliva in the preservation of a *p*H optimal for continued bacterial growth and metabolism.

The best substrates for the production of fatty acids by rumen bacteria are starches and sugars. Elsden, Hitchcock, Marshall and Phillipson (1946) found that the concentrations of volatile fatty acids in the alimentary tracts of the pig, rat, rabbit, horse, sheep, cow and deer were greatest in parts where bacterial activity was greatest, *i.e.*, in the paunch of ruminants and in the caecum and colon of all species.

The Digestion of Cellulose

As a result of observations with the microscope dating back many years it is generally agreed that in mammals the digestion of cellulose is brought about by micro-organisms. There is still difference of opinion on the question whether the protozoa found in the rumen can digest cellulose. There is little doubt that great weight was lent to the theory that protozoa can digest cellulose by the work of Cleveland (1928), who attributed, on the basis of his own microscopical and biochemical observations, cytoclastic powers to the protozoa (*hypermastigoda*) occurring in the hind-gut in certain termites. Cleveland believed that these *hypermastigoda* could even fix atmospheric nitrogen.

Cleveland's work has been reviewed by Mansour and Mansour-Bek (1934), who reject Cleveland's conclusions.

In spite of a report by Hungate (1943) that protozoa in the rumen can digest cellulose, other observations have tended to minimize belief in their cytoclastic powers. Thus Becker and Everett (1930) found that the growth of lambs in whose rumen the protozoa had been destroyed by a process considered to have had no detrimental effect on their health was slightly better than that of controls.

Van der Wath and Myburgh (1941) showed that starch in the feed of sheep with a rumen fistula disappeared just as quickly in the absence of the protozoa as in their presence.

It has, nevertheless, been shown (Johnson, Hamilton, Robinson and Garey, 1944) that protozoa separated in bulk from rumen contents are readily digestible and of high biological value. It is known also that rumen bacteria form a suitable food for isolated rumen protozoa (Hungate, 1943). It has therefore been suggested by Baker (1942, 1, 2) that in the ruminants protozoa form part of a food chain from which the host animal ultimately benefits. Such food chains are illustrated in Figures 1 and 2.

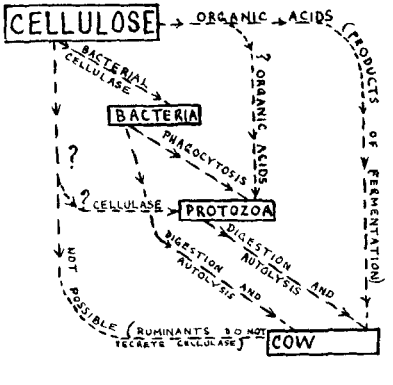

FIGURE 1.
FOOD CHAIN OF UTILIZATION OF CELLULOSE BY THE COW.

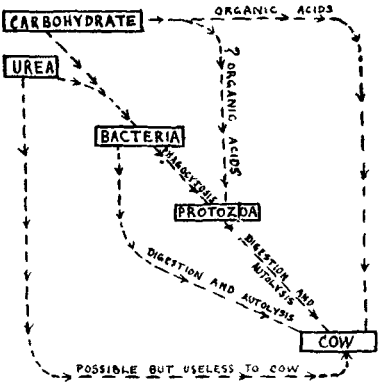

FIGURE 2.
FOOD CHAIN OF UTILIZATION OF UREA AND CARBOHYDRATE BY THE COW.

The benefits which the protozoa in ruminants confer upon their hosts still require elucidation. In view of the ready availability of acetic acid in protozoal metabolism (Doyle, 1943) it may be that the protozoa are purely commensal. Unless the digestibility of the protozoa is much greater than that of the bacteria, the entropy tax upon the process whereby bacterial protoplasm becomes protozoal protoplasm before final digestion would more than outweigh the benefit resulting from the increase in digestibility.

Herbivorous animals such as the horse and elephant come next to the ruminants in power of digesting cellulose. The pig is a poor third, while the chicken's power to digest cellulose is very small. In feeding farm animals Morrison (1938) puts roughage intakes, which would approximately represent intakes of cellulose, in the following descending order: cow and sheep, horse, pig. According to Nehring and Schramm (1939), sheep digest the fibre of hay better than rabbits, while rabbits digest the fibre of cereal grains better than pigs. In the cow, sheep, horse, goat and pig,

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bacteria are mainly responsible for cellulose digestion. The chicken, in common with other birds, gains access to the protoplasts locked up in the cell walls of plants by means of maceration in the crop and efficient grinding in the gizzard. The chicken, according to Mangold (1929), relies only to a very limited extent on the action of bacteria.

TABLE 4

CAPACITY OF PARTS OF THE GUT OF CERTAIN DOMESTIC ANIMALS EXPRESSED AS PERCENTAGES OF THE CAPACITY OF THE WHOLE GUT, TOGETHER WITH THE PERCENTAGE OF "FILL" OR RATIO OF THE TOTAL CAPACITY OF THE GUT TO THE BODYWEIGHT

Animal	Capacity of certain organs in terms of whole gut per cent.			Percentage "fill"
	Stomach	Colon and caecum	Small intestine	
Cow	66	12	22	12
Sheep	62	12	25	—
Horse	9	67	24	—
Pig	33	33	33	7·5

Figure 3 illustrates the differences in type of alimentary tract in the commoner farm animals. Table 4 gives some approximate figures for the capacities of parts of the tract expressed as fractions of the capacity of the total tract. I tried also to find out the relative percentage of "fill" in these four species, *i.e.*, the ratio of total capacity of gut to bodyweight, and to amplify the table to include the goat, dog, cat and chicken, but data were not easy to find.

Tables 5 and 6 show the extent to which cellulose, *i.e.*, the "crude fibre" of the analyst, is digested by animals eating a diet such as should be fed only to efficient digesters of cellulose.

TABLE 5

PERCENTAGE DIGESTIBILITY OF FIBRE (CELLULOSE) IN VARIOUS FEEDS BY BULLOCKS ON DIFFERENT PLANES OF NUTRITION

(Ritzman and Benedict, 1938)

Feed	Plane of nutrition	Digestibility of crude fibre Per cent.
Hay	Undernutrition	62
	Maintenance	49
Hay, maize meal and bran ..	Above maintenance	53
		52
Hay, linseed meal and bran ..	Above maintenance	59
		50

