RECENT WORK ON PROTEINS, WITH SPECIAL REFERENCE TO PEPTIDE BIOSYNTHESIS AND NUTRITIVE VALUE*

By A. E. BENDER, B.Sc., Ph.D., F.R.I.C.

Bovril Ltd.

THE protein field is so immense that only two aspects can be dealt with in the limited space available: the biosynthesis of the peptide bond (with which is associated protein 'turn-over' in the body) and the nutritive value of proteins. The first is of outstanding importance in the field of pure science, and the second is of great and increasing practical importance. Moreover, the two are closely related within the field of nutrition, which properly starts with a study of the soil in which food is grown and finishes with the cellular reactions in which that food, or its metabolites, participate.

Dynamic equilibrium

The most profound change that has come over our thinking in the protein field, and indeed in the whole field of metabolism, is the concept of dynamic equilibrium. Our earlier views on nitrogen metabolism were crystallized half a century ago by Folin,[1] who distinguished two types of protein catabolism in the body—one variable, the other constant. The constant or endogenous metabolism produces the urinary end-products of creatinine, neutral sulphur, ethereal sulphur and uric acid. The distinguishing character is that it is constant, being related to the basal metabolism of the body and continuing even when the diet is completely deficient in nitrogen. The other form of nitrogen metabolism is variable, reflects directly dietary changes, and gives rise in the urine to urea and inorganic sulphur. This is the exogenous metabolism.

It was assumed by Folin that protein synthesis came to an end when growth ceased in the adult. Borsook & Keighley[2] in 1935 suggested that protein synthesis continues even in the adult, and it is the confirmation of this 'continuing' metabolism that has given us a new outlook on the situation.

The possibility of 'labelling' the substances administered to the body so that their fate could be followed directly, despite their admixture with similar substances already present in the body, opened up completely new fields that could not otherwise have been explored. The technique had been used by Knoop 50 years ago, when he labelled his fatty acids with the phenyl group (which is difficult to metabolize), so that they could be identified in the urine, but this had obvious limitations. The work of Schoenheimer *et al.*[3, 4] with isotopically labelled amino-acids showed immediately and conclusively that our views of the growth and stability of the adult organism were quite wrong. By the use of isotopically labelled N-15 (^{15}N) amino-acids they showed that the exogenous metabolism of Folin is not static but dynamic. The nitrogenous groupings of the tissue proteins are constantly involved in chemical reactions, peptide links open, amino-acids are liberated, mix with others from the diet and tissues, and re-enter peptide links. All these reactions are balanced by an unknown regulator, so that the total amount of body material and composition do not change. The equilibrium appears to affect only the exogenous catabolism, for Schoenheimer showed that muscle creatine is dehydrated to creatinine at a constant rate and is not involved in any reactions in which C–C or C–N links are broken. Thus Folin's concept of two groups, the endogenous and exogenous metabolism, still holds.

We now visualize a metabolic pool of amino-acids (Fig. 1) which is in a state of dynamic equilibrium with circulating proteins, dispensable protein stores, and functional and structural cellular constituents. The pool is fed from the dietary proteins and overflows into the excretory pool and the energy-producing processes. The amino-acid pool takes part in irreversible reactions involving functional cellular proteins, the indispensable proteins and the non-protein nitrogen of endogenous catabolism.

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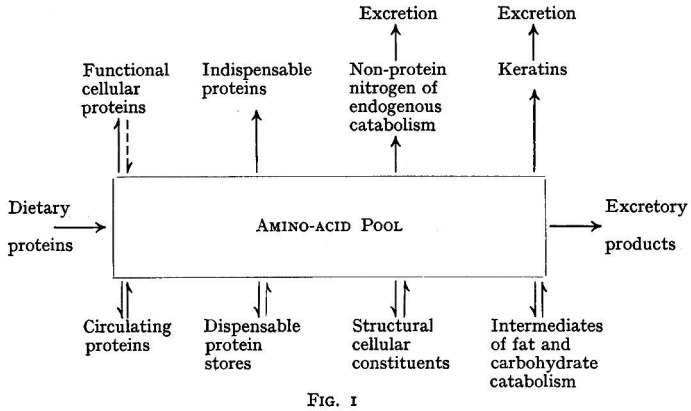

FIG. 1

If the intake of dietary amino-acids ceases, the pool is maintained by uncompensated contributions from, first, the dispensable protein stores, later, the structural cellular constituents and some of the functional cellular proteins, and, at a still later stage, from the circulating proteins.

In this way the continuous wastage of the indispensable protein and the non-protein nitrogen can be made good. The synthesis of keratins appears to go on continuously despite the absence of protein from the diet.

With an intake in excess of synthetic requirements the overflow is tapped off the pool and oxidized to produce energy, and the nitrogenous fraction excreted. It appears from results to be discussed later that entry into and exit from the amino-acid pool takes place in the balanced sets of amino-acids and that incomplete mixtures can neither be stored in the pool nor withdrawn from it.

By this means it is possible for the tissues to withstand external disturbing influences with less change than would be necessary in a fixed system.

Rate of protein turn-over.—The metabolic pool is small but is turning over at a rapid rate. This has been measured in many tissues by the use of isotopes[5] and found to be surprisingly large. For example, in the rat the proteins as a whole have a half-life of only 17 days, during which time they are resynthesized from the amino-acid pool. The rate of turn-over differs in different tissues. The liver proteins of the rat have a half-life of only 6–7 days, as have also the proteins of the plasma and the viscera. On the other hand, the carcass proteins are more stable and have a half-life of 21 days. The carcass proteins appear to consist of a small fraction with a high rate of turn-over and a large, metabolically inactive fraction ; collagen, in particular, turns over relatively slowly. In man the rate of metabolism is appreciably slower, the half-life of the liver and serum proteins being 10 days, of the proteins as a whole 80 days, and of the carcass 158 days. From their results, Sprinson & Rittenberg[6] and San Pietro & Rittenberg[7] calculated the size of the metabolic pool in man to be 0·5 g. of nitrogen/kg., and the total synthesis of protein to be 1·3 g./kg./day, or 90 g./day in the average man.

This high rate of metabolism can be observed directly by the injection of isotopically labelled amino-acids. Ten minutes after injection into mice only 3% of the material was left in the blood stream. The amino-acids had been incorporated at such rate that the visceral proteins achieved 75% of their maximum radioactivity in half an hour. The breakdown was equally rapid, as indicated by the expiration of radioactive carbon dioxide in the breath. This reached its maximum rate one hour after injection, at which time there was no activity left in the carcass.

The findings that proteins are synthesized and degraded with such amazing speed emphasizes the old question ' how are the proteins synthesized ? '.

Energy requirements.—One point can be readily disposed of, that is the question of the energy requirements of such a plethora of metabolism. Borsook[9] showed that if one peptide

bond is synthesized per equivalent of amino-acid turned over, the energy requirements would be only one three-hundredth part of the basal metabolic energy.

Biosynthesis of the peptides.—The synthesis of these proteins of the tissues is largely a question of the synthesis of the peptide bonds. By definition, an enzyme catalyses a reaction in both directions, so that proteolytic enzymes should be able to achieve synthesis. An early demonstration of something on these lines was made as long ago as 1901 by Sawjalow,[10] who obtained a protein-like precipitate, plastein, by treating a concentrated solution of peptone with gastric juice. More recently, Virtanen *et al.*[11] obtained a product of 40 amino-acid residues with a molecular weight of 2500–10,000, and Tauber[12] used chymotrypsin to obtain compounds with a molecular weight of 250,000–400,000. There is no adequate proof in this type of work that a peptide bond has been synthesized, and it is noteworthy that the reaction starts from protein hydrolysates and cannot be made to start from free amino-acid or even di- or tri-peptides. For these reasons this method of approach to the problem has yielded little information.

The more precise work that has been done in recent years has involved the use of simple models of the amino-acids and their derivatives.

One fundamental fact has been clearly established: the synthesis of a dipeptide from two amino-acids, which uses 3000–4000 calories, requires the assistance of the ubiquitous and omnipotent energy-rich phosphate bond (or, as we are now adjured to designate it, bond with a high free-energy of hydrolysis[13]). The intervention of the energy-rich phosphate is hardly surprising as it is the only source of energy known in all living cells, whether they be involved in muscular or osmotic work, or even glowing, like Photinus, the firefly, or producing a 500-v electric shock, like Torpedo, the electric eel.

This source of energy for peptide synthesis was first postulated by Lipmann,[14] in 1949, and has since been clearly demonstrated by the usual procedures of uncoupling the endergonic from the exergonic reactions, when dipeptide synthesis ceases. The synthesis of peptides has been demonstrated in a variety of tissues by the uptake of labelled amino-acids, e.g. bone-marrow cells and diaphragm,[15] the uptake of methionine by *Bacterium coli*,[16] of alanine by rat-liver slices.[17] The utilization of the added amino-acid ceases in the presence of such uncoupling agents as anaerobiosis, arsenate, azide, dinitrophenol, fluoride and cyanide.

After the synthesis of the smallest peptide, the dipeptide, for which the largest amount of energy is required, subsequent stages in the lengthening of the chain offer a choice of methods, either as alternatives or possibly occurring together.

The first of these involves the stepwise addition of further amino-acids, with the synthesis of a fresh peptide link, presumably resulting ultimately in a long polypeptide chain. The process has been demonstrated[18] by the use of simple derivatives of the amino-acids:

Benzoyl-L-tyrosine + glycineamide → benzoyl-L-tyrosinylglycineamide + water

This model reaction is carried out with the assistance of proteolytic enzymes, in this case with chymotrypsin. When glycineamide labelled with ^{15}N in the glycine nitrogen was used, the peptide isolated contained the labelled atom, but only in a yield of 1%. The reaction is endergonic and requires a supply of energy. The simplest method of supplying this is to choose reactants in which the product synthesized has a solubility less than that at equilibrium,[19] e.g. the glycineamide was replaced by glycineanilide, and the resultant benzoyltyrosylglycineanilide crystallized from the solution in 65% yield, the energy for the reaction being derived from the precipitation of the product.

It has been suggested by Fruton that this model may represent one of the methods by which peptides can be lengthened *in vivo*, namely by a condensation reaction coupled to removal of the condensation product by its relative insolubility, or by its participation in other reactions, or removal by circulation in the organism.

The second method of increasing the peptide chain involves the enzymically catalysed process of transpeptidation[20] from a supply of preformed peptide bonds which would supply the energy. Here a small amino-group is replaced by a larger peptide unit, e.g. the reaction has been demonstrated by treating benzoylglycineamide with isotopically labelled ammonia; in the presence of papain the ammonia is exchanged for the amino group:

$$C_6H_5{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CO{\cdot}NH_2 + N^*H_3 \rightarrow C_6H_5{\cdot}CO{\cdot}NH{\cdot}CH_2{\cdot}CO{\cdot}N^*H_2 + NH_3$$

Instead of the simple molecule of ammonia an amino-acid or peptide can be induced to replace the group at the peptide link; thus benzoxycarbonylglycyl-L-phenylalanine + diglycine = benzoxycarbonyltriglycine + phenylalanine. Cathepsin c, which has an optimum pH of 5 for hydrolysis, can catalyse transpeptidation at pH 7. If the α-amino group is not blocked, as in glycylphenylalanineamide, Cathepsin c will catalyse a transamination reaction in which the amide NH_2 of the glycylphenylalanineamide is replaced by another molecule of substrate to form a tetrapeptide amide, which reacts further to form an insoluble octapeptide of repeating glycylphenylalanineamide units.

The process of building a peptide chain by transpeptidation is linked with the three long-known and widely occurring compounds, glutamine, asparagine and glutathione. (Glutamine and glutamic acid account for 25–60% of the total amino-nitrogen in most animal tissues.) They have been suggested as the link between the formation of the peptide bond and the exergonic reactions in the oxidation of carbohydrates and fats. Energy is required for the synthesis of the two amides, glutamine and asparagine, from the corresponding free amino-acids, and for the synthesis of the tripeptide, glutathione. After this energy-consuming reaction they can carry out transpeptidations that result in the formation of new peptide bonds without any further expenditure of energy. Glutathione, which has for so long been regarded as a metabolite of key importance, was shown by Waelsch & Rittenberg,[21] with the aid of isotopic glycine, to have a half-life of only 2–4 hours, very much shorter than even the rapid turn-over of liver proteins which have a half-life of 7 days. Such intensive metabolism would certainly be suitable for a key substance in the synthesis of peptide bonds.

A different approach to the problem has been made by Dounce,[22] with nucleic acids. Apart from their function in controlling the order of arrangement of nucleic acids in the nucleic acid chains, it is suggested that the nucleic acids act as templates in governing the order of arrangement of amino-acids in peptide chains. The nucleic acid template, it is suggested, is first phosphorylated to yield a polyphosphate with a series of energy-rich bonds, which then interact with the amino-acids to form a nucleic acid–amino-acid complex. Linear polymerization of the amino-acids would form a peptide chain which simultaneously separates from the phosphonucleic acid. The specificity of the peptide is dependent on the nature of the original template of nucleic acid. This then requires that there must be at least as many specific nucleic acids in a cell as there are specific chain arrangements in the proteins of that cell, and Dounce suggests that there is an adequate number of these templates. He suggests that the enzyme that catalyses the interaction between the amino-acid and the phosphonucleic acid has three points of attachment to the nucleic acid chain, and calculates that with a molecular weight of 100,000 there can exist 4×10^{87} different nucleic acids, which should be adequate to account for all the proteins in nature, even though there are 1600 different proteins in the human body.

The template theory has little direct experimental evidence, but was advanced in the hope of pointing the way to experimental work that may provide evidence to solve the problem.

Thus there is ample evidence that much work has recently been done on the problem of synthesis of the peptide bond, though whether it constitutes an advance can only be determined by further work.

Protein nutrition

The need to be able to express the nutritive value of a protein rather than to think of all proteins as being equal has been slow in spreading from the laboratory to the fields of practical application, and it is as recent as 1951 that Blaxter[22a] suggested that animal feeding-stuffs should be used in relation to their amino-acid content, that is their actual nutritive value, rather than merely to their total protein content, or worse, '$N \times 6{\cdot}25$'.

To synthesize its specific proteins the cell must be provided with the required ingredients in the correct proportions. By definition, only the essential amino-acids need be so provided; the cell produces the non-essential amino-acids from its own resources so long as nitrogen in some form is available. If any one of the essential ingredients is present in inadequate amounts then it will limit the amount of protein that can be synthesized. Thus the limiting amino-acid will constitute a 'bottle-neck'. If one of the essential amino-acids is present to the extent of only 50% of the requirement then only 50% of the entire mixture can be used for synthesis and

the remainder is oxidized, or, for our present purposes, wasted. The protein would, in fact, have a biological value (BV) of 50, as only 50% would be retained in the body as newly synthesized tissue.

Until 1946 the BV of a protein could be determined only by long, laborious and relatively inaccurate biological measurements, but at that time Mitchell & Block[23, 24] suggested a new procedure derived from evidence collected over many years. It was already known that egg protein satisfied the amino-acid requirements of the experimental rat and even of man so fully that it approximated to the perfect protein with a BV of close to 100. Mitchell & Block suggested that a comparison of the amino-acid composition of the unknown protein with that of egg would yield a comparison of their biological values. They proposed the term ' chemical score ' (CS) for the content of the limiting amino-acid expressed as a percentage of that amino-acid contained in egg. (The original authors used both ' chemical rating ', which was the percentage deficit of the limiting amino-acid, and ' chemical score ', which was this value subtracted from 100. It is obviously simpler to use the content of the limiting amino-acid expressed as a percentage of the same amino-acid in the egg protein.)

Mitchell & Block showed that CS correlated well with biological values measured directly on animals, there being apparently a straight-line relation between these two values for 23 different proteins, with a correlation coefficient of 0·83 (Fig. 2). With the advent in recent years of improved methods of estimating the amino-acid content of proteins, such as microbiological, quantitative paper-chromatographic, isotope-dilution and resin-adsorption methods, it would have seemed that the BV of proteins could be deduced more accurately without the need for biological measurement.

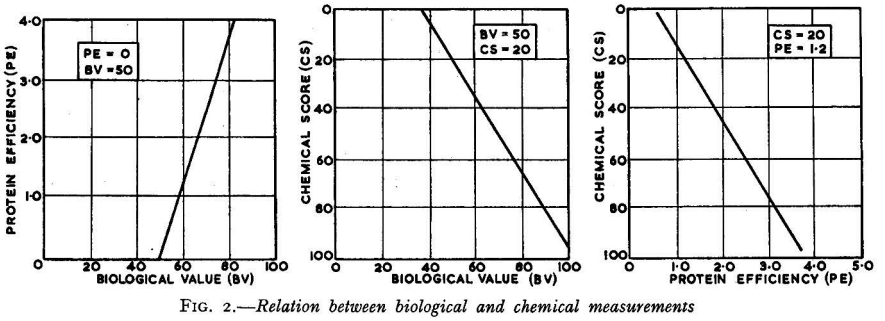

FIG. 2.—*Relation between biological and chemical measurements*

Recent work, however, has produced several reasons why we should *not* expect the amino-acid composition of a protein to predict the BV.

1. The amino-acid content of a protein is not necessarily the same as the mixture available to the metabolic mechanisms of the cell where they are to be used.

2. It is possible, at least in some circumstances, to alter the normal composition of some of the protein synthesized.

3. There are theoretical objections to the regression curve of Block & Mitchell.

4. Contrary to general expectation, the amino-acid content of a protein cannot be measured with any degree of accuracy by the usual standards of chemical analysis.

These real or apparent difficulties are discussed below.

1. *Enzyme-resistant links*

The protein that is analysed is the protein that is eaten, either by the experimental rat or by the final arbiter, the human being. But, after it has been eaten, the protein must undergo digestion, absorption and transport to the active metabolic surfaces. The question we have to consider is how the original protein compares with the products reaching their ultimate destination.

For analysis the protein is completely hydrolysed to its constituent amino-acids by some potent agent such as mineral acid, but during *in vivo* digestion the protein may not be completely hydrolysed, as some of the links may not be vulnerable to enzymic attack.

Geiger & Hagerty[25] showed that the maize protein, zein, would not support full growth

in rats even when enriched with amino-acids up to the level of casein. An enzymic digest of zein, containing 65% of free amino groups, plus arginine, lysine, histidine, trytophan and cystine, permitted growth of only 0·6 g. per day, but the acid hydrolysate produced excellent growth of 3·2 g. per day. The enzymic hydrolysis in the stomach and intestine does not yield products of the same availability as does acid hydrolysis in the laboratory.