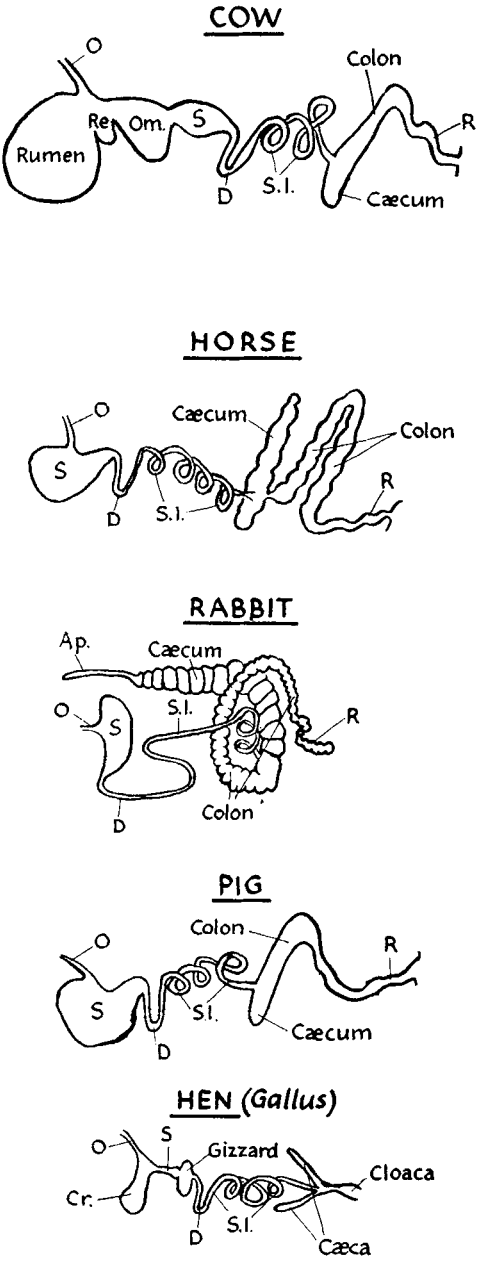

FIGURE 3.
ALIMENTARY TRACTS OF FARM ANIMALS.

Explanation of symbols: O. Oesophagus. Re. Reticulum. Om. Omasum. S. True stomach. D. Duodenum. S.I. Small intestine. R. Rectum. Ap. Vermiform appendix. Cr. Crop.

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Relative to the size of the animal the paunch of the ruminant is very large. Ruminants can therefore cope with far larger quantities of roughage than other animals and can be maintained in winter on hay alone. In summer, ruminants can thrive on grass and turn it into flesh or milk. Such production necessitates the digestion of the protoplasts of the grass or other plant material eaten. To do this a prior removal of the cellulose walls is necessary. Hence to get protein, fat and minerals from grass or hay entails the prior dissolution of cellulose. For this the bacteria of the paunch are responsible.

TABLE 6

PERCENTAGE DIGESTIBILITY OF CRUDE FIBRE IN HAY BY VARIOUS MAMMALS
(The results for the first four animals are from Ritzman and Benedict (1938) and for the last two from Voris, Marcy, Thacker and Wainio (1940))

Animal	Feed	Digestibility of crude fibre per cent.
Elephant ..	Coarse hay	38
Horse	Coarse hay	43
	Fine hay	47
Cow	Coarse hay	55
	Fine hay	76
Sheep	Fine hay	51
Ruminant ..	Not stated	53
Rabbit ..	Similar to above but not stated	17

As to the specific organisms responsible for the digestion of cellulose, there is, as has already been said, considerable divergence of opinion. This difference of opinion is due mainly to technical difficulties. Many investigators, such as Woodman and Stewart (1932) at Cambridge, Pochon (1935) in France, and Elsden (1945) at Cambridge, have isolated cultures of bacteria from rumen contents of sheep and cattle, and have found the organisms capable of hydrolysing cellulose slowly. The organisms, however, preferred more readily hydrolysed carbohydrates, such as sugars, to cellulose as a substrate; besides, the times required by the organisms to dissolve cellulose were very long. Evidently the digestion of cellulose in the rumen is much more efficient than any digestion *in vitro* so far accomplished. Again, volatile lower fatty acids are produced in the rumen in considerable amounts but, whereas in the rumen itself the predominant acid produced is acetic, *in vitro* the predominant acid is propionic, no matter whether the substrate is cellulose, glucose or lactic acid (Elsden, 1945). In rapid fermentation in the rumen, such as is produced by feeding clover or by adding sugar to the food, lactic acid is produced at first, but its rate of production declines

while that of acetic and propionic increases. From work at Cambridge and in South Africa it is safe to conclude that, with variation of the diet of sheep, not only does the type of fermentation change, but also the composition of the microflora. Thus, change from grass to lucerne hay favours an increase of yeasts such as *Schizosaccharomyces ovis* Quin, at the expense of iodophile bacteria (Quin, 1943).

In the present state of knowledge the best evidence for the kind of organism responsible for the digestion of cellulose in the rumen of the cow and sheep, and in the caecum of the horse and other herbivora, is provided by the use of the technique which Baker (1939) has employed, *i.e.*, direct microscopic observation with the petrological microscope. By such methods it can been seen that a type of streptococcus is responsible for the enzymic excavation of cavities in the cellulose walls of plant cells. During the production of such cavities the unattacked cellulose can be seen, with crossed nicols, to retain its anisotropy, while the cavities, the streptococci in them and a halo of cellulose undergoing attack become invisible because of their isotropic nature.

The digestion of cellulose is affected by the composition of the diet. Thus Johnson, Hamilton, Mitchell and Robinson (1942) have shown that increase of the non-fibre carbohydrate in the diet, the so-called N-free extractives, reduces the power of lambs to digest cellulose. Harris and Mitchell (1941) observed that urea increased the power of the sheep to digest cellulose.

It is believed by some that the rapid fermentation of carbohydrates other than cellulose by rumen bacteria or yeasts may be a cause of bloat in ruminants. There are, however, several other equally plausible rival theories as to the aetiology of bloat, which have been listed in a review now in the press (Owen, 1947).

Digestion of Cellulose in the Horse

According to Scheunert and Krzywanek (1929) the caecum is the chief site of cellulose digestion in the horse. They reported that efforts to isolate the responsible organism or organisms had not been successful at the time of writing, though Hopffe (1913) isolated an *Aspergillus* which he found also in the paunch of ruminants. Accompanying cellulose fermentation in the horse, as in the ruminant, are the evolution of CO_2, CH_4 and H_2 and also the production of organic acids. In the horse organic acids, *e.g.*, lactic acid, are also to a small extent produced in the stomach from recently ingested food mixed with saliva. The mixing of the contents of the horse's stomach is not very efficient, so that food is not subjected to the action of pepsin and hydrochloric acid until some time after ingestion. A horse normally digests some 30 to 50 per cent. of crude fibre. Digestibility is considerably reduced by surgical removal of the caecum. Cellulose digestion continues, but with diminishing intensity, as the food passes along the colon. Probably absorption of water and soluble nitrogenous substrates, and the production of too low a *p*H, are responsible for this diminution of intensity and serve to explain the paradox that, while both horse and cow have "fermentation vats" occupying some two-thirds of the capacity of the gut, the cow is the better utilizer of cellulose and can tolerate the larger intake of roughage. Scheunert and Krzywanek (1929) give figures showing the progressive

absorption of water as the food passes from caecum to rectum by way of the colon.

Digestion of Cellulose in the Pig

Such digestion of cellulose as occurs in the pig, which has by comparison with the horse a very limited capacity for dealing with roughage, takes place, according to Scheunert and Krzywanek (1929), in the colon as a result of bacterial action. The pig also harbours protozoa in its colon, but their function is not clear. Trautmann and Asher (1941, 1) used caecal and colonic fistulae in pigs to show that certain types of plant cellulose, *e.g.*, epidermal cells of the onion, are readily dissolved in a pig's gut in 6 to 8 hours, though artificial silk and cotton flax are unaffected after 6 days. Microscopical observation showed a coccus to be responsible. The same authors (Trautmann and Asher, 1939, 1941, 2) showed that cellulose is more efficiently digested in the rumen of the goat than in the pig's caecum and that it is dissolved extremely slowly in the caecum of the goat.

Digestion of Cellulose in Birds

Birds generally do not make use of cellulose, presumably because a heavy gut would be incompatible with flight. According to Mangold (1929), the bird relies more on mechanical than on chemical means to disrupt the cell walls of plant tissues. He regards the crop mainly as a storage organ necessitated by the bottleneck at the gizzard. After maceration in the crop the food is mixed with pepsin and hydrochloric acid in the proventriculus before being efficiently ground in the gizzard.

TABLE 7

PERCENTAGE DIGESTIBILITY OF CRUDE FIBRE (CELLULOSE) BY THE HEN
(Radeff, 1928, and Henning, 1929, quoted by Mangold, 1929)

Food	Digestibility of crude fibre per cent.	
	Normal hens	Hens with caeca removed
Barley	0	0
Wheat or rye ..	4·6 to 5·7	1·4
Maize	17·0	0
Oats	9·3	1·7 to 1·0

Nevertheless, some breakdown of cellulose does occur in the hen, and experiments described by Mangold (1929) show that the site of such breakdown is the paired caeca which in many birds are long, if not capacious, organs. Generally speaking, the more vegetarian the diet, the longer and more complicated are the caeca and the larger the large intestine. The ostrich, for example, has caeca over two feet long. In the domestic hen the caeca are 6 to 10 inches in length.

Bacterium coli is present in the fowl's intestine and abundant in the caeca. Since the caecal fluid has no digestive power, the breakdown of

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cellulose must be due to bacterial symbionts. That the digestion of cellulose by the domestic hen is not extensive, however, is shown by the fact that only one-sixth to one-sixteenth of the cloacal droppings come from the two caeca.

Radeff (1928) found, as shown in Table 7, that caecectomy completely destroyed or considerably reduced the power of hens to digest cellulose. Radeff's observations were confirmed and extended by Henning (1929). Further indirect evidence of cellulose digestion was adduced by Roeseler (1929), who showed that caecal faeces contained much less crude fibre than non-caecal ones. Mangold (1929) quotes many estimates of the hen's power to digest cellulose. They vary widely and there is no point in quoting them, because important items, such as the percentage of cellulose in the whole diet and the duration of the period of feeding, are not given and, on analogy with the ruminant, both these factors would considerably affect a symbiotic microflora.

The Formation of Protein

On the formation of protein from non-protein nitrogen I do not intend to say much, because the subject has already been described to the Society at a London meeting by Smith (1945). Data from milk yields of cattle in Germany, the United States of America and Britain, and from growth rates of lambs and calves, leave little doubt as to the efficiency of urea, together with readily available carbohydrates, preferably in the form of starch, in reducing the ruminant's requirement of protein. There is also abundant evidence provided by Smith and Baker (1944) and by Baker (1943) that multiplication of iodophile bacteria in the rumen is associated with the transformation of non-protein nitrogen into protein and with the accumulation of bacterial polysaccharide. The question of the relative quantitative importance of the following processes: the production of lower fatty acids in the rumen, the formation of protein, and the production of bacterial polysaccharide, has been answered by McNaught and Smith (1946, unpublished), using their technique of incubation of rumen contents *in vitro*. As substrate for the bacteria, urea and maltose were used. After incubation 89·6 per cent. of the maltose could be accounted for as follows: bacterial polysaccharide 14·7 per cent., volatile fatty acids 34·7 per cent., lactic acid 8·5 per cent., carbon dioxide 13·7 per cent., methane 4·2 per cent. and protein 13·8 per cent.

A more difficult question is where the rumen bacteria get their methionine. Methionine is the essential form in which sulphur is ingested by the mouse, rat, dog, man, and chicken, *i.e.*, all the species so far investigated, though it can be partly replaced by cystine. Kellermann (1938, 1, 2, 3) in South Africa gave elementary sulphur to sheep but produced only symptoms of toxicity. A recent American experiment on this subject may be mentioned tentatively. Loosli and Harris (1945) gave lambs isocaloric diets containing (*a*) no urea, (*b*) urea, (*c*) urea and sulphate or (*d*) urea and methionine. The lambs showed better nitrogen retention with, than without, urea. When they received methionine as well, they showed still better retention. An isocaloric diet (*e*) containing linseed meal gave the biggest retentions. This experiment, however, requires confirmation, for only 5 lambs were used in all. If confirmed, this result is interesting, because it sets a limit to the synthetic powers

of rumen bacteria and thus runs counter to the theory of Miller and Morrison (1942) that all protein for ruminants has very nearly the same biological value because it all goes through the same bacterial form before digestion. There are many objections to Miller and Morrison's theory. In my opinion, in cattle fed on a high plane of nutrition most of the protein is obtained in the orthodox way; as the plane of nutrition is lowered, bacterial synthesis assumes a greater relative importance. Such considerations may explain the failure of some investigators to demonstrate the utilization of urea by ruminants.

The Symbiotic Synthesis of Vitamins

Early in the history of the water-soluble B vitamin, which eventually evolved into the vitamin B complex, two discoveries were made whose close connexion was not at first realized. One was the demonstration by Bechdel, Eckles and Palmer (1926) in the U.S.A. that cattle did not need water-soluble vitamin B because it was made for them by symbiotic bacteria in the rumen. The other was the discovery of the phenomenon of refection in the laboratory rat by Fridericia (1926) in Denmark. Refection is a sudden change in a rat which renders it proof against deficiency of B vitamins. We now know that refection is due to symbiotic organisms, yeasts and bacteria, inhabiting the rat's caecum, so that the refected rat's metabolism resembles that of a normal ruminant as far as its requirement of B vitamins is concerned. An account of refection in the rat and its analogy to rumen physiology has already been given by Kon (1945).

Cattle can synthesize in their rumen vitamin B_1, riboflavin, pantothenic acid, vitamin B_6, nicotinic acid, biotin and also vitamin K (Goss, 1943), as judged by increases of these substances in rumen contents, the increases being many times greater than the concentration in the rations used. Hunt, Kick, Burroughs, Bethke, Schalk and Gerlaugh (1941) reported failure to demonstrate rumen synthesis of vitamin B_1, contrary to other workers' findings, but found synthesis of the other members of the vitamin B complex. An explanation of the discrepancy may perhaps be found in the observation by Bhagvat and Devi (1944, 1, 2, 3) that certain cereals and pulses contain a substance capable of destroying vitamin B_1. The substance was not the Chastek factor, for it was not enzymic in nature. Definite evidence as to the organisms responsible for synthesis of vitamins in the rumen is still needed.