Similarly it has been shown[26] that after 24 hours' autoclaving of pork, only 1% of the cystine that is present can be released by trypsin *in vitro*, and even after as little as 5 minutes' autoclaving 60% of the cystine cannot be liberated enzymically. Lea & Hannan[27] have recently investigated the interaction between proteins and sugars, the Maillard reaction, and provided further evidence that an amino-acid can be shown to be present chemically, but not be biologically available. When a mixture of 4 parts of glucose to 1 part of casein was heated at as low a temperature as 37°, at 70% r.h., 67% of the lysine appeared to be lost, according to enzymic methods, in 5 days. Acid hydrolysis, however, revealed that only 20% was lost, the remainder being present in some unavailable form. An amount of 24% of the methionine appeared to be lost in 5 days, but acid hydrolysis showed that it was still present in its original amount. These results were verified biologically by Henry & Kon,[28] who measured the BV of the casein–glucose mixture before and after this gentle heat treatment. The original mixture had a BV of 78; after 5 days it had fallen to 62, and after 30 days to 39. The digestibilities at these three times were 99, 97 and 91.

The result of this and other work on the effect of heat on proteins shows that damage of three types can be caused. There is the interaction between the amino-acids themselves, occurring in the absence of carbohydrate, resulting in an enzyme-resistant link, which is hydrolysed by acid. Lysine, aspartic acid and glutamic acid are chiefly affected in this way. There is the interaction between chiefly lysine, methionine, cystine, histidine, threonine, glycine and aspartic acid and certain carbohydrates, forming enzyme-resistant but acid-vulnerable links. And there is the interaction between free amino groups and sugars which results in actual destruction of the amino-acid. Thus in the first two cases the chemical analysis, involving an acid hydrolysis, would give a wrong picture of the nutritive value of the protein.

Digestion.—It has long been known that proteins vary in digestibility, the better ones being about 95% digested. Kuiken & Lyman[29] found that all the essential amino-acids of roast beef were available to the rat, as measured by excretion in the faeces. On the other hand, the proteins of cottonseed flour were only 85% digestible, and the individual amino-acids varied from 65% for lysine to 93% for arginine. What we do not usually know is which fraction of the protein we are losing in the undigested matter, whether it is part of the limiting amino-acid, or the relatively unimportant non-essential amino-acids, or part of the whole protein.

Some experiments of Denton & Elvehjem[39] add further information on this subject. These authors hydrolysed three proteins, beef, casein and zein, enzymically *in vitro*, and measured the rates of liberation of the amino-acids. Considerable differences were shown by the different proteins. Three-quarters of the arginine was liberated from all three proteins by pepsin alone, and little more by a mixture of pancreatic and duodenal enzymes; half of the phenylalanine of beef was released by pepsin but almost none of that of zein. Nearly all the methionine of beef but only half of that of casein and zein were liberated after 24 hours of enzyme treatment. Tryptophan was freed at a very late stage; from zein, none was freed after 4 hours, 50% in 10 hours and 100% in 24 hours. On the other hand, beef and casein released 75% of their tryptophan in 3 hours and no more after 24 hours.

A different approach was made by Guthneck *et al.*,[31] who used the weight regain of protein-depleted rats to measure the availability of lysine. A basal diet complete in all respects but lacking lysine was fed; increments of lysine produced a weight regain proportional to the lysine added. Ninety per cent. of the lysine of dried egg and skim milk was utilized, 84% from fresh meat and 70% from cooked meat, and 71% from cereals and legumes. With casein it was shown that 76% of the lysine was used, 7% excreted and 16% unaccounted for. Any lysine liberated at such a rate or such a time as not to be available at the right place and time for protein synthesis to ensue, would presumably be oxidized for energy and the nitrogen excreted in the urine; this would be unaccountable.

The site of liberation of the amino-acid appears to be of some importance, as illustrated by

experiments with soya protein. This material is limited by methionine, and either heat or the addition of cystine increases its BV. Cystine has no effect on the BV of heated soya, so it would appear that the effect of heat is to make more methionine available. Yet faecal analysis shows no change in the amount of methionine absorbed from soya after heating. Carroll *et al.*[32] showed that in the terminal 20% of the small intestine only 33% of the raw soya bean was digested whereas 79% of the heated material was digested. Thus it seems clear that the site and rate of digestion are of importance to the usefulness or otherwise of the protein fed.

After digestive hydrolysis those amino-acids that have survived the hazards of liberation from the protein are absorbed from the intestine into the blood stream. We have known for a long time[33] that the amino-acids vary considerably in their rates of absorption, and there is a recent finding that the presence of certain amino-acids in the intestine affects the rate of absorption of others. This may well alter the rates of arrival of the different amino-acids at the synthetic centres and so complicate the picture further.

The rate of arrival of the amino-acids at the enzyme assembly seems to be a factor of special importance. An understanding of the mode of synthesis of the peptide bond would help this problem considerably. Christensen & Streichey[34] measured the concentration of several of the amino-acids in various tissues, both in the fasting state and after feeding amino-acids. They found that high plasma levels of certain amino-acids interfered with the cellular activity for concentrating other amino-acids inside the cell. Thus the concentrations of glycine in the plasma resulted in greatly diminished levels of amino-acid in the cells, and a high plasma nitrogen resulting from feeding amino-acids other than glycine reduced the ratio of cellular to plasma glycine. The authors suggested that excessive concentrations of certain amino-acids in the plasma might decrease the rate of protein synthesis relative to breakdown, by altering the critical ratio of amino-acids present in the cell for synthesis of the protein. Indeed it has been shown that excess of certain amino-acids can be toxic, e.g. casein supplemented with excess of methionine causes dogs to go into negative nitrogen balance.[35] This concept of the correct balance of amino-acids implies that not only might the non-essential amino-acids be of importance, but that an excessive level of one of the essential amino-acids might be as important as the low limiting level of another.

There is considerable evidence[36, 37] that a complete set of amino-acids must be presented simultaneously to the enzyme system before protein synthesis can occur at the maximum speed. Animals that grew readily on diets containing all ten essential amino-acids failed to grow and eventually died when the amino-acids were fed in two portions each of five amino-acids 12 hours apart. As the two sets of amino-acids were fed at closer and closer intervals the animals showed an improved rate of growth, which was, however, always inferior to that achieved on a mixture of all ten amino-acids. This suggests that there is not even a temporary storage of incomplete mixtures of amino-acids, and no means of drawing on the amino-acid pool for the missing ones. The finding was extended by Laverton & Gram,[38] who showed that college women were in lower nitrogen balance when their proteins were separated than when they were consumed in a mixture at a single meal. On the same total protein intake, with a breakfast lacking animal protein, the subjects were in negative nitrogen balance ; when milk was moved from lunch to breakfast nitrogen balance became positive with a lower urinary excretion.

From this it follows that, theoretically at least, it should be possible to find two proteins of identical total chemical composition and thus of identical CS, but with different rates of liberation and perhaps of absorption of the amino-acids, so that they are presented to the cell at different rates and so have different BV.

2. *Abnormal proteins*

So much for the first objection raised. The second objection, the formation of abnormal proteins, has been demonstrated in special circumstances. Albanese[39] maintained infants on a diet lacking tryptophan, methionine or *iso*leucine and showed a fall in nitrogen retention and a 29% fall in plasma proteins in 10–16 days. When the diet was composed of wheat gluten, which is deficient in lysine, there was still a fall in nitrogen retention but no change in plasma-protein level. What was observed was a steadily increasing arginine content of the plasma proteins above normal as this amino-acid replaced the missing lysine. There was thus formation

of an abnormal protein in the plasma. Stepwise increments of lysine added to the diet slowly replaced the arginine and restored the plasma proteins to their normal composition. Such an occurrence would give a CS below the BV.

3. *The relation of chemical score to biological value*

A cursory inspection of Mitchell & Block's regression curve relating CS to BV suggests that the position and the slope of the line are not what might be expected, i.e. that a protein completely deficient in any one of the essential amino-acids would have a BV of zero as well as a CS of zero. (The finding that incomplete mixtures of amino-acids can reduce the urinary nitrogen output[40, 41] might have some bearing on this point, but the mechanism of the effect has not been satisfactorily explained.) At the other end of the scale it is more than reasonable to suppose that a protein with its full complement of amino-acids, i.e. with a CS of 100, should have a BV of 100. Thus the regression line would be expected to have a slope of 45° and each BV would correspond to the same numerical CS. The regression line of Mitchell & Block, produced from experimental data, does not correspond with this theoretically expected line. The fact that a protein with a CS of zero has a BV of 39 (although the only protein of this value tested was gelatin with a BV of 25) was explained by the authors to represent a protein which was adequate for maintenance but inadequate for growth. This distinction is unacceptable as, by definition, BV is the percentage of the protein retained by the body, and if 39% can be retained despite the fact that one of the essential amino-acids is missing, then the obvious question is 'in what form is this incomplete mixture retained in the body?'.

A point in support of the above criticism is that it has recently been shown[42] that the BV of gelatin does coincide with its well-established CS, and is, as expected, zero. The commonly found literature value of 25 was determined not on gelatin alone, but on a mixture with other proteins. This establishment of a value for gelatin produces a serious discrepancy in the otherwise admirably regimented line of data of Mitchell & Block. Either gelatin is an exception or the given regression line requires revision.

An examination of the three related regression curves given by Block & Mitchell[24] also suggests a discrepancy (Fig. 2). The relation between protein efficiency and BV shows that animals cease to grow, i.e. no protein efficiency can be measured, when the BV is 50 or less. The BV/CS relation shows that BV 50 corresponds to a CS of about 20. The third graph correlating CS with PE shows that this corresponds to a protein efficiency of 1·2 instead of zero. Even with the very wide limits of experimental error that must be permitted in these biological measurements this discrepancy throws doubt on the validity of these relationships.

4. *Amino-acid estimation*

The fourth obstacle to the chemical prediction of BV, that the measurements of the constituent amino-acids cannot be made sufficiently accurately, might well appear to be nonsensical in view of the large number of analyses reported in the literature, and the general acceptance of these values. It is, in fact, the very large number of such assays that has thrown them into disrepute, because there are now enough of them to show that they do not agree.

A test between the Universities of California and Wisconsin set out to establish the reliability of the microbiological assay.[43, 44] A larger test, organized by the Rutgers Bureau of Research, investigated both the amino-acid estimation and biological evaluation.

In the first test 41 samples were analysed for all ten essential amino-acids by both laboratories. It was found that within one laboratory the coefficient of variation could be as good as 3·5%, as with leucine, or as bad as 12%, as with methionine. Between the two laboratories the coefficient of variation ranged from 5·2% for histidine and 17·5% for tryptophan, with a mean of 8·9%. In this test the correct result was not known.

Rutgers experiment.—Twenty-five laboratories co-operated in a four-year project, from 1946 to 1950, involving the amino-acid analysis of six selected proteins, the analysis of a known standard mixture of pure amino-acids and the biological assessment of the proteins.[45] The point of interest is the analysis of the known mixture.

The answers for synthetic cysteine varied from + 71% to − 22·7% of the true answer, and at the other end of the accuracy range methionine varied only between − 1·1% and + 8·8%

of the correct result. Of 14 different amino-acids only 5 were estimated within ± 10% of the correct result, and 4 more were within ± 20%; the rest were far out.

If it is remembered that these analyses were carried out by laboratories particularly highly skilled in their techniques, which in many cases were originated by themselves, and aware of the outstanding importance of their measurements, then it would appear that very few amino-acids can be estimated with any degree of accuracy.

Estimations of the amino-acid content of the referee proteins hydrolysed centrally showed even greater variations. Some determinations might be said to have shown 'good' agreement, as with arginine in casein, which varied between 3·1 and 3·9%, histidine in peanut 1·1–1·6%, histidine in wheat gluten 1·0–1·8%. Others were in poor agreement, e.g. cystine in peanut varied between 0·4 and 1·2%; cystine in gluten 1·1–3·6%; leucine in gluten 4·4–10·0%; trytophan in egg albumen 0·2–1·3%.

It has generally been thought that the chief reason for different results obtained in different laboratories was due to variations in methods of hydrolysis, but in this test the proteins hydrolysed in each laboratory showed no worse results than the proteins hydrolysed centrally and distributed to each laboratory for analysis.

These results are akin to those reported by King,[46] who showed that in simple routine analyses of blood constituents, as carried out daily by skilled hospital-laboratory technicians, variations of up to 400% resulted.

With all these possible sources of error it is surprising that Mitchell & Block found any correlation between CS and BV. Nevertheless such a correlation was found. This would suggest that the various objections raised are within the limits of experimental error, or otherwise insignificant, or perhaps that a series of errors cancel each other out, or even that the results are fortuitous.

Proposed revision of CS/BV relation

It would be of inestimable value to be able to forecast the BV of a protein, or more practically of the mixture of proteins that constitutes any particular diet, from its chemical composition. For, despite the difficulty in measuring the amino-acid content of proteins, the advent of newer methods, such as isotope dilution and resin chromatography, promises more precise data, quite apart from the more accurate exploitation of the older methods.

The calculations of Mitchell & Block[24] were based on three sets of data, each of which might be subject to some considerable error, as indeed the authors themselves pointed out. Measurements of the amino-acid content of the various proteins, their BV, and, most critical of all, the measurement of the amino-acid content of the standard egg protein, were collected from the literature and the most acceptable values used. In most cases approximations to the truth were doubtless obtained by shrewd choice from the literature where similar estimations had been made by many groups of workers; for egg protein the values used were described as 'best available'. Despite criticism of the Rutgers experiment it seems reasonable to consider the conjoint values obtained for the amino-acid content of egg protein. Twelve laboratories analysed the same material and the Rutgers Bureau exercised some degree of selection of the results, the consequence of which was that 111 results were discarded out of a total of 656. It is fair

Table I

Analysis of egg protein, g. of amino-acid/16·0 g. of nitrogen

	Mitchell & Block[24]	Rutgers[45]	Edwards[47]
Histidine	2·1	2·1	3·6
Lysine	7·2	6·1	7·8
Trytophan	1·5	1·1	1·6
Phenylalanine	6·3	5·6	6·1
Cystine	2·4	2·3	
Methionine	4·1	3·2	3·9
Methionine + cystine	6·5	5·5	
Threonine	4·9	4·9	4·9
Leucine	9·2	9·0	9·7
*iso*Leucine	8·0	6·2	7·0
Valine	7·3	7·0	7·2

to assume that the averages compiled from these selected results are the best figures available for egg protein. These data are shown side by side (Table I) with those used by Mitchell & Block, and several differences are observed. Every difference lowers the standard set by egg to any protein that tries to emulate its perfection. (To add weight to the criticism of amino-acid estimations the Table includes the analysis of egg protein reported by Edwards *et al.*,[47] who claim that their results were thoroughly checked and corrected for the recovery of added pure amino-acids.) If the chemical scores of the various proteins are re-examined there are several amendments to be made (Table II). (Data and biological values are mostly those of Mitchell & Block.) It will be seen that in some cases not only is the CS raised, but the limiting amino-acid is changed.

Table II

Chemical scores and limiting amino-acids

	Mitchell & Block[24]		Proposed revision		
	CS		CS		BV
Beef	71	methionine + cystine	83	valine	76
Beef liver	70	*iso*leucine	84	methionine + cystine	77
Egg albumin	69	lysine	78	threonine	83
Milk	68	methionine + cystine	80	methionine + cystine	90
Lactalbumin	66	methionine	84	methionine	85
Beef kidney	65	methionine + cystine	76	methionine + cystine, valine	77
Beef heart	65	*iso*leucine	80	methionine + cystine	74
Casein	58	methionine + cystine	69	methionine + cystine	69
Sunflower seed	53	lysine	62	lysine	65
Heated soya	49	methionine	60	valine	75
Oats	46	lysine	54	lysine	66
Yeast	45	methionine + cystine	53	methionine + cystine	63
Rice	44	lysine	53	lysine	75
Maize germ	39	methionine	50	methionine	78
Sesame	39	lysine	46	lysine	71
Wheat germ	38	*iso*leucine	49	*iso*leucine	75
Whole wheat	37	lysine	44	lysine	67
Cottonseed	37	,,	44	,,	64
Flaxseed	35	,,	41	,,	78
Maize	28	,,	33	,,	54
White flour	28	,,	33	,,	52
Groundnut	29	methionine	31	threonine	58
Blood meal			18	*iso*leucine	5
Gelatin	0	tryptophan	0	tryptophan	0
Wheat gluten			34	lysine	37
Blood + *iso*leucine			47	methionine	28
Blood + casein			60	*iso*leucine	44

However, it must be recognized that it would require only a very small change in the analytical figures to change the apparently limiting amino-acids.

The discrepancy between the limiting amino-acids found by different analysts is extremely well shown in Table III with egg albumin as the protein. There are three useful analyses of this protein, the figures selected by Mitchell & Block, the selected data of the Rutgers experiment, and some recent figures of Lewis *et al.*[48] In each case they can be compared with the egg values of Block & Mitchell[24] or of Rutgers,[45] making six possible chemical scores. The score sheet shows one vote for lysine, three for threonine and none for methionine ; two forecasts predict that none of these is limiting. A short test[49] was carried out to ascertain which forecast

Table III

Amino-acid analysis of egg albumin according to different authors expressed as a percentage of the same amino-acid in whole-egg protein according to different authors

Whole-egg protein analysis from	Mitchell & Block[24]			Rutgers[45]		
Albumin analysis from	Mitchell & Block[24]	Rutgers[45]	Lewis[48]	Mitchell & Block	Rutgers	Lewis
Lysine, %	69	100	89	82	excess	excess
Threonine, %	78	100	82	78	100	82
Methionine	excess	excess	excess	excess	excess	excess
Limiting amino-acid	lysine	?	threonine	threonine	?	threonine

would be verified by the rat. Values for net protein utilization were determined on (1) egg albumin, (2) egg albumin + lysine, (3) egg albumin + lysine + threonine, (4) egg albumin + methionine. If the second showed no improvement and the third did, then threonine would be the bottle-neck. (The purpose of supplementing with the two amino-acids instead of threonine alone was to raise the BV by an amount sufficient to show clearly.) The results were (1) albumin 85; (2) + lysine 85; (3) + lysine + threonine 86; and (4) + methionine 97. Thus none of the predictions was correct.