Synthetic processes in the rumen present features of similarity to, and differences from, synthetic processes occurring in refection. Thus, protein synthesis in the rumen seems to be better with starch than it is with the more soluble sugar or the less soluble cellulose, though cellulose varies according to its origin. Beet-pulp cellulose, which Woodman and Stewart (1932) found to be readily attacked by rumen organisms, is a good adjuvant of dietary ammonia given as a source of protein to cattle (Millar, 1944). In view of the fact that sugars are good sources of energy for rumen bacteria growing *in vitro* (Smith, 1945), it would seem likely that synthetic processes in the rumen are favoured by a carbohydrate, such as starch, which yields to bacterial attack more readily than cellulose but which does not pass out of the rumen as quickly as a water-soluble sugar. The advantage of a solid carbohydrate, such as

cellulose or raw starch, is that, as a former colleague of mine puts it, it gives the bacteria "something to sit on".

The similarity to refection is obvious. Refection in the rat occurs when uncooked starch, particularly potato starch, is given. It is inhibited by substituting sugar for starch, or by dextrinizing or cooking the starch. An interesting observation is that of Hopkins and Leader (1945), that refection can be prevented or inhibited by giving rats cellulose in the form of filter paper.

Evidence as to the synthesis of vitamins in chickens (Ansbacher, 1944) is indirect. That such synthesis is attributable to symbiotic micro-organisms is at present only a hypothesis, the truth of which is strongly favoured by analogy with the rat and the ruminant. No opinion can be given as to the possible role of micro-organisms in covering the horse's requirement of B vitamins, experimental evidence being as yet inadequate (Pearson, Sheybani and Schmidt, 1944).

Possible Effects of Symbiotic Micro-organisms not already Cited

The beneficial effect on non-ruminants resulting from cooking soya beans (Everson, Steenbock, Parsons and Cederquist, 1943) is in contrast with the absence of such benefit in ruminants (Miller and Morrison, 1944). Perhaps the bacteria in the rumen are as effective as cooking in inhibiting the effect of the toxic substance in soya beans.

In some circumstances ingested materials may become toxic as a result of bacterial action in the rumen. The well-known toxicity of nitrate for ruminants is an example; the rumen contents reduce the nitrate to the very toxic nitrite (Olson and Moxon, 1942).

Recently it has been found that bacteria may benefit not only the host animal but also animals which eat the faeces of the host. Thus much waste of maize occurs in the U.S.A. through the feeding of uncrushed maize to fattening bullocks. This waste is made good by allowing poultry and pigs to eat the dung of the cattle (Ritzman and Benedict, 1938). Rubin and Bird (1946, 1, 2) have found that such dung contains an unidentified substance which stimulates the growth of hens. The dried dung is a suitable addition to poultry rations.

In conclusion it may be said that in feeding farm animals, as in feeding human beings, we must attend to the needs not only of the animal itself but also of its beneficent symbionts.

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Comparison between Direct Microscopical and Pure-Cultural Methods of Observation of Micro-organisms

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The classical Koch Conn postulates (Conn, 1917) for the establishment of a causal relationship between a micro-organism and some form of

normal or morbid process include both the actual demonstration of the micro-organism in the substrate and its isolation in pure culture. It is clear, therefore that, *in principle*, cultural and direct microscopical techniques are complementary rather than mutually exclusive disciplines. Yet, *in fact*, not only may it be impossible to meet these ideal requirements but, even where no difficulty arises, the scope and character of the information gained from cultural and from microscopical studies vary with the nature of the problem. This situation has long been familiar to soil microbiologists (Waksman, 1932) and has led to an extensive elaboration of direct methods. Similar problems are, however, encountered in the microbiology of the digestive tract, particularly in herbivora, where a mixed microbial population is intimately concerned with the breakdown of the ingested plant material. For instance, not only the rumen ciliates but also several representative species of iodophile bacteria present in the rumen and caecum have never been grown on artificial media, let alone isolated in pure culture. Consequently, the elucidation of the microbial factors concerned in the synthesis of protein from non-protein nitrogen in the rumen requires, as Dr. J. A. B. Smith and myself (Smith and Baker, 1944) discovered early in our investigation, a systematic application of direct microscopical methods, including a counting technique. Exclusive or premature resort in such connexions to the traditional plating-out procedures may result, even when a wide range of media is employed, in an entirely unreliable estimate of the kinds and numbers of micro-organisms concerned.

Descriptive Notes on Figure 1

1. Decomposition of starch. Starch granule (potato) in process of decomposition in caecum of guineapig. The granule is entirely surrounded by a dense mass of iodophile bacteria (vibrios). Iodine prep. × 500.
2. Decomposition of cellulose. Portion of epidermis of grass from rumen of sheep in polarized light. The decomposition of the region surrounding the guard cells is disclosed through loss of birefringence. The cutinized guard cells remain intact. Stephenson's medium × 500.
3. Decomposition of cellulose. Fragment of plant material from rumen of sheep in polarized light. Decomposition disclosed through loss of birefringence. Stephenson's medium plus iodine × 500.
4. Same preparation as 3, with nicols uncrossed. The iodophile bacteria responsible for decomposition are disclosed surrounding the margins of the affected regions of the substrate.
5. *Amylosarcina maxima*. A member of the free macro-iodophile population of the rumen of the ox which synthesizes bacterial starch from soluble carbohydrates. Iodine prep. × 800.
6. *Amylobacter radians*. Free macro-iodophile population, rumen of ox. Iodine prep. × 500.
7. Micro-organisms associated with disintegration of desquamated epithelium in rumen of lamb (3 days). Gram prep. × 800.
8. Decomposition of cellulose. Plant hair in polarized light, from caecum of horse. Disintegration disclosed through loss of birefringence. The outer margin and base of the hair (cutinized) remain intact. Stephenson's medium × 400.
9. Bacterial invasion of lymph nodules of appendix of normal rabbits. The micro-organisms entirely infiltrate the interstices of the tissue and are taken up in masses by the macrophages. A single macrophage replete with bacteria is seen in the centre. Gram stain × 800.
10. *Amylococcus*. Free macro-iodophile population, rumen of ox. Iodine prep. × 800.
11. *Amylospirillum*. Free macro-iodophile population, rumen of ox. Iodine prep. × 800.

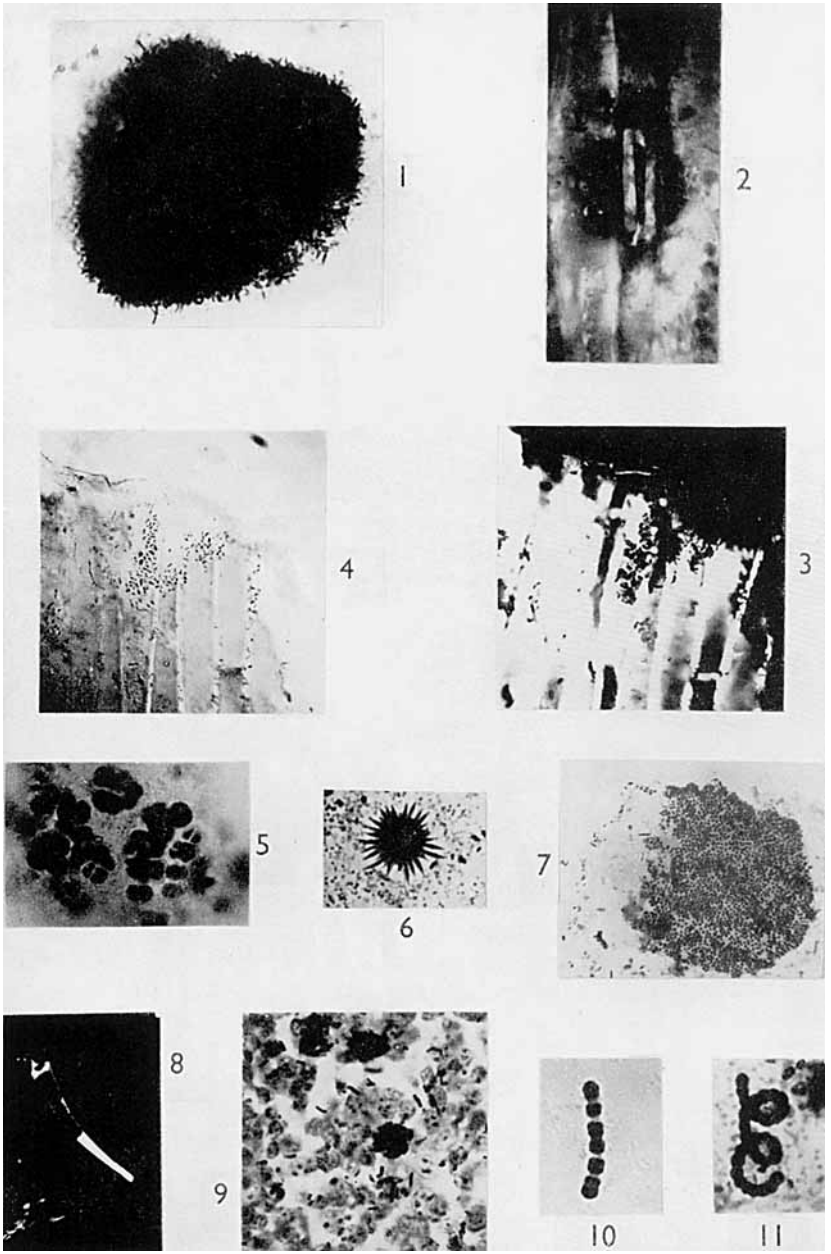

FIGURE 1. EXAMPLES OF DIRECT MICROSCOPICAL EXAMINATION.

[*For description, see p. 200.*

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We may, however, encounter also problems of an altogether different complexion, where the direct method is not merely forced upon us by the absence hitherto of any alternative but lays claim to consideration as an independent method of inquiry. Such is the state of affairs wherever it is necessary to gain insight into the actual relationships established *in vivo* between a mixed microbial population and its natural habitat, particularly where the latter embraces a substrate which is not merely a polyphasic, but a structurally organized, system. The natural fodder plants ingested by the herbivora clearly fall into this category, as is sufficiently apparent even if, ignoring the cell contents, we confine attention to the cellulose skeleton. Indeed, to regard this skeleton as so much crude fibre through which a variable quantity of lignin, cutin and wax is uniformly dispersed, is to ignore the distinctive features of the system. For not only are the so-called encrusting substances allocated to particular histological structures, but their distribution in the cell wall itself may be strictly regional (Bailey, 1938). The cellulosic matrix also possesses an intrinsic micellar organization, the actual configuration of whose main and secondary valencies may exert an influence on the distribution of the micro-organisms and the form and alignment of the enzymic cavities which they produce (Bailey and Kerr, 1937; Bailey and Vestal, 1937, 1, 2; Baker and Martin, 1937; Baker, 1939). The plant substrate therefore embraces macroscopic, microscopic, and submicroscopic levels of organization, the progressive stages of whose dismemberment in the gut cannot be exhaustively resolved by methods of bulk analysis. Since, moreover, the details of this organizational hierarchy differ from species to species and are subject to alteration with age, climate and manurial conditions, it is evident that their elucidation has a direct bearing on the practical problems of animal nutrition and welfare. Thus, to state the matter simply, in order to discover the factors governing the digestibility of grass or clover we must take into account the organization of the entire blade of grass or clover leaf as a gross and fine structural complex.

In approaching this problem a start was made some time ago through the introduction of the polarizing microscope and histochemical methods in the examination of the gut contents of herbivora. Since the techniques employed were described at a previous meeting of this Society (Baker, 1945) it must suffice to recall that disintegration is disclosed through loss of birefringence, the responsible micro-organisms being observed *in situ* on uncrossing the nicols (Figure 1). At Helsingfors Vartiovaara and Roine (1942) have confirmed the role of the iodophile types associated with this process, and specifically assert that, although they could be maintained in mixed culture, all attempts at pure cultural isolation have so far failed. Here also, therefore, to ignore the independent resources of the microscope is to court blank ignorance in regard to primary features of the cytoclastic process. What is next required is an equally satisfactory method for the microscopic detection of encrusting substances such as lignin, cutin and wax. Histochemical procedures, to be sure, are available, but they leave much to be desired in regard to scope and elegance. Far more promising appears to be the observation of the intrinsic and secondary fluorescence of the affected structures in ultraviolet light according to the technique elaborated by Haitinger (1938)

for botanical use. Such methods have already been applied by Eichler (1935) to the fungal decomposition of timber, and their application to the examination of plant residues in the gut presents no obvious difficulty.

Quite as important as the co-ordination of optical and histochemical methods, however, is the full utilization of the combined resources of the method of direct microscopical observation with the fistula technique designed by Phillipson (Phillipson and Innes, 1939). In a plan of investigation now under way at this Institute it is proposed to apply these methods in combination to the examination of a series of plant fibres, such as raw cotton, degreased cotton, flax, ramie, hemp and jute, selected with reference to differences in content of hemicelluloses, pectic substances, lignin, cutin, and wax. In this way the factors determining the intrinsic digestibility of the crude fibre components will be isolated from the disturbing influences introduced by the presence of cell contents in the fresh vegetable material. Thus a preliminary simplification of the problem is effected, and it is conjectured that the data assembled will afford insight into the changes occurring in the more complex substrates of actual fodder plants, to the examination of which we shall then proceed. During this work a renewed attempt will be made at pure cultural isolation of the microbial agents, with the knowledge gained in advance that, if we meet with success, we shall be dealing not with casual passengers in the gut but with micro-organisms whose role in the process of disintegration has been independently established by direct microscopical examination of the affected material.