The new regression line calculated from what are being assumed as improved data (Table II) is expressed by the equation $y = 23{\cdot}7 + 0{\cdot}74x$ (Fig. 3). (Some unpublished biological measurements are included, namely whole blood protein, blood protein supplemented with 8% of *iso*leucine, a mixture of equal parts of blood and casein, and wheat gluten. The amino-acid figures are those of Mitchell & Block.) The new regression line has a correlation coefficient of 0·82, and runs from 24 to 98. The graph also shows the original line of Mitchell & Block, which

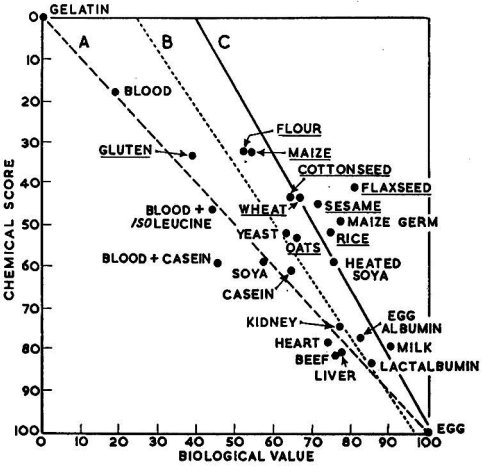

FIG. 3.—*Relation between biological value and chemical score (underlined proteins are limited by lysine)*

Curve A Theoretically 'perfect' relation
Curve B Revised relation
$y = 23{\cdot}7 + 0{\cdot}74x$
$r = -0{\cdot}82$
Curve C Original relation of Mitchell & Block
$y = 102 - 0{\cdot}634x$
$r = -0{\cdot}86$

runs from 39 to 102, and the theoretical 0–100 line. It is possible that the deviation of the new line from the theoretical is due to inaccurate data. It will be observed that it is a group of vegetable proteins, maize, white flour, wheat, cottonseed, sesame, maize germ, rice and oats, that have a higher BV than would be expected from CS and so draw the line away from the 0–100 position. It is noteworthy, and may be significant, that seven of these eight proteins are limited by lysine. In fact if we ignore the ten proteins that lack lysine the regression curve comes still closer to that expected, running from 14 to 98 ($y = 14{\cdot}4 + 0{\cdot}84x$, $r = 0{\cdot}88$).

As in these cases the BV was higher than the CS there is no question of unavailable amino-acids. An unavailable amino-acid produces a BV lower than CS. Still clinging to the simplest theory of protein metabolism, namely that the BV depends upon the essential amino-acids available, one possible suggestion might be that our standard egg contains chemically more lysine than is needed for the synthesis of rat tissues. If some of the lysine of the egg were unavailable, and all of the lysine of another protein were available, the latter protein might have a lower CS but the same BV, or at any rate a BV higher than its CS, which is the case of the exceptions noted above.

There is a little evidence on this subject. Mitchell[50] supplemented egg protein with various synthetic amino-acids and, judged on growth, only lysine showed a small but significant effect. This may mean either that some of the lysine of egg is indeed unavailable, or that the rat requires more lysine than is present, as distinct from available, in egg, and this would reduce all CS in which lysine is limiting. Guthneck *et al.*[31] showed, by weight-regain of protein-depleted adult rats, that only 90% of the lysine of dried egg was available. There is evidence

that the lysine *content* of egg is at least adequate or may be present in slight excess. Table IV shows the ratios of the amino-acids, in a diet composed of synthetic amino-acids, required by the growing rat or for repletion in the protein-depleted rat, with tryptophan as unity. The most recent paper, by Steffee *et al.*,[51] criticizes the other five ratios as being unbalanced because growth obtained on them was not optimal. The Table includes the ratios of the essential amino-acids in egg protein, both according to the values quoted by Mitchell & Block and by Rutgers. If Steffee's criticism is justified, and his own figures are more reliable than the others, then egg would appear to contain a surplus of lysine, and the improved growth reported by Mitchell must be due to lack of availability of some of the lysine. If Steffee's criticism is not valid, then there are still three authors to two in favour of this.

Table IV

Required ratios of essential amino-acids for young or protein-depleted adult rats, compared with egg protein

	A	B	C	D	E	F	Egg B & M	Egg R
Tryptophan	1	1	1	1	1	1	1	1
Histidine	1·5	2	1·1	2·5	1·5	1·1	1·4	1·9
Methionine	2·8	3	1·6	3	3	1·5	2·7	2·9
Threonine	3·1	3	1·3	6	1·8	1·7	3·3	4·4
Phenylalanine	3·2	2·5	3·5	3·5	7·5	3·4	4·2	5·1
Valine	3·6	3·5	3·6	7	2·5	3·5	4·9	6·4
Lysine	4·1	5	3·1	6·3	3·8	3·1	4·8	5·5
*iso*Leucine	4·3	2·5	3·1	5	2·3	1·8	5·3	5·6
Leucine	5·2	4·5	2·7	4·5	3·3	4·0	6·1	8·2

A Steffee *et al.*[51]
B Rose[52]
C Albanese & Irby[53]
D Kinsey & Grant[54]
E Albanese & Frankston[55]
F Kornberg[56]
B & M Block & Mitchell[24]
R Rutgers[45]

With BV already so high and growth rates already near optimum for egg, it might be difficult to prove this point biologically. A chemical approach might, however, be possible. The Van Slyke amino-nitrogen estimation on whole unhydrolysed proteins measures the side-chain amino groups of lysine plus the smaller number of terminal amino groups, which can be measured by the fluorodinitrobenzoate method. This appears to be related to the available nitrogen as shown by the casein–glucose complexes of Lea & Hannan.[27] A lysine determination on an acid hydrolysate would yield the total lysine in the protein. If egg, as distinct from other proteins, showed a difference between these two values it would provide evidence that some of the lysine of egg is unavailable and therefore all proteins lacking lysine are chemically underrated.

If this proves to be so then the speculation that the CS/BV relation holds from 0 to 100 is somewhat strengthened, although still but a speculation. Biological proof is required. This might be done by measuring the BV of mixtures of synthetic amino-acids of known CS covering the range of 0–100. Alternatively it has been claimed[57] that treatment of a protein with Raney nickel will remove all the methionine and cystine without otherwise affecting it. If this is so, and it must be admitted that up to now attempts to reproduce this effect have been unsuccessful, then the BV of incrementally supplemented protein after this treatment should trace the regression line faithfully from beginning to end. Another alternative is the use of deaminated protein which should lack all available lysine.[58]

A new biological method

The glib proposal to carry out such a large number of BV estimations might have involved a lifetime of work if it were not for an abbreviation recently introduced.[59] It can be shown mathematically that the definition of BV laid down by Thomas & Mitchell could be reduced to a simple formula as follows:

Let nitrogen intake on test diet $= I_f$; nitrogen intake on non-protein (or 4·5% egg protein) diet $= I_k$; total faecal nitrogen $= F$; metabolic faecal nitrogen $= M$; urinary nitrogen $= U$; endogenous urinary nitrogen $= E$; then, by definition,

$$\text{Net protein utilization (N.P.U.)} = \frac{I_f - (F - M) - (U - E)}{I_f - F - M} \times \frac{I_f - (F - M)}{I_f}$$

$$= \frac{I_f - F + M - U + E}{I_f}$$

Subtract I_k/I_f from both sides of the equation, then

$$\text{N.P.U.} - \frac{I_k}{I_f} = \frac{(I_f - F - U) - (I_k - M - E)}{I_f}$$

$I_f - F - U$ is the gain in nitrogen by the animal fed on a protein diet = final body nitrogen − initial body nitrogen = $B_f - B$ (body weight at start of experiment = B; on non-protein diet = B_k; on protein diet = B_f). $I_k - M - E$ is the change in nitrogen incurred by feeding non-protein diet = $B_k - B$.

$$\text{N.P.U.} - \frac{I_k}{I_f} = \frac{(B_f - B) - (B_k - B)}{I_f}$$

$$\text{then} \qquad \text{N.P.U.} = \frac{B_f - B_k + I_k}{I_f}$$

A measurement is required of the body nitrogen of the same rat both fed and starved of protein. This ideal condition is approached by using sets of litter-mates under these two conditions and assuming that the fed rats would have behaved as their deprived siblings. In practice, with four litters of eight rats it is possible to arrange eight experimental groups, as we have found that four rats are adequate in each group. One group is fed on a non-protein diet, adequate in all other respects, for ten days, to provide the value for body nitrogen on protein-free diet (B_k); each of the other groups is fed on a test protein. Ten days appears to be sufficiently long, after which the rats are killed and their bodies analysed for total nitrogen. This relatively simple determination has been reduced to a mere dry-weight determination by the finding that there is a constant relation between the water and nitrogen content of the rat, dependent only upon age. The nitrogen can be calculated from the water measurement from the previously established relation and then only the rats' consumption of nitrogen is required in order to calculate the net protein utilization. The BV can be calculated from the digestibility. It is thus possible to measure seven biological values in ten days, or, with an overlapping routine, seven each week. This renders feasible the large number of BV estimations that would be necessary to examine the relation between CS and BV.

Results from this abbreviated method agree very well with measurements made on the same materials by the orthodox procedure,[60] and also with the accepted literature values.

The limiting amino-acid can be determined in any one set of experiments since, excluding arginine, there are nine essential amino-acids for the rat, and commonly eight rats in each litter. The test protein is fed unsupplemented, and also with triple combinations of all nine essential amino-acids, making six diets, which, together with the non-protein standard group, can be accommodated by four eight-rat litters. The results are read in the way shown in Fig. 4. The two triads containing methionine cause an increased BV of casein; no other triads have any effect, and methionine is thus the limiting amino-acid.

Casein alone 60

Histidine	Lysine	Tryptophan	61
Methionine	Threonine	Leucine	84
Valine	*iso*Leucine	Phenylalanine	60
80	60	61	

FIG. 4.—*Biological value of casein supplemented with triads of amino-acids. Values underlined show improvement*

The desirability of expressing the feeding value of animal feeding-stuffs in terms of BV, at least for pigs and poultry, rather than merely as N × 6·25, was discussed above. It is, of course, out of the question for the agricultural or analytical chemist to carry out full-scale determinations of BV, but, if this rapid method were shown to be applicable to mice, it would be relatively simple for even a purely chemical laboratory to feed two groups, each of four mice, on the test diet and a non-protein diet for a week, and then to measure their dry weight.

In conclusion it might almost be claimed that the biological measurement of the nutritive value of proteins can be carried out more rapidly and more accurately than estimations of amino-acids.

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THE LIGNIN FRACTION OF ANIMAL FEEDING-STUFFS. IV.*—The Preparation of 'Reference' Lignin by Extraction with Ethyl Acetoacetate

By F. E. MOON† and A. K. ABOU-RAYA

Ethyl acetoacetate was found to be a satisfactory solvent for lignin, but not highly selective. The purest 'ester lignins' were obtained from the residues after alkali extraction, and when mature plant materials were used these lignins showed little variation in methoxyl content. It is suggested that the percentage of methoxyl in pure lignin is not affected by plant maturity and that the true lignin present in a crude preparation may be determined from the methoxyl content by the use of an appropriate conversion factor.

Introduction

The determination of the lignin contents of plant materials by the attempted removal of all other substances and the weighing of the residues gives results that are far from satisfactory. Even when corrected for ash, there is still an unpredictable degree of contamination in the residue, due to both nitrogenous and non-nitrogenous substances, so that the true lignin content remains highly conjectural. From the results given in an earlier paper[1] it appears that the total methoxyl in the crude lignin residue may be the best measure of the true lignin content, but although this may be useful in comparative work, such as digestibility measurements, it cannot be used to determine the absolute lignin content of any sample without a knowledge of the methoxyl content of the pure lignin. Moreover, it is generally believed that the methoxyl content of lignin is not constant, but varies with the age of the plant[2–4] and with the species;[5] the evidence on these points is far from conclusive, however. It was considered that, before further progress could be made with methods for the determination of lignin, some lignins of as high a purity as possible should be prepared for reference purposes.

Methoxyl and nitrogen contents were taken as the most reliable criteria of the purity of the various preparations. Crude lignin invariably has a high nitrogen content, but until there is satisfactory evidence to the contrary it is reasonable to assume that pure lignin is nitrogen-free. Meyer & Bondi[6] believe that the lignin molecule of plants contains nitrogen to the extent of 1–1·5%, but we have made about 20 different preparations of acid lignin from rye-grass hay and found them to have nitrogen contents varying from 0·3 to 5·6% according to the pretreatment, the nitrogen content of the material treated with 72% sulphuric acid and the degree of contamination with non-nitrogenous substances; the nitrogen in lignin preparations is thus as variable as the mineral content and may equally be regarded as being due to contamination. Nitrogen content alone, however, is an inadequate index of purity, since contamination with carbohydrates may be quite considerable. If suitable pretreatments are employed to remove methoxyl-containing substances other than lignin, then the methoxyl content of the final preparation may serve as an index of freedom from carbohydrate contamination, and preparations with maximal methoxyl and minimal nitrogen contents are presumably the most pure.

Brauns' extraction procedure for wood[7] did not appear suitable for young plant material and, although the treatment employed is a mild one, the 'native lignin' obtained would only represent a very small fraction of the total lignin. The alkali extraction procedure of Bondi & Meyer[5] appeared more promising, being milder than that usually employed with wood yet giving a relatively high yield. Although this method produced from oat straw (0·35% of nitrogen) a lignin containing 17·5% of methoxyl and only 0·47% of nitrogen, with other plant materials containing more nitrogen the lignins obtained were much more contaminated, that from young clover (4% of nitrogen) containing 7·79% of nitrogen and only 1·8% of methoxyl. The possibility of obtaining a relatively pure lignin from the pulp remaining after alkali extraction was also examined, but treatment with 72% sulphuric acid was found to yield preparations of relatively poor quality. The method of Lemmel,[8] employing ethyl acetoacetate in the presence of hydrochloric acid, was therefore investigated as it seems to have received very little attention hitherto.

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† Present address: The University, Glasgow, W.2

Preparations of 'ester lignin' (lignin extracted by ethyl acetoacetate)

All samples were pretreated with ethanol–benzene and materials derived from young plant growths were also digested with acid pepsin. For ester extraction the pretreated materials were moistened with concentrated hydrochloric acid (1 ml./g.), and ethyl acetoacetate (5–10 ml./g.) was added; the mixture was stirred and filtered and the residue washed with more ester. The filtrate was diluted with eight volumes of warm water to dissolve the ester and precipitate the lignin, which was filtered off and washed with water. After being dissolved in slightly warmed 0·5N-sodium hydroxide solution the lignin was reprecipitated and coagulated by acidifying and warming.

Preparations nos. 1 to 6 in Table I were obtained from simply pretreated samples and the analytical figures indicate a variable degree of contamination. Only the lignin from oat straw (no. 1) had a low nitrogen content, but even this had a methoxyl content lower than that of an alkali lignin from the same straw. It was concluded that ethyl acetoacetate is not a highly specific solvent for lignin, and that if it is to be used to obtain a relatively pure 'reference' lignin more efficient pretreatment is necessary. Further preparations (nos. 7–23, Table I) were therefore made from the pulps remaining after alkali extraction, and the pulp ester-lignin from oat straw (no. 7) was found to contain less nitrogen and more methoxyl than the ester lignin

Table I

Composition of ester lignin preparations

Prep. no.	Starting material	Pretreatment with 0·5N-NaOH* solution	Nitrogen in lignin, %	Methoxyl in lignin, %	Methoxyl in corrected† lignin, %
1	Oat straw	None	0·34	14·7	15·0
2	Mature lucerne	,,	—	2·3	—
3	Faeces from sheep fed on rye-grass hay	,,	1·75	11·7	13·1
4	Faeces from sheep fed on timothy hay	,,	1·20	12·6	13·6
5	Immature lucerne	,,	—	4·0	—
6	Faeces from sheep fed on pasture	,,	3·52	8·1	10·0
7	Oat straw	Overnight at 85°	0·17	17·9	18·1
8	Mature lucerne	1 h. boiling	0·41	18·1	18·6
9	Faeces from sheep fed on timothy hay	Overnight at 85° + 30 min. boiling	0·31	17·3	17·7
10	Immature lucerne	1 h. boiling	0·40	16·8	17·2
11	Immature Italian rye-grass	1 h. boiling	—	11·7	—
12	Immature Italian rye-grass‡	Overnight at 85° + 30 min. boiling	0·76	13·6	14·3
13	Immature cocksfoot	1 h. boiling	0·70	9·9	10·4
14	Immature cocksfoot‡	Overnight at 85° + 30 min. boiling	2·05	11·2	12·8
15	Young oats	40 min. boiling	—	9·0	—
16	Young oats	1 h. boiling (1·25N-NaOH)	—	12·1	—
17	Young timothy	40 min. boiling	—	13·2	—
18	Young clover	40 min. boiling	1·47	9·3	10·1
19	Faeces from sheep fed on pasture	Two periods of 8 h. at 85°	1·21	8·3	9·0
20	Ester pulp from No. 19	None	0·85	12·5	13·5
21	Ester pulp from No. 19	40 min. boiling (1·25N-NaOH)	0·65	14·6	15·1
22	Faeces from sheep fed on pasture	1 h. boiling (1·25N-NaOH)	1·05	13·7	14·1
23	Faeces from sheep fed on pasture	1 h. boiling (1·25N-NaOH) then overnight with 0·5N-NaOH then 30 min. boiling	0·73	16·1	16·9

* 1·25N-NaOH solution used in preparations nos. 16, 21, 22 and 23
† Corrected lignin = ester lignin minus lignin-nitrogen × 6·25
‡ Pretreated with pepsin and trypsin

(no. 1) obtained without alkali pretreatment. With mature lucerne the ester lignin (no. 8) yielded by the alkali pulp was comparable with that obtained from oat straw and in marked contrast with the low-quality lignin obtained by ester extraction without alkali pretreatment (no. 2).

The claim[5] that legume lignin contains only about 5% of methoxyl is thus refuted and the low values commonly obtained must be attributed to the greater contamination of preparations made from legumes. In the present work it was found that legume lignin was more soluble in ethyl acetoacetate than in alkali. Thus the total methoxyl present in the alkali lignin obtained from mature lucerne was found to be only about 6% of the total methoxyl in an acid lignin preparation from the same material, whereas the ester lignin obtained after the alkali extraction contained 21% of the total lignin methoxyl. This low solubility in alkali will inevitably lead to a high degree of contamination in alkali lignin preparations but will also increase the yield of ester lignin from the alkali pulp, with a consequent reduction in the contamination of the ester lignin.