One or two points now remain which in conclusion may be worth a moment's attention. The first is that, in addition to that of cellulose, the decomposition of starch granules can be studied by the methods outlined above. Secondly, the types of micro-organism encountered on cellulosic material in herbivora may be demonstrated microscopically also in the human colonic and caecal contents, and may be responsible for the known increase in digestibility of crude fibre in man associated with some clinical conditions (Baker and Palmer, 1946). A third and more important point is that the organized substrate may include, as well as ingested material, the actual tissues of the animal. Thus, in rabbits, Dr. Enticknap and I (Baker and Enticknap, 1943) were able to demonstrate microscopically that, in the normal animal, the iodophile bacteria of the caecum invade the vermiform appendix in vast numbers and are taken up there by the macrophage system of the nodules. More recently, again, in examining the gut contents of lambs from ewes on high and low planes of nutrition, it has been observed that the desquamated epithelium of the rumen is the natural habitat of a dense microbial population entirely distinct from the milk microflora and isolated from it, in the young animal, by the action of the oesophageal reflex.

It would be out of place to discuss the significance of these and the preceding observations here. My object in citing them is to illustrate the actual scope and applications of the direct method by reference to concrete examples. In comparing the direct method with the traditional cultural methods I would suggest the following conclusions. Pure-cultural and direct methods play a complementary role in the elucidation of the changes occasioned in the gut by microbial agencies. Wherever an organism can be studied in pure culture this should manifestly be done.

Where it is not possible, however, valuable information may still be gained by direct microscopical examination, particularly where a counting technique can be employed. Direct methods are in any case essential to the control of pure-cultural work and to the independent investigation of the changes occurring in complex organized substrates. Throughout, the greatest advantage may be expected to arise from their combination with the fistula technique and methods of bulk analysis.

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Food Yeast

Dr. A. C. Thaysen (Microbiological Research Institute, St. Claire, Port of Spain, Trinidad)

Since I am not engaged in nutritional work I cannot discuss the nutritional aspects of food yeast, a subject which, I understand, is still under debate.

When I was asked to speak on food yeast I took it to mean that members of The Nutrition Society might be interested to hear a brief account of the process which has been evolved for the large-scale manufacture of this protein and vitamin concentrate. Before giving this account, I am tempted, as a mere microbiologist, to draw attention to the widespread use of micro-organisms in nature as food, for it is not always realized that bacteria, fungi including yeast, and many other micro-organisms are ingested by a multitude of hosts including certain forms of virus as well as many highly organized plant and animal species. A bacteriophage, for instance, is dependent on the availability of a definite species of bacterium for its growth. So are members of the family of *Myxobacteria*, which feed exclusively on certain other bacteria. They would appear to kill these by an antibiotic substance and to digest them by proteolysis. Protozoa are largely dependent on micro-organisms for food, as are also the larvae of some insects. Leguminous crops require the partnership of bacteria for normal development, and so, it would seem, do all herbivorous animals. I drew attention at an earlier meeting of The Nutrition Society to the rate at which micro-organisms develop in the rumen of the ox and mentioned then that at least some 400 to 600 g. of dry microbial substance are being produced and digested daily by this animal (Thaysen, 1945).

I shall not attempt to interpret these facts nutritionally, but I should like to emphasize their ecological significance. Were it not for the almost astronomical rate of reproduction of micro-organisms, a multitude of the hosts mentioned would be deprived of their means of existence. The rate of reproduction of certain types of yeast, to take but one example, is such that, starting with half a ton of actively growing cells, some 32,000 tons of fresh cells containing some 3500 tons of protein can be produced within 24 hours, if reproduction proceeds under optimum conditions. This can be compared with the rate of growth of the living tissue of an ox weighing approximately half a ton. When adequately fed, such an animal will produce about 2 lb. of meat in 24 hours, with a protein content of less than 1 lb.

No higher form of living organism, either plant or animal, can approach this phenomenal rate of reproduction and of synthesis of protein. Moreover, it is significant that yeast and many other micro-organisms build up their protein from inorganic sources of nitrogen. As a method of protein production, therefore, the cultivation of yeast is unique, particularly since the conversion of inorganic nitrogen to protein approaches the theoretically possible maximum.

In the ordinary method of yeast manufacture nothing like full advantage is taken of the maximum rate of reproduction. If the weights of the initially introduced yeast and of the final yield are determined it is found that the increase during 9 hours' growth amounts to a threefold or fourfold gain in weight. The estimated theoretical increase should be about sixty-four fold.

Under conditions of slow growth the age of the individual cell must vary to a very much greater extent than when growth is rapid. As a consequence the slowly growing cells will show considerable variation in cell content and cell structure. This may not necessarily be of importance in the production of baker's yeast, but will certainly be important in a process of food-yeast manufacture, where standardization of the product as regards protein and mineral and vitamin content is of primary importance.

For these reasons it appeared desirable to explore whether it might not be possible to secure a closer approximation to optimum growth conditions in food-yeast manufacture than is normally the case in baker's-yeast production. Incidentally, if this were possible it would also ensure a greater output of yeast from a given size of plant.

The growth rates of microbial cultures vary with the age of the cultures. Buchanan and Fulmer (1928-30) have discussed this in their well known textbook and have given a graphical representation of the various rates. It is clear that the growth curve falls into at least four clearly recognizable sections. Of these, three are of special interest to our discussion: the initial growth-accelerating section; the period of logarithmic growth; and the section of negative growth acceleration.

The rate of reproduction can be calculated by means of the following formula, given by Buchanan:

$$g = \frac{t \times \log 2}{\log b - \log B}$$

where g represents the generation time, the time taken for a mother cell to produce a fully-grown daughter cell, t is the time during which

growth has continued, and b and B, the total number of cells present in the culture at the end and at the beginning of the time under investigation.

With this formula it is possible to determine the average generation time of a germination carried out on the lines of a typical baker's yeast fermentation. It amounts to about 248 minutes; that is to say, one cell produces a fully grown daughter cell within that space of time.

The growth rate curve of such a fermentation is shown in Figure 1. It will be observed that, apart from experimental errors, the logarithm

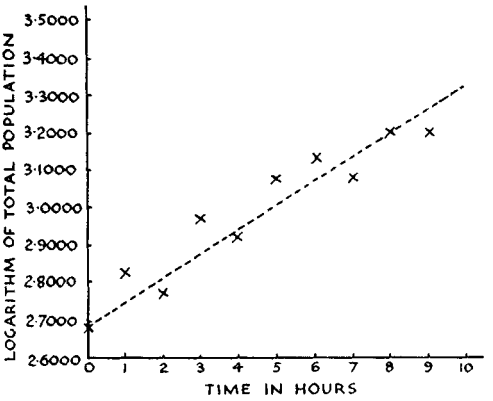

FIGURE 1. GROWTH RATE CURVE OF A TYPICAL BAKER'S YEAST FERMENTATION.

of the total population increases at a uniform rate throughout the fermentation, and that this curve clearly resembles one of the sections, the third, of the curve in Figure 2, which represents an identical fermentation started with a lower initial total cell population. This third section can be recognized as Buchanan's period of negative growth acceleration. The period of logarithmic growth, extending in Figure 2 between the end of the second hour and the end of the tenth, is absent from Figure 1.

If the aim is to speed up the growth of the food yeast organisms it would obviously be desirable to encourage the logarithmic phase of growth, not only during its recognized duration but throughout the fermentation.

Much time has been devoted to exploring whether this is possible. Though it cannot be claimed that the shortest generation time of 87 minutes, reached during the logarithmic phase, can be maintained over prolonged periods, it is quite possible to secure a stabilized growth rate of from 100 to 120 minutes. The output of yeast from a given plant capacity can, therefore, be double that obtainable by using the standard method of procedure. Moreover, the maintenance of the faster growth rate lends itself to a continuous method of production, which may be maintained over days and weeks by the simple procedure of withdrawing a predetermined volume of fermented wort, containing from 2 to 2½ per cent. of dry yeast, from the bottom of the fermentation vats, and replacing it at the top of the vessels with an equal volume of fresh wort in which the necessary food substances, sugar, nitrogen and phosphorus, are

dissolved. The longest period during which this continuous method of production has been maintained is 20 days, but this need not necessarily represent the maximum.

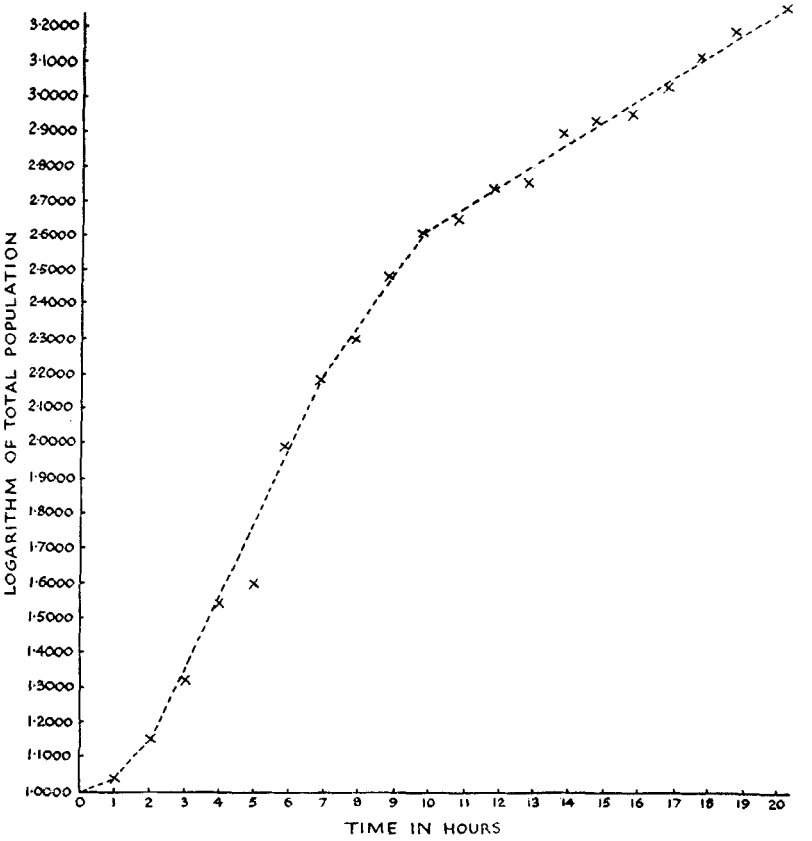

FIGURE 2. GROWTH RATE CURVE OF A TYPICAL BAKER'S YEAST FERMENTATION. Fermentation started with a lower initial cell population than in example in Figure 1.

In working such a continuous process on a factory scale, it has been found that there is little or no danger of the fermenting wort becoming infected with bacteria or other yeasts, even with fermenters open to the air, and in a tropical climate. The reason for this is undoubtedly that the reaction of the fermenting wort is kept at a *p*H between 3·2 and 3·5, a reaction which does not interfere with the growth of the food yeast organism, but checks that of most contaminating types. Another reason may be found in the dilution of any infection present by the rapid replacement of the fermenting wort.

It has been observed in practice that the rate of growth of the food yeast organism is materially slowed down by the presence of iron in the wort, in concentrations of 10 parts per million and over. For this reason it is important to ensure that the equipment used is made of

non-corrosive materials such as wood or stainless steel. The presence of copper in small quantities also interferes with the rate of growth.

A vital factor in maintaining growth is the availability of an adequate supply of air. A quantity of 140 cubic feet per minute, per 2000 gallons of fermenting liquor contained in vessels not more than 25 feet in height, would ensure the maintenance of the rate of growth aimed at, provided the air was dispersed into the liquid through ceramic blocks with apertures not exceeding 12 μ.

A continuous flow of yeast and fermented wort produced under such conditions can be delivered to a battery of centrifuges; in the first the bulk of the spent wort is removed and the concentrated yeast passed as a thick cream to a second centrifuge. In this the yeast cream is mixed with clean water to its original concentration and again separated from the bulk of the liquor. If necessary the washing can be repeated in a third centrifuge.

The washed yeast cream is delivered direct to internally heated roller driers, on which it is dried to form thin flakes. To facilitate subsequent packing the dried flakes are conveyed to a mill and broken down to a fine powder. It was originally planned to subject the washed yeast cream to spray-drying but, since this method is expensive, roller-drying was adopted. Neither method seriously affects the vitamin concentration of the yeast.

When care is taken to avoid the presence of residual sugars in the washed yeast cream, the dried yeast has a light straw colour and a faint meaty or cheesy taste. It is certainly not unpalatable.

The yield of dry yeast obtained amounts to almost 60 per cent., when calculated on the sugar used. Should it fall below 50 per cent., the growth rate has fallen materially below the figure aimed at, possibly owing to contamination with iron or with micro-organisms.

In order to detect a slowing in the rate of growth, adequate checks must be made during the fermentation. The growth rate is checked hourly by counting the number of yeast cells in 1 ml. of fermenting wort. From this number and the total volume of wort at the given time the rate of growth can be determined by Buchanan's formula. As a check on contamination with micro-organisms, stained preparations of the fermenting wort are made at intervals.

The type of carbohydrate used in large-scale production of food yeast has usually been the mixture of saccharose and invert sugar contained in sugar cane or beet molasses. There is no reason, however, why other hexoses, or even pentoses, such as xylose, should not be used. Hitherto molasses has been the cheapest raw material, but the war and post-war rises in the cost of molasses make it possible that other sources may become competitive. Perfectly good food yeast has been prepared from waste potatoes, bananas and other fruits, from carob beans, straw and even from sulphite waste liquor. With the last the rate of growth of the yeast is slow. It is to be remembered, however, that the use of starch necessitates a preliminary hydrolysis of the polysaccharide into glucose, a process which adds to the cost of production.

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Discussion

In opening, the Chairman suggested the following questions for discussion: What is the human position in relation to cellulose digestion? Cellulose-digesting organisms can be seen in human faeces but have not yet been successfully grown. Food remains only a short time in the human intestine, whereas in animals it remains for a longer period. This raises the question, how quickly does cellulose fermentation occur in an animal at 37° C.? Do we really acquire our vitamins directly from food or as a result of bacterial action?