The faeces from sheep fed on timothy hay also gave a pulp ester-lignin (no. 9) of high purity, comparable with the preparations from oat straw and mature lucerne. Less mature materials, however, gave lignins with lower methoxyl percentages and higher nitrogen contents, the only exception being the preparation from immature lucerne (no. 10), due to the resistance of this plant lignin to alkali treatment. The immature Italian rye-grass and cocksfoot used to obtain preparations nos. 11 to 14 were cut before flowering and contained 1·26 and 1·77% of nitrogen respectively, on a dry-matter basis. Although they were treated in the same way as the immature lucerne (containing 3·67% of nitrogen) they yielded pulp ester-lignins (nos 11 and 13) of much lower purity. By introducing pretreatment with pepsin and trypsin and reducing slightly the severity of the alkali treatment, preparations (nos. 12 and 14) were obtained with higher methoxyl percentages, although contamination was still evident. Young oats and clover gave pulp ester-lignins (nos. 15 and 18) with rather less than 10% of methoxyl but when the severity of the alkali treatment of oats was increased a small yield of ester lignin (no. 16) was obtained with 12·1% of methoxyl. The alkali pulp from young timothy (no. 17) was extracted with concentrated hydrochloric acid before the ester treatment and, although the yield of ester lignins was low, the methoxyl amounted to 13·2%. Thus it appears that although the methoxyl contents of pulp ester-lignins from young plants are lower than for comparable preparations from mature plants, the range of values obtained does extend into the range characteristic of more mature plants and the lower values are probably entirely due to greater contamination.

Faeces from sheep fed on pasture grass yielded pulp ester-lignin (no. 19) of low methoxyl content, comparable with the preparations from young oats (no. 15) and young clover (no. 18). When, however, the residual pulp was re-extracted with ester a second yield of lignin was obtained (no. 20) with a much higher methoxyl content; this preparation was superior to the ester lignin prepared without alkali pretreatment (no. 6) and also superior to comparable preparations of alkali lignin and pulp acid-lignin. By subjecting the pulp from the first ester extraction to a further treatment with alkali before re-extracting with ester a still purer lignin (no. 21) was obtained, with 15% of methoxyl. As it appeared that the mild alkali treatment used for preparation no. 19 was insufficient for effective removal of contaminants, more vigorous alkali extractions were used for preparations nos. 22 and 23, resulting in lignins of much greater purity.

As the pulp ester-lignins derived from immature pasture had methoxyl contents (corrected for nitrogen) varying from 9·0 to 16·9%, according to the steps taken to reduce contamination, it may be concluded that a low methoxyl content is not a true characteristic of lignin from young plant materials but merely a reflection of contamination, which is usually much greater in preparations from young plants. The pure lignin in immature plants may be identical with that in mature plants and there is no need to postulate a change during growth in order to account for variable methoxyl percentages more rationally accounted for by variable contamination.

'Reference' lignin

If it may be assumed that lignin does not undergo any change in methoxyl content with maturity, then it should be possible to determine the amount of true lignin in a preparation by

measuring the methoxyl content and so avoiding the quite considerable errors that have hitherto resulted, due to contamination with both nitrogenous and non-nitrogenous substances. In order to arrive at a valid factor for converting from methoxyl to lignin, however, it is necessary to determine the methoxyl contents of very pure lignin preparations, and the results presented above indicate that pulp ester-lignin prepared from mature plants may be the purest lignin obtainable and the best ' reference ' lignin from which to arrive at satisfactory methoxyl conversion factors. A number of preparations of ester lignin were therefore made from mature plants and the analytical figures of these are given in Table II. In view of the indications that the true nitrogen content of lignin is negligible, the methoxyl percentages in these preparations have been corrected by assuming the nitrogen to be present as protein (N × 6·25) contamination; any errors involved in this correction are likely to be small, as the amounts of nitrogen were usually very low. The corrected methoxyl percentages have then been used to calculate factors for converting methoxyl figures into their equivalents of true lignin.

Table II

Composition of ' reference lignins ' and methoxyl conversion factors for ' true lignin '

Prep. no.	Plant	Nitrogen in ester lignin, %	Methoxyl in ester lignin, %	Methoxyl in reference* lignin, %	Methoxyl conversion factor for true lignin
1	Italian rye-grass (*Lolium italicum*)	0·18	17·61	17·81	5·61
2	Cocksfoot (*Dactylis glomerata*)	0·34	17·34	17·72	5·64
3	Timothy (*Phleum pratense*)	0·12	18·00	18·13	5·52
4	Oats (*Avena sativa*)	0·17	17·90	18·10	5·52
5	Barley (*Hordeum distichum*)	0·14	18·82	18·98	5·27
6	Wheat (*Triticum vulgare*)	0·15	18·66	18·84	5·31
7	Red clover (*Trifolium pratense*)	0·59	17·89	18·57	5·38
8	Lucerne (*Medicago sativa*)	0·41	18·14	18·61	5·37
9	Pea (*Pisum arvense*)	0·33	17·81	18·18	5·50
10	Bean (*Faba vulgaris*)	0·17	19·89	20·11	4·97
11	Linseed (*Linum usitatissimum*)	0·15	16·66	16·82	5·95
12	Moor mat grass (*Nardus stricta*)	0·54	14·03	14·52	6·89
13	Scots pine (*Pinus sylvestris*)	0·05	15·35	15·39	6·50
14	Potato (*Solanum tuberosum*)	0·37	19·73	20·19	4·95
15	Bracken (*Pteris aquilina*)	0·22	14·86	15·07	6·64

* Reference lignin = ester lignin minus lignin-nitrogen × 6·25

With few exceptions the figures in Table II show relatively little variation in the methoxyl content of reference lignin, and the first six preparations, all from Gramineae, gave methoxyl conversion factors for true lignin varying only from 5·27 to 5·64, with an average of about 5·5. The legumes clover, lucerne and pea gave very similar conversion factors, although the preparation (no. 10) from bean haulm was rather richer in methoxyl, giving a conversion factor of 4·97. Linseed lignin contained less methoxyl, requiring a conversion factor of almost 6, and Scots pine and moor mat grass contained still less, requiring conversion factors of 6·5 and 6·9 respectively. It may be noted that the methoxyl content of the reference lignin from Scots pine was very similar to the figure (15·3%) obtained by Schubert & Nord[9] for native lignin from Scots pine. The lignin of soft woods has been extensively studied and is considered to be of lower methoxyl content than that of hardwoods owing to the absence of syringyl radicals from the

molecules. Bracken also gave a lignin of comparatively low methoxyl content, whereas potato haulms yielded a preparation with 20% of methoxyl, requiring a conversion factor of only 5·0. Thus it appears that there may be some differences in the methoxyl contents of the pure lignins in different plant species, although these differences are probably confined within fairly narrow limits. To obtain the true lignin content of any particular plant material it is therefore preferable to use a methoxyl conversion factor derived from a reference lignin obtained from mature plants of the same species. For the common pasture plants and cereals, however, a factor of 5·5 may reasonably be employed to calculate true lignin from the methoxyl present in lignin preparations freed from contaminating sources of methoxyl, as by the methods previously described.[1, 10] There will inevitably be some error in the use of this factor (as in the conventional use of the factor 6·25 to convert nitrogen to protein) but, if lignin methoxyl does not vary appreciably with plant maturity, any error will be approximately constant and much less than the errors liable to occur when lignin is determined by weighing, with no reliable means of assessing the extent of contamination with other substances.

The Edinburgh & East of Scotland College of Agriculture
13 George Square
Edinburgh, 8

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THE TOXICITY OF PHENYL BENZENESULPHONATE AND SOME CHLORINATED DERIVATIVES TOWARDS EGGS OF CERTAIN TETRANYCHID MITES

By A. H. M. KIRBY and W. H. READ

Certain chlorinated phenyl benzenesulphonates in current use for the control of mites infesting plants appear to function mainly as ovicides. 4-Chlorophenyl 4-chlorobenzenesulphonate and 2 : 4-dichlorophenyl benzenesulphonate have been selected as being the most effective by American workers, whereas 4-chlorophenyl benzenesulphonate appeared to be the most ovicidal of the esters examined in Britain.

This paper describes the results of replicated assays of the ovicidal activities of phenyl benzenesulphonate and certain chlorinated derivatives towards the glasshouse red spider mite, and of leaf-permeation studies involving summer eggs of the fruit tree red spider mite.

4-Chlorophenyl benzenesulphonate was found to be at least as ovicidal as 4-chlorophenyl 4-chlorobenzenesulphonate; differences between the five most potent compounds were significant at the LD_{50} level, but not at the LD_{95} level. Phenyl 4-chlorobenzenesulphonate and the esters containing three or four chlorine atoms were of low or negligible activity.

The ability of 4-chlorophenyl benzenesulphonate to exert ovicidal action against eggs on the under surfaces of leaves after its application to their upper surfaces exceeded that of 4-chlorophenyl 4-chlorobenzenesulphonate and that of phenyl benzenesulphonate.

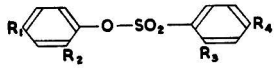
Chlorination in the *para*-position of the phenyl ring of phenyl benzenesulphonate enhanced ovicidal action, whereas *para*-chlorination in the benzenesulphonic ring reduccd it greatly. The effects of *para*-chlorination in both rings do not appear to be merely a summation of the effects of chlorination in either ring.

Introduction

There are several generations per season of most economically important phytophagous mites under temperate climatic conditions, and in heated glasshouses the number is considerably increased. These generations overlap ; consequently it is usually impossible to apply a chemical except when both eggs and other stages are present, and the applications of acaricides, which are toxic only to active stages, must be repeated at frequent intervals unless they are sufficiently persistent to destroy larvae emerging from eggs present at the time of application. Few acaricides are toxic to the eggs or sufficiently persistent except at levels either uneconomic, phytotoxic or hazardous to workers or consumers, and different substances may be required to destroy the eggs. One such substance is azobenzene, which was shown by Blauvelt[1] to provide substantial control of the two-spotted mite, *Tetranychus bimaculatus* Harvey, on roses in the U.S.A. Considerable use has been made in Great Britain of this ovicide, which is applied conveniently in glasshouses as aerosols and smokes, usually in conjunction with an organophosphorus acaricide.[2–4] It is, however, somewhat injurious to cucumbers.[5]

Azobenzene is also highly toxic to the summer eggs of the fruit tree red spider mite, *Metatetranychus ulmi* Koch, but unsafe on certain varieties of apple. Eaton & Davies[6] examined many compounds, differing from azobenzene in the nature of the group linking the benzene rings, for toxicity to the eggs and adult females of this mite. One of the most promising was diphenyl sulphone in which the azo-link is replaced by a sulphur atom carrying also two oxygen atoms. This compound has proved much safer on apple foliage than azobenzene and is in commercial use against the fruit tree red spider mite. Recently a report[7] has appeared of a further development along this line ; the bridging unit is $-S{\cdot}CH_2-$ and each phenyl ring carries a chlorine atom in the *para*-position. The corresponding sulphoxide and sulphone have also been claimed to be ovicidal.

Modification of the link between the phenyl groups by the insertion of a third oxygen atom between one phenyl group and the sulphone group of diphenyl sulphone yields phenyl benzenesulphonate [structure (1), where $R_1 = R_2 = R_3 = R_4 = H$]. Kenaga & Hummer[8] examined



(I)

phenyl benzenesulphonate together with other esters in which R_4 was chlorine or bromine and in which the phenyl ring had up to three substituent atoms or groups of various types. Their results, expressed as the 'minimal LD_{100} value' for each compound, showed that for phenyl benzenesulphonate the figures were 0·375% both for adult female mites and for eggs. Where R_1 = Br and R_4 = Cl the 'minimal LD_{100} value' for the eggs fell to 0·03% and, where $R_1 = R_2$ = Cl, to 0·007%. Compounds with chlorine at either R_1 or R_4 were not more active than the unsubstituted ester.

4-Chlorophenyl 4-chlorobenzenesulphonate, first marketed as a 50% wettable powder under the name 'Ovotran', has received extensive trial for the control of a number of mites in the U.S.A., and a further compound, 2 : 4 dichlorophenyl benzenesulphonate, not examined by Kenaga & Hummer, is also available commercially. Phenyl benzenesulphonate, the two monochlorinated esters (R_1 = Cl, and R_4 = Cl) and the dichloro-ester ($R_1 = R_4$ = Cl) were tested by Kirby & McKinlay[9] against the summer eggs of the fruit tree red spider mite. Kirby & Tew[10] have reported the activity of these and other esters towards the winter eggs of this mite. Although the winter eggs have usually proved more resistant than the summer eggs to ovicides, high kills of both types of egg were obtained with 0·1% of the dichloro-ester and of one monochloro-ester, 4-chlorophenyl benzenesulphonate. Good results were also obtained with these two esters in a pilot field-trial in 1953 at East Malling Research Station.[10a] A preliminary examination by Read & Smith[5, 11] of the relative toxicities of 4-chlorophenyl benzenesulphonate and 4-chlorophenyl 4-chlorobenzenesulphonate towards eggs of the glasshouse red spider mite, *T. telarius* L., which appears to be closely related to the North American species *T. bimaculatus* and for which the name *T. urticae* Koch is preferred by some,[12] indicated that the first

compound was the more potent ovicide. Confirmation of this was obtained in later work in which the ovicidal activities of other chlorinated phenyl benzenesulphonates were investigated. Lack of precision in the estimates of the LD_{50} and LD_{95} values, revealed by a statistical examination of the results of these tests, prevented accurate estimation of their relative potencies.

The European term *Tetranychus telarius* L. and the American term *T. bimaculatus* Harvey have often been considered synonymous, but McGregor[13] took the view that *T. bimaculatus* is a different species from *T. telarius* and other species of European mite which may or may not be separable from each other. The position is under active review by acarologists,[14] and an early decision is obviously desirable. If *T. bimaculatus* and the common glasshouse red spider mite of Britain are not identical, the results obtained by Kenaga & Hummer with the former species might not be applicable to the latter species as, indeed, had been proved for the fruit tree red spider mite.[9] The present authors therefore decided to examine the relative toxicities of as many esters of this class as were available towards the eggs of the glasshouse mite available at Cheshunt Experimental and Research Station, and a study was therefore undertaken of the relative activities of phenyl benzenesulphonate and the eight chlorinated derivatives given in Table I.

Table I

Data for compounds assayed against eggs of the glasshouse red spider mite

	Compound	Abbreviation	Melting point, °C
A	Phenyl benzenesulphonate	PBS	34·5–35·5
B	4-Chlorophenyl benzenesulphonate	CPBS	59·5–61
C	Phenyl 4-chlorobenzenesulphonate	PCBS	91–92
D	4-Chlorophenyl 4-chlorobenzenesulphonate	CPCBS	83–84·5
E	2 : 4-Dichlorophenyl benzenesulphonate	DCPBS	46·5–47
F	Phenyl 2 : 4-dichlorobenzenesulphonate	PDCBS	76·5–77
G	2 : 4-Dichlorophenyl 4-chlorobenzenesulphonate	—	117–117·5
H	4-Chlorophenyl 2 : 4-dichlorobenzenesulphonate	—	80–80·5
J	2 : 4-Dichlorophenyl 2 : 4-dichlorobenzenesulphonate	—	89·5–90

It is of interest to note that 4-chlorophenyl benzenesulphonate has been extensively and effectively used in Britain for the control of the fruit tree red spider mite on tolerant varieties of apple, and of the glasshouse red spider mite on tolerant glasshouse crops, in 1952 and 1953.

A remarkable feature of the field performance of these esters, against both the fruit tree and the glasshouse red spider mites, is the long period over which they remain efficacious. Since esters in general may be hydrolysed by biological systems, it seemed desirable to investigate the action, if any, of the hydrolysis products of these esters; those derived from 4-chlorophenyl benzenesulphonate and 4-chlorophenyl 4-chlorobenzenesulphonate were examined.

Some further experiments on the penetration of apple leaves by some of these esters are also reported below.

Experimental

Compounds employed

4-Chlorophenyl 4-chlorobenzenesulphonate was purified, by recrystallization from methanol, from a sample obtained from the American manufacturers. All the other esters were prepared at East Malling Research Station from the appropriate phenol and benzenesulphonyl chloride. Melting points were recorded by Kirby & Tew,[10] but are included in Table I. Five possible hydrolysis products, phenol, 4-chlorophenol, 2 : 4-dichlorophenol, benzenesulphonic acid and 4-chlorobenzenesulphonic acid, were obtained from commercial sources and purified by recrystallization before assay.

Bio-assay procedure

The ovicidal activities of the esters towards eggs of the glasshouse red spider mite were determined by the method described by Read & Wain.[15] Tests were carried out during the period 15 June to 15 September, as shown in Table II. The design employed was thus in balanced incomplete blocks. In this way each compound was tested once against every other

one, and, in all, four times; the exception was 2 : 4-dichlorophenyl 2 : 4-dichlorobenzenesulphonate, for which the fourth test was abandoned. However, as three of the compounds failed to cause any diminution in hatch except at the highest concentration used, the design was not fully achieved, and, in the analyses, the differences between occasions was ignored with a consequent loss of sensitivity.

Table II

Layout of bio-assays

Series 1		Series 2		Series 3		Series 4	
Compound	Date	Compound	Date	Compound	Date	Compound	Date
A, B, D	18/6	A, C, E	18/7	A, F, J	21/8	A, G, H	8/9
C, G, J	27/6	B, G, F	19/8	B, C, H	29/8	B, E, (J)	11/9
E, F, H	10/7	D, J, H	13/8	D, G, E	4/9	C, D, F	15/9

Results

Compounds containing three or four chlorine atoms were found to be of low ovicidal activity. In three tests with 2 : 4-dichlorophenyl 2 : 4-dichlorobenzenesulphonate there was no mortality at 0·5%, the highest convenient concentration for use in these tests. 4-Chlorophenyl 2 : 4-dichlorobenzenesulphonate was somewhat toxic at 0·5% but non-toxic at 0·25%.

2 : 4-Dichlorophenyl 4-chlorobenzenesulphate was inactive in one test at 0·2% and somewhat toxic at 0·4% in another test; the remaining tests indicated an LD_{50} value between 0·31% and 0·34% but the regression lines were of very doubtful linearity.

The remaining six esters gave high mortalities at the concentrations employed except phenyl 4-chlorobenzenesulphonate, which appeared to have an LD_{50} well above 0·5%, but it proved possible to calculate a dosage–mortality line and hence LD_{50} and LD_{95} values for this compound also.

Table III

Values for Y in probit line equations, with $\chi^2_{[3]}$ values, for all tests of compounds A to F

Compound	1st occasion	2nd occasion	3rd occasion	4th occasion
A	$2{\cdot}591x + 0{\cdot}471$ 0·34	$4{\cdot}013x - 0{\cdot}203$ 0·67	$2{\cdot}570x + 1{\cdot}333$ 9·95	$1{\cdot}377x + 3{\cdot}80$ 34·24
B	$1{\cdot}270x + 4{\cdot}030$ 5·56	$1{\cdot}374x + 5{\cdot}158$ 12·61	$2{\cdot}095x + 3{\cdot}791$ 1·84	$1{\cdot}896x + 3{\cdot}953$ 21·13
C	$4{\cdot}250x - 4{\cdot}460$ 27·04	$1{\cdot}123x + 1{\cdot}981$ 26·81	$2{\cdot}449x + 0{\cdot}432$ 9·53	$1{\cdot}007x + 2{\cdot}181$ 9·03
D	$1{\cdot}653x + 3{\cdot}154$ 0·89	$1{\cdot}828x + 3{\cdot}197$ 0·97	$1{\cdot}480x + 3{\cdot}192$ 3·18	$2{\cdot}438x + 2{\cdot}972$ 10·99
E	$3{\cdot}341x + 1{\cdot}400$ 0·87	$2{\cdot}813x + 1{\cdot}410$ 2·99	$2{\cdot}482x + 1{\cdot}804$ 59·31	$1{\cdot}966x + 2{\cdot}466$ 19·68
F	$4{\cdot}384x - 2{\cdot}350$ 13·17	$2{\cdot}620x - 0{\cdot}004$ 59·22	$3{\cdot}752x - 2{\cdot}845$ 0·41	$2{\cdot}804x - 1{\cdot}119$ 1·24

The individual dosage–mortality probit equations for phenyl benzenesulphonate (PBS), 4-chlorophenyl benzenesulphonate (CPBS), 2 : 4-dichlorophenyl benzenesulphonate (DCPBS), phenyl 4-chlorobenzenesulphonate (PCBS), 4-chlorophenyl 4-chlorobenzenesulphate (CPCBS) and phenyl 2 : 4-dichlorobenzenesulphonate (PDCBS), calculated from observed mortalities after correction for mortalities of controls, are given in Table III, and the mean regression coefficients together with LD_{50} and LD_{95} values are given in Table IV.

Phenol, 4-chlorophenol, 2 : 4-dichlorophenol, benzenesulphonic acid and 4-chlorobenzenesulphonic acid were not appreciably toxic at 0·5%.

Table IV

Mean dosages for 50 and 95% mortalities and regression coefficients

Compound	B	D	E	A	F	C	Significant difference
Mean LD_{50} log (molar × 10^4)	0·72	1·04	1·24	1·34	1·99	2·40	0·45
,, (per cent.)	0·014	0·033	0·053	0·051	0·30	0·67	—
Compound	B	E	D	A	F	C	—
Mean LD_{95} log (molar × 10^4)	1·73	1·88	1·96	2·06	2·47	3·43	0·80
,, (per cent.)	0·14	0·23	0·28	0·27	0·90	7·24	—
Compound	B	D	C	E	A	F	—
Mean regression coefficient (*b*)	1·66	1·85	2·21	2·65	2·89	3·34	Differences are not significant

Note: Compounds G, H and J were insufficiently ovicidal for a reliable computation to be made of regression lines

Leaf penetration

It was demonstrated by Kirby & McKinlay[9] that certain ovicides, including azobenzene, para-oxon and parathion, and two chlorinated phenyl benzenesulphonates (CPBS and CPCBS) could permeate leaves and kill eggs of the fruit tree red spider mite laid on the other side. That permeation could occur had been suggested by Blauvelt & Hathaway[16] to explain the success of CPCBS when used in the greenhouse as an aerosol which provides a deposit almost entirely on the upper surface of leaves. It seemed desirable to repeat this experiment with CPBS and CPCBS, and of interest to extend it to include the unchlorinated ester, PBS.

Solutions were prepared containing 5% of PBS, CPBS or CPCBS, and 1% of Triton X-100, in acetone ('AnalaR'). A quantity of 2 ml. of solution was added to 100 ml. of distilled water with magnetic stirring, and four apple leaves, each bearing about 200–600 summer eggs, on the lower surface, were painted with the suspension on the upper surface only using a small camel-hair brush and avoiding any overflow at the edges. The leaves were allowed to dry and then stored separately in standard 4-in. Petri dishes, with a ring of compressed paper pulp[16a] which was kept moist to keep the leaf as fresh as possible. The dishes were kept on the bench in a north-facing room, and final counts made on the 18th day after treatment. The results were subjected to an analysis of variance, using angles of equal information, and, re-transformed to percentages, are given in Table V. The data obtained by Kirby & McKinlay[9] have also been examined statistically and the re-transformed percentages included in the same Table. The values after correction by Abbott's formula are also given in each case.

Table V

Percentage mortalities of summer eggs of fruit tree red spider mite when toxicants were applied to the opposite side of the leaves bearing the eggs

1953 Experiment				1950 Experiment*			
Toxicant	Concn., %	Percentage mortality		Toxicant	Concn., %	Percentage mortality	
		Gross	Corrected ($c = 0$)			Gross	Corrected ($c = 0$)
CPBS	0·1	52·1	42·7	CPBS	0·1	73·3 †	69·1
CPCBS	0·1	18·1 †	2·0	Para-oxon	0·05	60·2	53·9
PBS	0·1	14·2	0·0	Parathion	0·05	42·3 †	33·1
Formulation control		16·4	0·0	CPCBS	0·1	41·1	31·7
Undipped control		11·4	—	Azobenzene	0·1	28·1 † †	16·7
				Diphenyl sulphone	0·2	14·5 †	0·9
				DMC‡	0·2	11·8	0·0
				Rotenone	0·01	6·3	0·0
				Formulation control		13·7	0·0
				Undipped control		8·1	—

* Derived from data obtained by Kirby & McKinlay (1951)
† Figures in brackets do not differ significantly ($P = 0·05$)
‡ 1 : 1-Bis-(*p*-chlorophenyl)ethanol

Discussion

Although it is possible to say that 2 : 4-dichlorophenyl 2 : 4-dichlorobenzenesulphonate was the least active of the nine esters tested in this series, and that the two esters containing

three chlorine atoms were not much more active, the other six have not been definitely distinguished from each other. Phenyl 4-chlorobenzenesulphonate appears to fall in much the same category as the tri- and tetra-chlorinated esters and to be substantially less active than the remaining five esters at high mortality rates.

With the LD_{95} as the criterion of activity, there is no significant difference between the ovicidal activities of phenyl benzenesulphonate (PBS), 4-chlorophenyl benzenesulphonate (CPBS) and the three esters containing two chlorine atoms each (CPCBS, DCPBS and PDCBS), although CPBS approaches significant superiority over PDCBS. The regression coefficients, which express the slope of the regression lines, indicate no definite differences between compounds; the line for PDCBS has a much steeper slope than those for CPBS and CPCBS, but variations between tests carried out on different occasions were so great as to make any difference in slope non-significant.

It is, however, not surprising that comparisons of the LD_{50} values for these five esters should indicate that PDCBS is significantly weaker than the other four. Moreover, it is evident that CPBS is significantly more ovicidal than DCPBS or PBS. CPCBS is nearly significantly less active than CPBS and more than PBS.

The results are in contrast with those obtained by Kenaga & Hummer,[8] who found CPCBS much more toxic to eggs of the two-spotted mite than all the other esters they examined. Seigler & Gertler,[17] however, found PBS and CPBS much more active against codling-moth larvae than were DCPBS and a number of other esters carrying various substituents in the phenyl ring. Unfortunately, esters chlorinated in the benzenesulphonic ring were not included in tests made by these workers.

Effect of chlorination

It is possible to make an analysis of variance on the mean regression coefficients, LD_{50} values and LD_{95} values for the four esters containing respectively no chlorine, PBS, one *para*-chlorine in the phenolic ring, CPBS, one *para*-chlorine in the benzenesulphonic acid ring, PCBS, and one *para*-chlorine in both rings, CPCBS, as these four compounds constitute a factorial design, thus:

	Benzenesulphonate	4-Chlorobenzenesulphonate
Phenyl derivative	PBS	PCBS
4-Chlorophenyl derivative	CPBS	CPCBS

It is therefore possible to analyse the effect of introducing one chlorine atom into the *para*-position of either ring, or into both rings. The mean LD_{50} and LD_{95} values for the two pairs, phenyl esters and benzenesulphonates, are set out in Table VI; the regression coefficients did not differ significantly for either pair.

Table VI

Effects of chlorination upon toxicity of phenyl benzenesulphonate

	Benzenesulphonates	4-Chlorobenzene sulphonates	Mean LD_{50}	Mean LD_{95}
Phenyl derivatives	PBS	PCBS	1·87	2·75
4-Chlorophenyl derivatives	CPBS	CPCBS	0·88	1·85
Mean LD_{50}	1·03	1·72	Sig. diff. = 0·36	
Mean LD_{95}	1·90	2·70		Sig. diff. = 0·68

Note: Significant differences are worked out for $P = 0{\cdot}05$

With regard to the effect on the LD_{50}, the insertion of a chlorine atom at the *para*-position of the benzenesulphonic group of the benzenesulphonates was to reduce activity, and this difference was highly significant at the 1% level. The insertion of a chlorine atom at the *para*-position of the phenolic ring in the phenyl compounds caused a marked increase in activity, significant at the 0·1% level.

The effect on the LD_{95} value is similar, but smaller. The means for benzenesulphonates differed significantly at the 5% level, as did the means for phenyl compounds, showing that *para*-chlorination of the phenolic ring was advantageous and that of the benzenesulphonic ring disadvantageous.

The activity of CPCBS is of special interest. Insertion of chlorine at the *para*-position in the phenolic portion of PBS lowers the LD_{50} from 1·34 to 0·72, i.e. by 0·62. Conversely, *para*-chlorination of the acidic portion raises the LD_{50} from 1·34 to 2·40, i.e. by 1·06. If these opposing effects were additive, an LD_{50} of 1·34 plus 0·44, i.e. 1·78, would be predicted for CPCBS. Similarly, the calculated LD_{95} would be 3·10, whereas the value found experimentally was 1·96. In both cases, the predicted values are greater than those for PBS, but the experimental values are less. Although this departure from an additive relation is not significant at the 5% level, it is considered that the effect of *para*-chlorination in both rings is not merely a summation of the effects of chlorination in either ring, but that there is a definite interaction. The evidence is insufficient to interpret the nature of this interaction, but it may be the chlorination in one half of the molecule affects the intrinsic toxicity, whereas chlorination in the other half affects penetration of the chemical to the site of action. The modes of action of PBS and its chlorinated analogues are unknown. The phenols and acids comprising the active esters may show no activity because they are incapable of penetrating the eggs, or because, should they penetrate, they are unable to reach vital sites of action. Alternatively the entire ester molecules may be necessary to form a bond with a specific receptor system.

Leaf penetration

In the experiment on permeation recorded here, the mortality due to CPCBS or to PBS did not exceed that due to the formulation mixture alone ($P = 0{\cdot}05$), but that due to CPBS was much greater ($P = 0{\cdot}001$). Similarly, in the earlier experiment,[9] the mortality due to CPBS was significantly greater than that due to CPCBS, but on that occasion both chemicals were relatively more effective, and even CPCBS caused a significant mortality. It is clear that CPBS has a considerable advantage over CPCBS from this point of view, but under favourable conditions CPCBS may have an appreciable effect through leaves; the value of PBS is doubtful, but seems likely to be small and certainly less than that of CPBS. Of the three other well-known mite ovicides tested, only azobenzene seems to have any action through the leaf and that is small; diphenyl sulphone and 1 : 1-bis-(*p*-chlorophenyl)ethanol (DMC) are inactive even when applied at 0·2%.

Conclusions

It should be emphasized that 4-chlorophenyl benzenesulphonate has proved equal or superior to 4-chlorophenyl 4-chlorobenzenesulphonate investigated in bio-assays employing the fruit tree red spider mite, both as summer eggs[9] and as winter eggs,[10] and eggs of the glasshouse red spider mite as reported above, in contrast with the complete superiority of 4-chlorophenyl 4-chlorobenzenesulphonate in the experiments reported by Kenaga & Hummer.[8] It would appear that for a number of pests such as the mites discussed and the codling moth it is only necessary to insert chlorine in the *para*-position of the phenolic ring of phenyl benzenesulphonate to achieve a satisfactory level of toxicity.

The commercial value of an ovicide is dependent, however, upon a number of factors besides ovicidal activity, including relative costs, absence of phytotoxicity or tainting, persistence and the degree of accompanying acaricidal action. The ability to permeate foliage is an added asset, especially where the ovicide is to be applied as an aerosol. It appears that phenyl 4-chlorobenzenesulphonate and phenyl 2 : 4-dichlorobenzenesulphonate are unlikely to be of practical value. The decision as to which is the best for any particular purpose of the four most active of these esters (phenyl, 4-chlorophenyl, and 2 : 4-dichlorophenyl benzenesulphonate and 4-chlorophenyl 4-chlorobenzenesulphonate) disclosed by these tests must be decided by extensive field trials. Kirby & Bennett (unpublished work), using several varieties of apples, and Read (unpublished work), using cucumbers, have established that phenyl benzenesulphonate is the least phytotoxic of these compounds, and that 4-chlorophenyl benzenesulphonate is the most likely to split the fruit and spurs on apple trees.

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East Malling Research Station
nr. Maidstone
Kent

and

Experimental and Research Station
Cheshunt
Hertfordshire

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THE EFFECT OF PROCAINE PENICILLIN ON THE GROWTH OF WEANED PIGS

By G. A. CHILDS and W. F. J. CUTHBERTSON

In a series of practical tests on farms in the United Kingdom, procaine penicillin (procaine benzylpenicillin) was found to increase the growth rate of weaned pigs and to improve the efficiency of food utilization.

Fortification of the diet with 10 g. or 15 g. of procaine penicillin per ton did not give significantly greater responses than did 5 g.; the economic level of addition may be less than 5 g. of procaine penicillin per ton under the conditions used.

Introduction

Extensive researches in the U.S.A., reviewed by Braude *et al.*,[1] have shown that a number of antibiotics are effective in promoting growth and feed conversion of pigs from weaning to bacon weight. The most commonly used of the antibiotics are 'Aureomycin' (chlorotetracycline) and procaine penicillin (procaine benzylpenicillin). A number of tests have been carried out in which these two antibiotics have been simultaneously compared.

Table I summarizes all the available published reports of trials in which the two antibiotics have been used in controlled tests, except those obtained at one institute where the control groups grew poorly; according to Braude *et al.*[1] the animals may have been atypical, probably owing to the presence of disease.

These researches show that procaine penicillin and chlorotetracycline were equally effective in promoting pig growth and feed efficiency, even though penicillin was used often at much lower concentrations than chlorotetracycline. The data also shows that the two antibiotics were equally effective when the pigs were offered either wholly vegetable rations or diets containing some animal protein.

Table I

American data on the effect of procaine penicillin and chlorotetracycline on growth (lb./day) and feed efficiency (lb. of food per lb. of body-weight gain) of pigs from weaning to bacon weight

Diet	Ref.	Control		Procaine penicillin			Chlorotetracycline		
		Growth	Feed efficiency	Addition, g./ton	Growth	Feed efficiency	Addition, g./ton	Growth	Feed efficiency
Vegetable	2	1·68	3·55	10	1·78	3·59	10	1·83	3·43
,,	3	1·14	—	8·2	1·19	—	8·2	1·21	—
,,	4*	1·46	3·23	20	1·58	3·29	20	1·67	2·98
,,	5	1·45	3·71	3	1·57	3·52	6	1·57	3·49
,,	6	1·36	3·81	2–15	1·34	3·50	9–16	1·37	3·36
,,	7	1·24	3·89	2	1·48	4·48	20	1·50	4·57
,,	8	1·24	3·89	10	1·45	4·42	—	—	—
,,	9*	1·00	3·16	10	1·19	2·94	10	1·18	2·95
Vegetable + animal	5	1·43	3·70	3	1·68	3·32	6	1·59	3·42
,,	5	1·07	3·52	3	1·20	3·19	10	1·42	3·24
,,	9	0·57	4·12	18	0·79	3·03	25	0·71	3·41
,,	9	0·59	5·04	18	0·86	3·96	25	0·84	3·81
,,	10	1·4	3·4	5	1·54	3·32	12·5	1·63	3·49
,,	10	1·4	3·4	5	1·45	3·35	12·5	1·53	3·50
Means of all results		1·22	3·72	7·8	1·36	3·53	13·7	1·39	3·47
Means of mixed protein		1·21	3·82	8·8	1·36	3·61	13·4	1·38	3·57

* Tests continued for a few weeks only

In view of the close similarity between the effects of the two antibiotics under American conditions, it was thought of interest to determine the responses obtainable with procaine penicillin on the growth of pigs under normal farm conditions in the United Kingdom, and also to estimate the minimum level of this antibiotic required in the food to produce a maximal effect on growth and feed-conversion rate. [A study of the effect of antibiotics on pig rearing at a number of U.K. research stations has been published by the Agricultural Research Council (A.R.C.).[11]] It is likely that the effects obtained may depend quantitatively on a number of factors, e.g. diet, method of management, hygiene and breed; thus tests have been carried out at a variety of different establishments under conditions typical of British pig-rearing practice.

Experimental

In all tests the pigs used were derived from home-reared litters and put on experiment shortly after weaning at nine weeks of age.

The animals in each litter were weighed individually and divided into groups matched as accurately as possible for litter origin, sex and weight. These sets of pigs were then allocated at random to the different treatments. In most instances all the pigs on one treatment were kept in single pens and group-fed, i.e. normal farm practice was employed. This procedure was adopted not only because it is the usual method, but also because there was generally an insufficiency of accommodation, labour, pigs of the same age, or equipment to make practicable any serious departure from the farm routine. This method allows of no estimate of inter-pen variance, and special care was therefore taken to use closely comparable pens and equipment for each of the test-groups at any one establishment and to randomize the position of the test pens. The pigs were weighed at weekly or fortnightly intervals and records were kept of all food consumed.