Mr. Baker: Some data are available from clinical observations. For example, Hurst (1919) showed that there is an increase in the amount of cellulose digested in some types of constipation. In one case, digestion was increased by as much as three times. In a second case, where an agar preparation had been given with a view to increasing the bulk of the faeces, there was, in fact, no increase in bulk, but the patient's temperature rose to 108° F. The human colon contains organisms similar to those found in the ruminant, but their significance is probably very small because the food is retained in the human intestine for so short a time.

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The Chairman: Is there any enlargement of the intestinal tract in vegetarians?

Professor R. C. Garry (Department of Physiology, University College, Dundee): In the 1914-18 war certain German surgeons observed that the large intestine of Russians was much more developed than that of Germans (unpublished observations).

Dr. I. Leitch (Imperial Bureau of Animal Nutrition, Bucksburn, Aberdeen): In ruminants, an absence of roughage in early life has been found to alter the development of the intestine. Is the intestine of predominantly plant-eating native tribes known to differ from that of habitually carnivorous tribes, in respect of length or lumen diameter?

Dr. J. M. Shewan (Torry Research Station, Department of Scientific and Industrial Research, Aberdeen), asked about the intestines of fish, said: There are few accurate data, but the haddock has 10 caeca, in contrast with herring and mackerel, which have 200. These differences may be related to different feeding habits.

Mr. H. Pritchard (Highbury, Brancote Gardens, Bromborough, Cheshire): It is important to know whether absorption from the lower colon can contribute to the nutrition of the animal.

Professor Garry: The question of absorption from the large intestine s extremely important. I am impressed by the fact that the epithelium in absorptive areas is characterized by columnar structure with a striated border. What is the structure in the rumen, abomasum, and other parts of the ruminant's digestive tract?

Mr. Baker: I agree as to the importance of the relation of epithelial structure and absorption. For example, the caecum and colon of the horse possess villi and columnar epithelium. On the other hand, the rumen, which has stratified epithelium, allows of the absorption of some materials only, including acetates.

Dr. Owen : It has been shown that absorption of water in the horse can take place right down to the rectum and is greatest there. If water is absorbed, will not water-soluble substances also be absorbed ? For example, phenolic products are excreted in the urine which must have been derived by absorption from the bowel.

Professor Garry : It is important to distinguish between diffusion and normal, natural absorption. Diffusion may go on at any point from the mouth and nose downwards, but should be contrasted with metabolic absorption, which, I believe, is always associated with a striated border of the epithelium. One cannot assume that water absorption means absorption of water-soluble substances ; for example, there is selective absorption of the hexose sugars.

In a general discussion, the opinion emerged that it was important to differentiate between diffusion and absorption and that some of the evidence with regard to absorption from the lower bowel was not of necessity scientifically established.

The Chairman : On the question of bacterial synthesis of vitamins, how are these vitamins made available to the host ? Is it necessary that the organisms should die and be disintegrated ? It is not possible for the intestine to absorb an intact organism. What is the role of phagocytosis in making vitamins available ?

Mr. Baker : Smith (1946) produced as much as 500 g. of bacterial sediment from incubated rumen material. This sediment was fed to rats and was found to have a high coefficient of digestibility.

REFERENCE

Smith, J. A. B. (1946). Private Communication.

Dr. Owen : Nutrients may become available in three different ways : by enzymic digestion, by autolysis, and after digestion by protozoa. The protozoa themselves are readily digested by intestinal enzymes.

Dr. E. R. Dawson (Research Department, Distillers Co., Ltd., Great Burgh, Epsom) : American work shows that if live, fresh yeast is ingested it may absorb vitamin B_1 from the system or from other food.

Dr. Thaysen : Yeast has a cellulose cell wall which is difficult to destroy.

Miss M. Andross (College of Domestic Science, Park Drive, Glasgow, C.3) : Could food yeast be used to increase the protein content of bread ?

Dr. Thaysen : This has been done and the result was an excellent loaf of very agreeable flavour.

Mr. S. A. Price (Research Laboratories, Vitamins, Ltd., 23, Upper Mall, Hammersmith, London, W.6) : Why is *Torula* used for the manufacture of food yeast instead of *Saccharomyces* ? Is it a question of palatability ? American work has shown that by using the second type of yeast, vitamin B_1 content might be increased seven or eight times. Although *Lactobacillus arabinosus* is successfully used for the assay of nicotinic acid, it cannot be used for vitamin B_1 or riboflavin. Could it be genetically modified to yield mutants capable of use for assay of the last two vitamins ?

Dr. Pontecorvo : These mutants could be produced, but it would be preferable to select a simple acid-producing organism and attempt to derive mutants from it suitable for all assays.

Dr. Thaysen : The use of *Saccharomyces* leads to a more "yeasty" taste, and an organism is wanted which will attack xylose as well as dextrose. That is why *Torula* is employed ; we may yet find a better organism.

Dr. Leitch : Swedish work (Malm, 1945) suggests that yeasts merely concentrate the vitamins available in the medium.

REFERENCE

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Dr. D. P. Cuthbertson (Rowett Research Institute, Bucksburn, Aberdeen) : Is there any theory of the causation of "bloat" in cattle ? In the hydrolysis of proteins before assay, is there any danger of racemization ? In the breakdown of carbohydrate by rumen organisms in artificial culture, would the production of fatty acids have been increased if the fatty acids had been removed or allowed to disappear as they were formed ?

Dr. Owen : There are several theories about bloat. Some interference with belching may cause it. Sudden access to fresh young pastures deficient in roughage may lead to excess fermentation and bloat. Clark and Quin (1945) have shown that cyanogenetic glucosides are not to blame. There are other theories, such as the belief that saponins in young grass alter the consistency of the rumen contents in such a way as to interfere with belching (Quin, 1943).

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Dr. E. G. White (Rowett Research Institute, Bucksburn, Aberdeen) : It has been established that if there is plenty of roughage there is less bloat. In dogs also, excessive production of gas in the stomach as the result of a heavy meal can produce torsion of the stomach so that both the duodenal and oesophageal openings are obstructed.

Mr. Price : I should like to stress the importance of possible racemization during protein hydrolysis. For example, in tryptophane assay the hydrolysis must be carried out in an alkaline medium ; this results in complete racemization and hence it is necessary to double all results obtained in the succeeding assay.

Dr. Owen, replying to *Dr. Cuthbertson's* third question: During the incubation the media had to be buffered but, with better buffering, volatile fatty acid figures might have been relatively higher. In the rumen the large bulk of saliva acts as a buffer.

Dr. Thaysen : Conditions may be very different *in vivo* and *in vitro*, and care in interpretation is necessary.

Dr. White, referring to the availability of bacterial protein : The cow and the horse can break down cellulose and build up bacterial protein and fatty acids. In the cow, bacterial protein is formed in the rumen and may possibly be broken down again in the small intestine. In the horse, the formation of bacterial protein can take place only in the caecum. Where can it be broken down and absorbed ?

Mr. Baker : We must not overlook the possibility of autolysis playing a part as well as enzyme digestion in making bacterial protein available. Autolysis can go on with great speed in both the rumen and the caecum.

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