In all tests food was offered as meal. Fresh batches were prepared at approximately monthly intervals; the same batches of ingredients were used in the preparation of both the basal rations and those containing procaine penicillin, the latter rations being made up by

incorporating procaine penicillin (supplied as a mixture with oyster-shell meal containing 5 g. of procaine penicillin per lb.) at the stated level in the basal ration.

The equipment, diets, method of feeding and other relevant details at each establishment are shown in the Appendix.

Results and discussion

The main results of each test are summarized in Table II, which demonstrates the mean rate of gain and feed conversion for the different groups of pigs over the period of study. The weighted mean results obtained in all of the tests are presented in Table III.

Table II

The effect of procaine penicillin on the mean weight gain and feed-conversion ratios (lb. of food per lb. of live-weight gain) of pigs maintained under normal U.K. farm conditions on rations containing some animal protein

Farm	Expt. No.	Breed	Initial age, weeks	Test period, weeks	No. of litters used for test	No. of pigs per group	Procaine penicillin, g./ton	Mean initial wt., lb.	Mean wt. gain, lb./day and S.E. of mean	Food, lb./lb.of live-wt. gain
A	1	W.S.B.	11	16	1	6	0	69	1·05 ± 0·050	4·16
						6	3	66	1·245 ± 0·013	3·75
B	2	L.W.	15	8	2	4	0	82·4	1·88 ± 0·068	3·68
						4	15	80·8	1·96 ± 0·037	3·41
C	3	L.W. × W.S.B.	9	6	8	15	0	35·6	0·961 ± 0·020	4·09
						15	15	35·2	1·04 ± 0·021	3·80
D	4	W.S.B.	8	20	3	6	0	25·7	1·23 ± 0·042	4·37
						6	15	27·7	1·33 ± 0·022	3·70
D	5	W.S.B.	8	18	3	9	0	27·0	0·62 ± 0·067	5·07
						9	5	27·5	0·87 ± 0·060	4·21
						9	15	26·7	0·88 ± 0·069	4·17
E	6	L.W.	11	12	4	10	0	51·0	1·42 ± 0·048	3·28
						10	5	49·2	1·49 ± 0·047	3·19
						10	10	54·3	1·50 ± 0·053	3·36
E	7	L.W.	10	14	3	10	0	52·7	1·35 ± 0·054	3·87
						10	5	50·0	1·38 ± 0·046	3·55
						10	10	48·0	1·41 ± 0·061	3·53
F	8	L.W.	13	14	3	6	0	51·0	1·36 ± 0·050	4·06
						6	5	52·5	1·45 ± 0·100	3·78
						6	10	52·1	1·48 ± 0·050	3·85

L.W. = Large White
W.S.B. = Wessex Saddle Back

Table III

Weighted mean weight gain (lb./day) and food-conversion ratio (lb. of food per lb. of live-weight gain) of control and procaine penicillin-fed pigs maintained under U.K. farm conditions on diets containing some animal protein

Treatment	Weight gains (lb./day and S.D. of mean)		Feed efficiency (lb. of food per lb. of live-wt. gain)	
	All expts.	Omitting expt. 5	All expts.	Omitting expt. 5
Control	1·156 ± 0·018	1·247 ± 0·019	4·08 ± 0·19	3·92 ± 0·14
Penicillin-fed	1·278 ± 0·015	1·375 ± 0·016	3·70 ± 0·09	3·59 ± 0·07
Weighted diff.	0·132 ± 0·023	0·116 ± 0·025	0·38 ± 0·10	0·30 ± 0·08
A.R.C. data[11]				
Control	1·17 ± 0·013		3·72 ± 0·038	
Penicillin-fed	1·29 ± 0·013		3·45 ± 0·038	

The results show clearly that procaine penicillin supplementation consistently improved growth and feed efficiency, though, as was to be expected with groups of the size employed, there were some differences between tests. There is also an indication that the effects of the antibiotic may be somewhat different from farm to farm. On the average a mean growth improvement of 0·132 lb. per day and an economy of 0·38 lb. of food consumed per lb. of live-weight gain were observed.

In a number of these experiments the effects of supplementation with different levels of procaine penicillin were compared. The mean results of these tests (Experiments 5, 6, 7 and 8, Table II) show that there is no significant difference between the benefits obtained on supplementation with 5, 10 or 15 g. of procaine penicillin per ton of diet; there is thus no advantage

to be gained by supplementing the ration with more than 5 g. of procaine penicillin per ton of food, and it may well be that even lower levels of procaine penicillin might prove satisfactory.

The results of trials carried out at the request of the Agricultural Research Council were published[11] shortly after the completion of the work reported here. The mean results of our trials carried out on six different farms are similar to those noted by the A.R.C. workers, both in the degree of benefit obtained and in the observation that the improvement differs from farm to farm. The growth rates in lb. of live-weight gain per day also agree closely with those noted for control and penicillin-fed groups in the A.R.C. tests, but the average feed-conversion rates in the present tests were somewhat higher. This is not surprising, for in most of the tests described here the rations normally used on the farms were employed, for the aim of these trials was to determine the effect of feeding procaine penicillin to pigs under conditions typical of United Kingdom practice, rather than to find out what might happen in the better-than-average dietary and environmental circumstances that pigs might be reasonably expected to enjoy at the establishments used for the A.R.C. tests.

Appendix

Farm A

This was a mixed farm with the experimental animals housed in concrete sties. The diet was prepared from an indifferent batch of mixed oats, barley and wheat to which 8% of fish meal was added. The same batches of basal ration were used for both control and experimental groups of pigs. Immediately before the test was begun all pigs were treated with sodium fluoride to eliminate worms.

Method of feeding.—The meal was offered as a wet mash and given according to appetite, i.e. such amount as the pigs would consume in half an hour.

Farm B

This was a large farm specializing in pig production and carrying a stock of about 3000. The test was carried out in a well-constructed brick building. Conditions of hygiene were fair, but the buildings were cold (the tests were carried out during the winter). Before the experiment all pigs used in the test were treated with sodium fluoride to eliminate worms.

Diet.—Details are:

Dried yeast (extracted)	0·5 cwt.	Beans	0·5 cwt.
Maize meal	2·0 ,,	Chalk	0·25 ,,
Middlings	5·0 ,,	Salt	3·5 lb.
Wheat meal	4·5 ,,	Ferrous sulphate	2·0 oz.
Barley meal	6·0 ,,	Copper sulphate	2·0 ,,
Fish meal	1·0 ,,	Vitamin D_3 concentrate	
Grass meal	1·0 ,,	(80,000 i.u./g.)	1·0 ,,

Feeding method.—Food was offered as a wet mash and given according to appetite.

Farm C

A mixed farm carrying about 300 pigs. The experimental animals were housed in pens in a 'Danish-type piggery'. Hygienic conditions and management were average.

Diet.—Details are:

Wheat offals	20%	Barley meal	15%
Wheat meal	15%	Malt culms	5%
Maize flakes	10%	Proprietary protein and mineral	
Maize meal	5%	concentrate	5%
Copra meal	10%	Proprietary protein concentrate	15%

Type of feeding.—Meal was offered as a wet mash. The pigs were rationed according to Ministry formula but may have been underfed.

Farm D

This was a medium-sized piggery carrying a few hundred animals. Immediately after weaning the experimental pigs were treated with sodium fluoride to eliminate worms. The pigs were at first kept in pens indoors and moved after 12 weeks into portable sheds and runs.

In experiment 4 the pigs were given for the first 4 weeks:

Oats	42·5%	Flaked maize	5%
Barley	25%	Peas	7·5%
Grass meal	7·5%	Meat and bone meal	5%
Weatings	5%	Minerals	2·5%

From this time onwards the animals were given:

Oats	40%	Flaked maize	7·5%
Barley	10%	Peas	17%
Grass meal	6·5%	Meat and bone meal	6·5%
Weatings	10%	Minerals	2·5%

Feeding.—Dry meal to appetite was fed. In experiment 5 the pigs selected were from particularly poor litters, reared in wooden arks on concrete runs and after 12 weeks transferred to pens in a 'Danish-type piggery'. These animals were thus reared outside during the worst of the winter months (October to January); this and their low initial weight probably account for the poor growth of all groups.

Diet.—Details are:

Barley	49%	Dried grass	4·7%
Oats	23·3%	Meat and bone meal	6·5%
Weatings	2·3%	Mineral mix	2·5%
Peas	11·7%		

Farm E

This was a large well-organized establishment in which the pigs were kept in clean concrete pens in warm wooden buildings under conditions of hygiene much better than average.

Diet.—Details are:

Fine wheat offal	40%	White fish meal	10%
Barley meal	35%	1 oz. of cod-liver oil per week	
Flaked maize	15%		

Feeding.—This was Danish type, i.e. a small amount of water splashed on to the food in the troughs. Diet was given according to appetite.

In experiment 6 the animals were allotted to the different treatments at random, irrespective of litter origin or sex. In experiment 7 the composition of the litters was such that only six of the pigs in each group were accurately matched for litter and sex, so the weights of those six animals in each group were used for calculation of weight gains, but all ten pigs contributed to the feed-conversion figures.

Farm F

This was a pedigree farm in which the test animals were housed in converted brick buildings which were warm and dry. All animals on this farm were in first-rate condition. The experimental pigs were treated with sodium fluoride to eliminate worms before use in the test.

Diet.—Details are:

Wheat offal	40%	White-fish meal	10%
Barley meal	35%	1 oz. of cod-liver oil per week	
Flaked maize	15%		

Cod-liver oil was eliminated from the diet and fish meal reduced to 5% during the last three weeks of test

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Greenford
Middlesex

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STUDIES ON EGG SHELLS. IV.*—The site of deposition of radioactive calcium and phosphorus

By C. TYLER

Egg shells from birds fed with either radioactive calcium or phosphorus have been examined by taking autoradiographs of tangential sections. The autoradiographs for calcium show a distinctive form of layering, which can be correlated with time of laying. This is interpreted as indicating that the supply of calcium to the shell gland may be made up entirely of food calcium or entirely of bone calcium or of varying proportions of the two, depending upon the time of day that a particular layer of the shell was being deposited. The autoradiographs for phosphorus indicate that most of it is in the outermost layer of shell, with some of it actually on the surface.

It has been shown by Spinks, Berlie & O'Neil[1] that radioactive calcium fed in the diet of laying hens quickly appears in the egg shell. After an observation kindly pointed out to the author by Dr. D. H. Tomlin, that the deposition of radioactive calcium in an egg shell as shown by an autoradiograph was apparently in distinct layers, it was decided to study the matter further.

Experimental

Birds and feeding

Two Rhode Island Red × Light Sussex pullets in full lay were fed each day with 120 g. of a typical laying ration to which was added 5 g. of calcium carbonate and 0·5 g. of sodium chloride. This ration was made into a crumbly mash with 100 ml. of water containing 6·4 microcuries (μc) of radioactive calcium (^{45}Ca) in solution. Drinking water was supplied *ad lib.* The birds were fed at 9.30 a.m. and observation showed that in most cases the food was all eaten by 11 a.m., so that practically no food was available from 11 a.m. one day to 9.30 a.m. the next day. The ration containing radioactive calcium was fed for seven days and all eggs were collected, the time of laying being recorded to within a few minutes, except for eggs laid before 8.30 a.m.

* Part III: *J. Sci. Fd Agric.*, 1953, **4**, 587

These were almost certainly laid between 6 a.m. and 8.30 a.m. The same ration without radioactive calcium was then fed for another seven days and eggs were collected and recorded as before. Table I shows the egg records and time of laying.

Table I

Egg records and time of laying

Day	Birds			
	1	2	3	4
1	10.30 a.m.	—	2.25 p.m.	11.45 a.m.
2	3.25 p.m.	8.45 a.m.	—	3.30 p.m.
3	—	9.05 a.m.	8.30 a.m.	—
4	8.30 a.m.	10.00 a.m.	8.30 a.m.	10.15 a.m.
5	10.15 a.m.	4.00 p.m.	8.50 a.m.	1.30 p.m.
6	11.30 a.m.	—	10.25 a.m.	—
7	3.45 p.m.	8.30 a.m.	10.45 a.m.	8.30 a.m.
8	—	8.30 a.m.	—	10.25 a.m.
9	8.45 a.m.	8.55 a.m.	—	12.15 p.m.
10	10.20 a.m.	9.25 a.m.	10.10 a.m.	3.25 p.m.
11	11.35 a.m.	9.05 a.m.	—	—
12	3.30 p.m.	9.40 a.m.	10.45 a.m.	8.30 a.m.
13	—	11.30 a.m.	10.00 a.m.	11.05 a.m.
14	9.00 a.m.	—	10.25 a.m.	12.10 p.m.

Birds 1 and 2 received radioactive calcium for the first time at 9.30 a.m. on day 1 and for the last time at 9.30 a.m. on day 7 ;. birds 3 and 4 received radioactive phosphorus on the same plan

An exactly similar experiment was carried out at the same time with two other birds and feeding about 6μc of radioactive phosphorus (^{32}P) instead of calcium. Actually the dose was 7μc the first day and this had fallen, by decay, to 5μc on the seventh day.

Preparation of the shells

As the eggs were laid they were broken and the shells washed and dried, great care being taken not to contaminate the outside of the shell with either egg content or water.

A portion of the shell, convex face downwards, was then placed at the bottom of a $\frac{1}{2}$ in. × 2 in. flat-bottomed tube and covered with liquid ' Kalladoc '. A few particles of benzoyl peroxide were added, and the tube was stoppered and placed on top of an electric oven (at about 40°) for two or three days. The liquid polymerized and, on breaking the glass tube, a piece of shell embedded in a cylinder of hard transparent plastic was obtained. By grinding the bottom end of the plastic cylinder on a geological grinding wheel, with a fairly fine abrasive, the plastic was ground away until the convex surface of the shell was just reached. This took only a few seconds and was indicated by the presence of a minute white spot on the otherwise brown shell surface. Grinding was then continued carefully until a transparent centre appeared in the circle, or oval, of ground shell surface. At this stage the sections were examined under the microscope by transmitted light and grinding was finished off on a glass plate with a finer abrasive, constant reference being made to the microscope. The ' end point ' was reached when the shell was ground through to a hole at the centre. Fig. 1 gives a diagrammatic view of such a section as seen in plan and vertically through it. Clearly the flat surface of the specimen exposes, in turn across the section, all layers of shell from outermost to innermost.

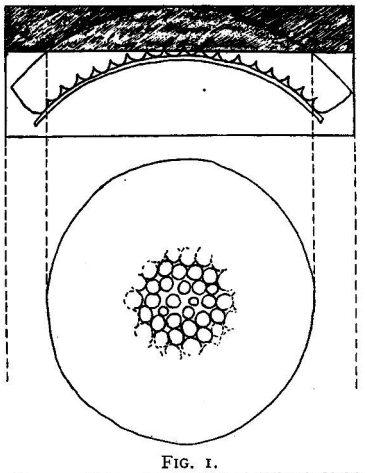

FIG. 1.

The upper diagram shows a piece of shell embedded in plastic, the shaded portion having been rubbed away. The lower diagram is the plan of this, namely a tangential section. The plastic is not shown in the lower diagram

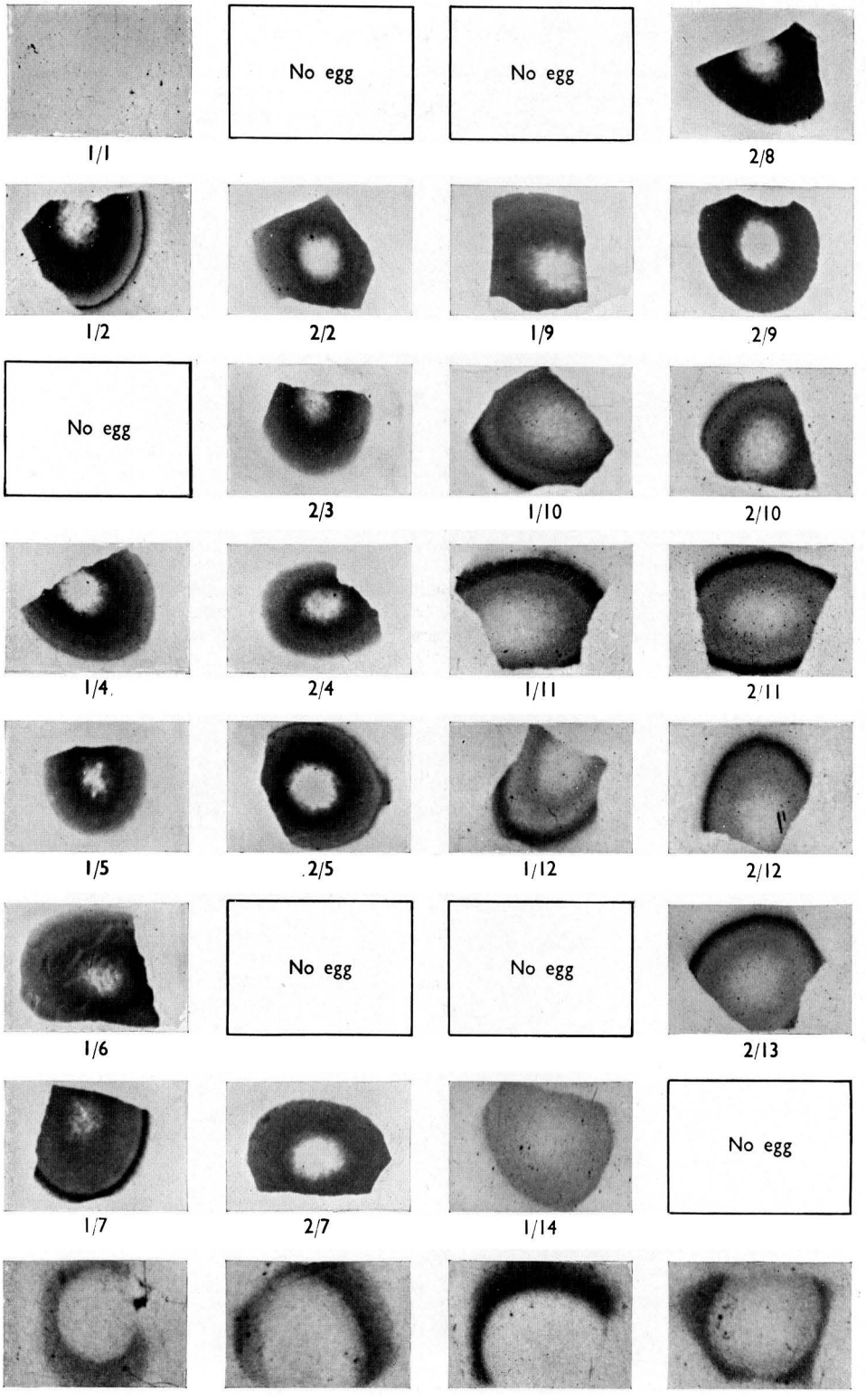

FIG. 2.

The autoradiographs in the bottom row are from shells laid by hens fed with radioactive phosphorus. The rest refer to calcium and are numbered according to bird and day of laying. Magnification: × 3

Preparation of autoradiographs

Autoradiographs were obtained by placing the ground surface of each specimen in contact with a piece of *X*-ray film for a period of one week. Ilford ' Industrial C ' film was used for the specimens from the radioactive calcium experiment, but the faster and coarser-grained ' Industrial G ' film was necessary for those containing radioactive phosphorus, because of their extremely low activity. This accounts for the poor quality of the corresponding autoradiographs. It was found that sufficiently good contact between film and specimen was achieved by covering the film with thin cardboard and binding this to the specimen with adhesive tape. All films were developed together by a standard procedure, and prints made from them are shown in Fig. 2.

Local variations in autoradiographic reaction in any particular print may be strictly compared with corresponding variations of activity, but, since exposure times were not the same for all prints, no guide is given in comparing intensities between different shells in Fig. 2.

Results and discussion

Calcium

With bird 1, the first egg laid (1/1) showed no radioactivity. This indicates that no measurable amount of calcium from the food fed at 9.30 a.m. had been deposited on the shell of this egg laid exactly one hour afterwards. Thus the time taken for food calcium to reach the blood, plus the time taken between the last deposit of calcium on the shell and oviposition, is at least one hour. The next egg (1/2) was laid at 3.25 p.m. and there is in the autoradiograph a dark inner layer, shading off to almost white, followed by a dark outer ring. Now an egg shell takes about 20 hours to form, and, according to Burmester,[2] its rate of formation after the first three or four hours is uniform. Thus the deposition of this shell must have been started at about 7.30 p.m. the previous day, i.e. about 10 hours after feeding the first dose of radioactive calcium. Thus when shell formation started there would be radioactive calcium present in the blood and also as a new deposit in the bone. From the work of Bloom, Bloom, & McLean[3] this deposit would almost certainly be the readily mobilizable medullary bone. It is reasonable to suppose that as shell formation progressed the newly deposited bone would be most readily removed and would contribute to the layers on the inside of the shell. However, this radioactive bone calcium is limited in quantity and hence with later layers of shell it would be more and more diluted with non-radioactive bone calcium until, just before the next feed, the deposit would be almost non-radioactive. As soon as the radioactive calcium from the next feed reached the blood it would be available for shell formation ; thus at this stage bone calcium would make little, if any, contribution, and a strongly radioactive outer layer would be laid down. The autoradiograph obtained gives strong evidence to support this suggested sequence of events.

It will be seen that the egg laid by bird 1 on day 7 (1/7) was also laid in the afternoon and that the picture is substantially the same. The gradual fading shown in the shell laid down during the night and early morning is not so pronounced and this is probably because by this time the bones are well charged with radioactive material. Nevertheless, the outer ring is still quite marked. Bird 2 laid only one egg (2/5) in the afternoon, and again the autoradiograph is quite clear in showing the shading from inside to outside with the dark ring on the outside.

As to shells 1/4, 1/5, 1/6, 2/2, 2/3, 2/4 and 2/7, this group in general shows a gradual fading from inside to outside and clearly represents the same picture as before without the dark ring on the outside. This is to be expected since they were all laid in the morning and hence started to form between about 12 noon and 3 p.m. on the previous day when the supply of radioactive calcium from the food was good, but they were then laid before the current day's supply became available.

Eggs 1/4, 2/2, 2/3 and 2/7 were laid before 9.30 a.m. and hence could not have received calcium from the current day's supply, so no dark outer ring could be expected. Eggs 1/5 and 2/4 were laid at 10.15 a.m. and 10 a.m. respectively. This period of, at most, 45 minutes between feeding and oviposition is scarcely sufficient to allow for any radioactive calcium from the food to be deposited on the shell, and support for this comes from shell 1/1. It is therefore not surprising that again no dark outer ring is present. The other egg of this set, namely 1/6, was laid at 11.30 a.m. This is two hours after feeding and the absence of the dark outer layer

suggests that calcium deposition ceases a considerable time before oviposition, or else that absorption of calcium is not as rapid as was thought. The former suggestion seems to be the more reasonable.

With these shells there is some indication that the gradations from inside to outside are less clearly marked in the later eggs. This is probably due to the gradual build-up of radioactive calcium in the bones.

As to the eggs laid after withdrawing radioactive calcium from the ration, it is clear that with eggs 1/10, 1/11, 1/14, 2/10, 2/11, 2/12 and 2/13 there is a markedly darker ring on the outside, with, usually, a less dark ring on the inside. These eggs were all laid between 9 a.m. and 11.35 a.m. and not in the afternoon. When interpreting these results it must be remembered that it is now the bone that provides radioactive calcium and not the food. Thus these egg shells will, in the last stages of deposition, i.e. generally between 8 and 10 a.m., be relying almost entirely on bone calcium, which is radioactive, and this accounts for the ring on the outside. Similarly the shading on the inside will be due to radioactive calcium from the bone, but during this period non-radioactive food calcium going directly to the shell, or indirectly *via* the bone, will dilute the radioactive calcium to different degreees in different layers. Once more there is strong evidence that eggs laid up to 11.30 a.m. have none of the current day's food calcium in them, when that food is fed at 9.30 a.m.

The eggs laid during the morning of the first two days after withholding radioactive calcium, namely 1/9, 2/8 and 2/9, appear to be anomalous. However, these three eggs were all laid early in the morning and it may be that they were held over for a longer time than usual after shell formation was complete. The afternoon egg 1/12 has a dark outer ring indicative of a morning egg and a similar explanation could be advanced here. However, it must be stressed that such an explanation is very tentative and unsatisfactory until further evidence is forthcoming.

Phosphorus

All the phosphorus results are of the same type, irrespective of the time of laying, and it is clear from the selection given in Fig. 2 that most of the radioactive phosphorus is on or near the surface of the shell, with only a little spread fairly evenly throughout the body of the shell. The reason for the shading-off towards the outside is because the darkest part with the sharply defined inner edge corresponds to a thin line of exposed shell surface, but as this surface buries itself deeper in the plastic (see Fig. 1) the intensity is reduced because it becomes more and more difficult for the radioactivity on the surface to affect the photographic film through an ever-thickening layer of plastic.

Table II

Radioactivity of a piece of egg shell after standing in 100 ml. of 0·1N-sodium hydroxide solution : from a bird fed with radioactive phosphorus

Days in NaOH	Count per minute*
0	113
1	95
2	81
5	73
7	74

* Corrected for decay

As an additional test a piece of untreated shell was placed directly under a Geiger counter and the count per unit time recorded. The shell was then placed in cold 0·1N-sodium hydroxide solution for some time but taken out at intervals for counting. After being corrected for decay the values shown in Table II were obtained. It would appear that some of the radioactive phosphorus is on or near the surface of the shell and is readily soluble in cold dilute alkali.

Conclusions

It must be admitted at the outset that the results are only semi-quantitative and that some are anomalous ; nevertheless there is strong evidence to support the idea that the dark rings and shading observed on the autoradiographs are capable of a straightforward explanation.

It is well known that both food and bone calcium are usually utilized in the formation of an egg shell because the bird rarely, if ever, absorbs sufficient food calcium during a period of shell formation to supply all that found in the egg shell.[4] Now, since shell secretion is at a constant rate, it follows that, when a bird is absorbing food calcium, the amount coming from the bone will be correspondingly reduced, whereas, when no food calcium is being absorbed, the contribution of the bone to shell formation will be greater. At any particular moment the supply

of calcium to the shell gland will be almost all food calcium, or all bone calcium or varying proportions of the two. All these events take place *via* the medium of the blood. Thus at certain times the food calcium may raise the blood level to such a degree that there is sufficient calcium for shell formation and also some left over to be deposited on the bones; however, when food calcium cannot keep pace with removal of calcium by the shell gland, then, in an attempt to maintain blood levels, bone calcium will be mobilized.

Generally, it would appear that, *under our conditions of feeding*, with birds eating most of their food between 9.30 a.m. and 11 a.m., eggs laid about 3 p.m. have a layer of calcium on the outside derived chiefly from the food, whereas eggs laid in the morning before about 11.30 a.m. have the outer layer of calcium derived from the bone. The innermost layers may be derived entirely from either food or bone calcium depending upon when these were laid down, and the middle layers will usually consist of varying proportions of food and bone calcium.

For phosphorus, the first point seems to be that although a little is spread fairly evenly throughout the shell, most of it is on or near the surface. This is opposed to the view of Romanoff & Romanoff.[5] The second point is that some of this phosphorus on the outside is readily soluble in alkali and it is suggested that this fraction is not really part of the shell but is adventitious in character. It is well known that when shell formation takes place there is a heavy excretion of water-soluble phosphorus.[6, 7] When the egg is laid it passes out through the same vent as the faeces and urine and it would be surprising if it did not pick up a quantity of phosphorus on its surface at this stage. The evidence certainly points in this direction.

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Department of Agricultural Chemistry
The University
Reading

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MYCOLOGICAL FORMATION OF FAT. I.—Media Conducive to Formation of Fat from Sucrose by *Aspergillus nidulans, Penicillium javanicum* and *Penicillium spinulosum*

By A. M. GAD and T. K. WALKER

Fat production by the moulds *Aspergillus nidulans, Penicillium javanicum and Penicillium spinulosum* has been studied and media conducive to high productivity have been developed. The highest percentage yields of fat on sucrose utilized were 13·2 from *A. nidulans*, 7·9 from *P. javanicum* and 6·0 from *P. spinulosum*, the last-mentioned mould requiring corn-steep liquor (C.S.L.) (0·5% w/v) for this result. In general, the levels of concentration of sodium phosphate and of magnesium sulphate in the medium have been shown to be highly critical factors.

Introduction

The purpose of the work described here was to determine the particular concentrations of certain nutrients most favourable to formation of fat from sucrose by three moulds that previously had given good results in glucose media. These previous studies with glucose as source of carbon were undertaken by Woodbine, Gregory & Walker[1] and by Garrido & Walker.[2 3]

Experimental methods

The moulds were *Aspergillus nidulans* Eidam, *Penicillium javanicum* v. Beyma and a strain of *Penicillium spinulosum* that was isolated as a casual contaminant from a culture of *Mucor albo-ater* in the laboratory at Manchester.

Composition of the media

By incorporating $NaH_2PO_4,2H_2O$ and $MgSO_4,7H_2O$, both at three concentrations, in different portions of a basal medium of inorganic salts, nine solutions were prepared. The particular levels of $NaH_2PO_4,2H_2O$ and of $MgSO_4,7H_2O$ that were adopted included in each case two very low concentrations and one high concentration. The low concentrations of these two salts, as well as the fixed concentration of K_2SO_4 in the basal medium, were selected on the basis of certain results obtained by Steinberg[4] in the course of studies of the nutrition of mould fungi, and by Laborey,[5] who worked in the same field. Steinberg determined the minimum quantities of certain inorganic salts (so-called dibasal solution) that would suffice for maximum development of mycelial felt. The high concentrations were based on the inorganic media used by Prill, Wenck & Peterson,[6] because preliminary experiments in our Laboratory had confirmed the effectiveness of these media for promoting formation of fat in moulds.

The components of the basal medium, which were never varied in quantity, were (g./100 ml.): NH_4NO_3, 0·300; K_2SO_4, 0·022; $FeCl_3,6H_2O$, 0·016; $ZnSO_4,7H_2O$, 0·005. All the inorganic salts were B.D.H. 'AnalaR' grade, and distilled water was used for preparation of the solutions. Sucrose (A.R. grade) was added at the rate of 17 g./100 ml. to all these solutions. After adjustment of the pH value to 6·8 with sodium hydroxide the solutions were dispensed, 25 ml. in each of a number of 100-ml. conical flasks. In this manner each of the nine media was divided between fifteen flasks so that each of the three moulds could be grown in quintuplicate. The flasks and their contents were then sterilized in steam at 10 lb. for 15 minutes. In other experimental series the effects of corn-steep liquor (C.S.L.) were studied by adding it at the rate of 0·5% w/v to similar flasks of some of these media. The C.S.L. had total solids content of 32%.

Inoculation, incubation and methods of analysis

Inoculation was performed as described by Woodbine *et al.*[1] and, after incubation at 25°, usually for a period of 8 or 9 days, the contents of the flasks were treated as reported by these authors[1] and the yields of fat were estimated. In every case the combined contents of a quintuplicate set of flasks were used for each estimation of fat. The results are set out in Tables I, II and III. The standard deviations are given immediately underneath the figures of mean felt weight (5 felts).

Discussion

A. nidulans

As shown in Table I, the weights of sucrose utilized, of felt developed and of fat synthesized all increased as the concentration of $NaH_2PO_4,2H_2O$ was raised from 0·036 g. to 0·730 g./100 ml. The only effect that resulted from an increase in the concentration of magnesium ions in the absence of C.S.L. was a progressive rise in the percentage of fat in the felt. The best medium for production of fat was that in which both phosphorus and magnesium were present at their highest levels. Addition of C.S.L. to this last medium increased the consumption of sucrose but did not increase further the yield of fat. On the other hand, the addition of C.S.L. to two other media containing phosphate at its lower levels caused in both cases remarkable enhancement of general mycological activity, including increases in the yields of fat. This

suggests that in these two instances the effects of the C.S.L. were caused in part at least by the additional phosphate that it contributed to the media.

Table I

Formation of fat by A. nidulans *in surface culture*
25 ml. in 100-ml. conical flasks; 8 days at 25°; initial sucrose 4·25 g./flask

Basal medium with addition of			Sucrose utilized/ flask, g.	Mean felt wt., g.	Fat wt., g.	Fat, g./100 g. of dry felt	Fat, g./100 g. of sugar utilized
$NaH_2PO_4,2H_2O$, g./100 ml.	$MgSO_4,7H_2O$, g./100 ml.	C.S.L., g./100 ml.					
0·036	0·037		1·95	0·58 (± 0·03)	0·06	10·4	3·1
0·036	0·050		1·99	0·52 (± 0·025)	0·08	15·4	4·0
0·036	0·500		2·40	0·73 (± 0·03)	0·20	27·4	8·3
0·073	0·037		2·43	0·79 (± 0·05)	0·13	16·4	5·3
0·073	0·050		2·47	0·80 (± 0·02)	0·16	20·1	6·4
0·073	0·500		2·52	± 0·75 (± 0·02)	0·18	24·0	7·1
0·730	0·037		2·96	0·94 (± 0·04)	0·26	27·7	9·0
0·730	0·050		2·98	0·96 (± 0·04)	0·28	29·2	9·3
0·730	0·500		2·87	0·93 (± 0·07)	0·38	41·9	13·2
0·036	0·500	0·500	3·73	1·30 (± 0·10)	0·38	29·2	10·2
0·073	0·500	0·500	3·83	1·30 (± 0·08)	0·33	25·4	8·6
0·730	0·500	0·500	3·70	1·21 (± 0·09)	0·38	31·4	10·3

The final pH values of the cultures lay between 5·2 and 6·5 without C.S.L., and between 5·5 and 6·5 when C.S.L. was used

Table II

Formation of fat by P. javanicum *in surface culture*
25 ml. in 100-ml. conical flasks; 9 days at 25°; initial sucrose 4·25 g./flask

Basal medium with addition of			Sucrose utilized/ flask, g.	Mean felt wt., g.	Fat wt., g.	Fat, g./100 g. of dry felt	Fat, g./100 g. of sugar utilized
$NaH_2PO_4,2H_2O$, g./100 ml.	$MgSO_4,7H_2O$, g./100 ml.	C.S.L., g./100 ml.					
0·036	0·037		3·98	0·98 (± 0·07)	0·26	26·5	6·5
0·036	0·050		3·99	1·07 (± 0·07)	0·28	26·0	7·0
0·036	0·500		3·33	0·83 (± 0·06)	0·17	21·5	5·1
0·073	0·037		4·03	1·12 (± 0·03)	0·30	26·6	7·4
0·073	0·050		4·05	1·16 (± 0·03)	0·32	28·0	7·9
0·073	0·500		4·18	1·08 (± 0·04)	0·26	24·4	6·3
0·730	0·037		3·98	0·96 (± 0·07)	0·22	22·9	5·5
0·730	0·050		3·69	1·19 (± 0·08)	0·22	18·3	5·9
0·730	0·500		3·78	0·99 (± 0·07)	0·26	26·3	7·0
0·036	0·500	0·500	4·25	0·77 (± 0·04)	0·13	16·9	3·0
0·073	0·500	0·500	4·25	0·96 (± 0·05)	0·16	16·6	3·7
0·730	0·500	0·500	4·25	1·15 (± 0·08)	0·25	21·7	6·0

The final pH values of the cultures lay between 3·1 and 2·5 without C.S.L., and between 5·5 and 6·7 when C.S.L. was used

P. javanicum

Results for this organism are shown in Table II. It is probable that the medium most suitable for promoting fat synthesis in this mould is the one that contains phosphate at 0·073 g./100 ml. and magnesium sulphate at 0·050 g./100 ml. The use of C.S.L. was detrimental in two out of the three instances in which it was added to the medium. In the third instance, addition of C.S.L. resulted in formation of more felt but did not raise the yield of fat.

P. spinulosum

As seen from Table III, the greatest percentage weight of fat on felt as well as the greatest actual weight of fat was afforded when both phosphate and magnesium sulphate were present at their lowest levels. Addition of C.S.L. to the media was beneficial in all cases, its use resulting in greater consumption of sugar and in notable enhancement of the yields of felt and of fat in the felt. This enhancement could not have been caused by the additional quantities of phosphate and of magnesium brought into the media *via* the C.S.L. because *P. spinulosum* gave better yields of fat at the lower levels of these two ingredients; so it may be presumed that in corn-steep liquor there is present some other factor, at present unknown, which is capable of stimulating both growth and fat synthesis in this mould.

Table III

Formation of fat by P. spinulosum *in surface culture*
25 ml. in 100-ml. conical flasks; 9 days at 25°; initial sucrose 4·25 g./flask

Basal medium with addition of			Sucrose utilized/ flask, g.	Mean felt wt., g.	Fat wt., g.	Fat, g./100 g. of dry felt	Fat, g./100 g. of sugar utilized
$NaH_2PO_4,2H_2O$, g./100 ml.	$MgSO_4,7H_2O$, g./100 ml.	C.S.L., g./100 ml.					
0·036	0·037		3·09	0·78 (± 0·09)	0·16	20·5	5·1
0·036	0·050		2·95	0·89 (± 0·05)	0·115	12·9	3·9
0·036	0·500		3·22	0·86 (± 0·05)	0·13	15·0	3·9
0·073	0·037		3·15	0·77 (± 0·01)	0·08	10·3	2·5
0·073	0·050		3·27	0·96 (± 0·06)	0·10	10·3	3·0
0·073	0·500		3·41	0·85 (± 0·05)	0·09	10·5	2·6
0·730	0·037		3·52	0·82 (± 0·04)	0·12	14·6	3·4
0·730	0·050		3·66	1·15 (± 0·03)	0·11	9·6	3·0
0·730	0·500		3·62	0·88 (± 0·04)	0·11	12·5	3·0
0·036	0·500	0·500	4·13	1·25 (± 0·06)	0·25	19·7	6·0
0·073	0·500	0·500	4·12	1·26 (± 0·07)	0·22	17·4	5·3
0·730	0·500	0·500	4·19	1·29 (± 0·06)	0·22	17·1	5·3

The final pH values of the cultures lay between 2·5 and 3·4 without C.S.L., and between 3·4 and 5·1 when C.S.L. was used

Summing up, *A. nidulans* is the most effective of these three moulds as a producer of fat from sucrose, the best yield of fat from *A. nidulans* being appreciably greater than the highest yield afforded by *P. javanicum* and more than double that from *P. spinulosum*. The experiments have also shown that, whereas *A. nidulans* requires for fat synthesis a medium very rich in PO_4^{3-} and Mg^{2+}, moderate concentrations of these ions are more favourable to formation of fat in *P. javanicum*, and to obtain the best results from *P. spinulosum*, phosphate and magnesium sulphate must each be present in very low concentration. Similar relationships between the levels of these ions and the abilities of these three moulds to synthesize fat from glucose had been noted in earlier work by Garrido & Walker,[3] from whose work the following figures are taken. In a comparison of the suitabilities of sucrose and glucose as substrates for the production of fat by the three moulds, glucose is superior to sucrose, the respective

yields of fat per 100 g. of carbohydrate utilized being: *A. nidulans*, from glucose 17·2 g., from sucrose 13·2 g.; *P. spinulosum*, from glucose 9·6 g., from sucrose 5·1 g.; *P. javanicum*, from glucose 8·5 g., from sucrose 7·9 g. Experiments carried out recently in this Laboratory, but not yet published, have indicated that, with some moulds, fructose is less suitable than glucose as a source from which fat can be elaborated, and this finding may be related to the yields of fat from glucose and from sucrose that are cited above.

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Division of Industrial Biochemistry
Faculty of Technology
University of Manchester

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CONNECTIVE TISSUE OF MEAT. IV.*—Comparison of Methods for Determining Collagen in Meat

By L. H. LAMPITT, L. C. BAKER and K. P. BROWN

Collagen was determined in samples of fresh, frozen and freeze-dried silverside by determining hydroxyproline in the acid hydrolysate of (i) the meat, (ii) the direct aqueous autoclave extract of the meat, and (iii) the aqueous autoclave extract of the residue left after preliminary extraction of the muscle tissue with dilute alkali or acid; collagen was also determined in (iii) as the loss of weight when the residue was autoclaved with water.

In Part III of this series a comparison was made of methods for determining collagen in tendon dissected from shin of beef and veal, using in particular tendon prepared in the form of defatted, freeze-dried powder. It was concluded that the most accurate estimation of collagen was obtained by the determination of hydroxyproline in the acid hydrolysate of an aliquot of the aqueous autoclave extract of the tendon powder; any elastin present in the tendon should remain undissolved.

It was considered doubtful whether a similar method could be applied successfully to meat. When meat was autoclaved with water approximately half the solids dissolved; an aliquot of the aqueous extract, therefore, contained a large amount of non-collagenous protein and the amino-acids derived from this protein on hydrolysis would, it was expected, be so much in excess of the hydroxyproline that the result would be a lowering of the density of the colour developed (see Baker *et al.*[2]). On the other hand it was known that if meat were first extracted in a blender with 0·1N-sodium hydroxide solution to remove the bulk of the muscle protein, a little collagen would be dissolved at the same time. For tendon powder, however, it was found that less collagen was dissolved when the powder was (*b*) blended with water and then made alkaline or (*c*) stirred up with 0·1N-sodium hydroxide solution rather than when it was

* Part III: *J. Sci. Fd Agric.*, 1954, **5**, 226

(*a*) blended with 0·1N-sodium hydroxide solution. It was therefore possible that extraction of meat by method (*b*) or (*c*), and subsequent determination of hydroxyproline in the acid hydrolysate of an aqueous autoclave extract of the residue, might constitute the most satisfactory method of determining collagen in meat. The investigation of these various possibilities forms the subject of the present paper.

A major difficulty in the analysis of meat is the preparation of a homogeneous sample; the results for replicate determinations of collagen in finely chopped meat showed greater variation than was desirable. For this reason analyses were also made on samples of powder prepared by freeze-drying finely chopped meat with subsequent grinding; some of the powder was extracted with ether to give samples of defatted, freeze-dried meat powder.

In Part I of this series[3] dilute hydrochloric acid was found to be an unsatisfactory reagent for the extraction of muscle protein from meat because it failed to extract the protein completely. The use of dilute hydrochloric acid for dissolving muscle protein was, however, recommended by Bate-Smith.[4, 5] It was suggested that a possible reason for this contradiction was that Bate-Smith used freshly killed material, whereas, in the experiments described in Part I, commercial meat (stored for an unknown period in the frozen state) was used. Experiments are now reported in which dilute hydrochloric acid and dilute sodium hydroxide are compared for extracting muscle protein from silverside from a freshly slaughtered animal.

Experimental

Materials

The meat (mostly silverside), after removal of any surface skin or fat, was prepared for analysis as described in Part I of this series.[3] Some of the finely chopped meat was freeze-dried and passed through a Raymond (hammer) mill, using a $\frac{1}{16}$-in. sieve; part of this dry powder was extracted with ether in a Soxhlet apparatus and then exposed to the air to allow the solvent to evaporate. The fat extracted from the powder was estimated and from this and the determined moisture contents of the meat, freeze-dried powder and defatted freeze-dried powder the results could be calculated on a non-fatty-solids basis.

Methods

(i) *Direct acid hydrolysis of meat.*—Finely chopped meat is difficult to dissolve in a limited amount of acid. In the experiments described, 10 g. of this material was boiled under reflux with 40 ml. of 6N-hydrochloric acid for about 4 hours; in this time solution was complete except for the separation of humin. The suspension was diluted with 6N-hydrochloric acid to 50 ml. and a representative 5 ml. was autoclaved in a sealed tube for 6 hours at 25 lb. pressure. The acid hydrolysate, after cooling, was neutralized, diluted to 25 ml. and a 1-ml. aliquot used for colour development.

(ii) *Autoclaving with water followed by acid hydrolysis of the aqueous extract.*—Two methods were used for obtaining an aqueous autoclave extract.

(*a*) Finely chopped meat, 10 g., was suspended in 100 ml. of water and heated with stirring to coagulate the muscle protein. The suspension was autoclaved for 6 hours at 25 lb. pressure and the clear extract decanted through glass wool into a 200-ml. graduated flask; the residue was autoclaved for a further 2 hours at 25 lb. pressure with 50 ml. of water and the extract decanted into the flask. The residue was washed with water and the extract and washings were diluted to 200 ml.

(*b*) Finely chopped meat, 10 g., was treated in the blender with 150 ml. of water for 30 seconds and the suspension washed into a beaker with a further 100 ml. of water. The suspension was heated with stirring to coagulate the muscle protein and then autoclaved for 8 hours at 25 lb. pressure. The extract was decanted through glass wool into a 250-ml. graduated flask and the residue was washed with water. The washings were added to the extract in the flask, which was then diluted to 250 ml.

For the determination of hydroxyproline, 20 ml. of each of the extracts was, after acidification, evaporated to dryness and the residue autoclaved with 2 ml. of 6N-hydrochloric acid for 6 hours at 25 lb. pressure in a sealed tube. After being cooled and neutralized the hydrolysate was diluted to 25 ml. with water and 1-ml. aliquots were used for colour development.

(iii) *Acid hydrolysis of the aqueous extract obtained by autoclaving with water the residue left after preliminary extraction of meat with a reagent to remove muscle protein.*—The preliminary extraction of the meat with a reagent to remove muscle protein was carried out as described previously (cf.[3], p. 371). In the next stage of the determination, namely the extraction of the residue by autoclaving with water (to convert the collagen into soluble gelatin), it is now preferred to use two autoclaving treatments, each of 3 hours' duration, rather than one treatment for 6 hours. Weighing the dried residue before and after the autoclaving treatments gave the weight of ' collagen ' ; the use of 0·1N-sodium hydroxide solution for the preliminary extraction conformed to the modified method of Lowry, Gilligan & Katersky. Determination of hydroxyproline in the aqueous autoclave extract by the method already described also gave a result for the collagen content of meat.

For the extraction of muscle protein, cold 0·1N-sodium hydroxide solution was the reagent most generally used but some comparative experiments were made with hydrochloric acid at pH 2·0. Alternative methods of treating the meat with the reagent were also investigated: (*a*) The meat was blended for 30 sec. with 0·1N-sodium hydroxide solution. (*b*) The meat was blended for 30 sec. with water and concentrated sodium hydroxide solution was then added to give a final strength of 0·1N. (*c*) The meat was rubbed up (not blended) with 0·1N-sodium hydroxide solution.

Results

(*i*) *A comparison of results obtained by methods* (i), (ii) *and* (iii)

The meat used for this experiment was a piece of silverside, finely chopped ; the results are given in Table I.

Table I

Comparison of collagen contents of meat by methods (i), (ii) *and* (iii) *; results are expressed as percentages of meat solids*

Direct acid hydrolysis	Aqueous autoclave extract					
	After extraction with 0·1N-NaOH					
I	II	III		IV		V
	No preliminary extraction	Blended for 30 sec. with NaOH		Blended for 30 sec. with water, and NaOH then added		NaOH stirred into meat
By H-P detmn.	By H-P detmn.	By H-P detmn.	Wt. diff.	By H-P detmn.	Wt. diff.	By H-P detmn.
2·74	2·76	2·97	3·17	3·04	2·98	
2·98	2·77	3·01	3·07	3·48	3·70	
2·78	2·17	2·79*				
2·68	2·55	2·69*				
		3·31*				3·00*
		3·41*				2·95*
Average						
2·80 $^{+\ 0\cdot18}_{-\ 0\cdot12}$	2·56 $^{+\ 0\cdot21}_{-\ 0\cdot39}$	3·03 $^{+\ 0\cdot38}_{-\ 0\cdot34}$	3·12 ± 0·05	3·26 ± 0·22	3·34 ± 0·36	2·98 ± 0·02

H-P : Hydroxyproline

* Residue from preliminary extraction not dried, weighed and autoclaved with water, but dissolved in 6N-hydrochloric acid and hydrolysed under pressure

It will be seen that for finely chopped meat some of the individual results varied by more than ± 10% from the mean. However, the mean results by methods (i) and (ii)—i.e. columns I and II—were lower than those obtained by method (iii) and by estimating hydroxyproline ; this confirmed the view that in methods (i) and (ii) the presence of excessive amounts of amino-acid derived from muscle protein resulted in a lowering of colour density in the hydroxyproline estimation.

(*ii*) *A comparison of results on fresh and freeze-dried meat by methods* (i), (ii) *and* (iii)

Two samples of silverside were used for this experiment and the analytical results are recorded in Table II.

Table II

	Total solids of fresh meat	Freeze-dried meat		Defatted freeze-dried meat solids	Non-fatty solids (g.) in	
		Solids	Fat		fresh meat, 10 g.	defatted freeze-dried meat, 2·5 g.
Sample I	24·9	89·5	9·2	87·9	2·23	2·2
Sample II	28·6	92·6	19·3	88·1	2·26	2·2

The results obtained in the determination of collagen are recorded in Table III.

Table III

Comparison of collagen contents of fresh and freeze-dried meat; results are expressed as percentages of non-fatty solids

Direct acid hydrolysis By H-P detmn.	Aqueous autoclave extract					
	No preliminary extraction By H-P detmn.		After blending with 0·1N-NaOH for 30 sec.			
			By H-P detmn.		Wt. diff.	
I	II	III	IV	V	VI	VII
Defatted freeze-dried meat	Defatted freeze-dried meat	Freeze-dried meat	Fresh meat	Defatted freeze-dried meat	Fresh meat	Defatted freeze-dried meat
1 4·76	4·45	4·05	3·24	4·41	3·98	5·97
		4·05	3·75	4·45	3·87	14·0
			3·16		3·36	
Average			3·38	4·43	3·74	10·0
2 4·62	4·60	4·70	4·31	4·50	4·93	5·91
		4·80	4·02	4·57	4·25	6·33
			4·29		4·38	
Average			4·21	4·53	4·52	6·12

The results obtained by the hydroxyproline method indicate: (*a*) that better duplicates were obtained with freeze-dried or defatted freeze-dried meat (columns III and V) than with fresh meat (column IV); (*b*) that with defatted freeze-dried meat satisfactory agreement was obtained by methods (i), (ii) and (iii)—(columns I, II and V); and (*c*) that the results obtained with fresh meat were lower than those obtained with defatted, freeze-dried meat.

(*iii*) *A comparison of the results obtained by using acid and alkaline reagents for the extraction of muscle protein in method* (iii)

(*a*) *Fresh and frozen meat.*—For this experiment a part of the silverside was taken from a beef carcass within a few hours of slaughter in order to compare the efficiencies of dilute acid and dilute alkali for the extraction of muscle protein from freshly slaughtered animals. The silverside was chopped and the following reagents were used for extracting the muscle protein by treatment in a blender for 30 seconds: hydrochloric acid at pH 2, 0·1N-hydrochloric acid and 0·1N-sodium hydroxide solution. In order that the acidity of the extracting acid should be at pH 2, 10 g. of finely chopped meat was initially blended with 150 ml. of 0·03N-hydrochloric acid, using a further 100 ml. of 0·03N-hydrochloric acid for washing out the container; the residue left after centrifuging was washed with 0·01N-hydrochloric acid. The results for collagen are given in Table IV.

From the results obtained by estimation of hydroxyproline it can be seen that: (i) The replication of results was reasonable except occasionally when a result notably higher or lower than the average was obtained; this appears to be characteristic for fresh or frozen meat and is attributed to sampling difficulties. (ii) The results obtained on the fresh meat and on the meat stored frozen for 6 weeks were in reasonable agreement.

Of the results obtained by weight difference, those for fresh meat after extraction of muscle protein by blending with 0·1N-sodium hydroxide solution (average 3·74%) were in reasonable agreement with those obtained by estimation of hydroxyproline, but those obtained after extraction with hydrochloric acid were higher than those obtained by estimation of hydroxyproline, somewhat higher for hydrochloric acid at pH 2·0 (average 4·11%), and much higher for 0·1N-hydrochloric acid (average 10·8%). From this comparison it is concluded that

Table IV

Comparison of results on freshly killed and frozen stored meat, with acid and alkaline reagents for preliminary extraction; collagen contents are expressed as percentages of non-fatty solids

		Direct acid hydrolysis	Aqueous autoclave extract									
			No preliminary extraction	Preliminary extraction								
				By hydroxyproline detmn.					By weight difference			
		H-P	H-P	0·01N-HCl	0·1N-HCl	0·1N-NaOH	Water then NaOH	Stirred with NaOH	0·01N-HCl	0·1N-HCl	0·1N-NaOH	Water then NaOH	Stirred with NaOH
Freshly killed meat	1			3·12	3·77	3·19			3·98	10·7	3·51		
	2			3·36	3·63	3·92			4·08	10·2	4·04		
	3			3·43	3·80	3·48			4·27	10·4	3·76		
	4			3·36	3·65	3·27			4·12	12·0	3·64		
Average				3·32	3·71	3·46			4·11	10·8	3·74		
Meat stored frozen for 6 weeks	1			3·65		3·76			4·42		4·17		
	2			3·61		3·52			4·47		4·10		
Average				3·63		3·64			4·45		4·13		
Meat stored frozen for 6 months	1	4·87	3·42			4·05	4·26	3·35			4·10	5·12	4·46
	2	4·10	3·17			3·57	4·24	6·28			3·73	4·69	7·45
	3	4·64	3·94			3·88	4·21	4·30			4·44	4·84	4·92
	4	3·46											
Average		4·27	3·51			3·83	4·24	4·64			4·09	4·88	5·61

hydrochloric acid at pH 2·0 or at 0·1N is not suitable for the extraction of muscle protein when collagen is to be estimated by the weight-difference method.

After the meat had been stored in the frozen state the percentage of collagen found by all methods tended to rise. The rise in the results by the weight-difference method was quite likely to be due to an increase in the non-collagenous material not removed by the preliminary extraction with acid or alkali, but which became soluble on autoclaving with water. The rise in the results obtained by the hydroxyproline method is less easy to understand; it may be that the small amount of collagen dissolved in the preliminary treatment with the reagents for extracting muscle protein becomes less, or that the collagen is more completely dissolved on autoclaving with water. In this connexion it is interesting to note that, in the results obtained on the meat stored for 6 months, those obtained by direct acid autoclaving are higher than those obtained on aqueous autoclave extract (see also Table I), suggesting that the dissolution of the collagen on autoclaving is not quite complete.

(*b*) *Freeze-dried meat powder.*—The results obtained are given in Table V.

Comparing the results obtained on freeze-dried meat (not defatted) by the hydroxyproline method, (*a*) with those obtained on freshly killed meat (Table IV), fairly good agreement was obtained: after blending with acid at pH 2·0, 3·23% compared with 3·32%, after blending with 0·1N-sodium hydroxide solution, 3·78% compared with 3·46%; and (*b*) with those obtained on meat kept frozen for 6 months (Table IV), agreement was also fairly good: after blending with 0·1N-sodium hydroxide solution, 3·78% compared with 3·83%, and after stirring with 0·1N-sodium hydroxide solution, 4·48% compared with 4·64%. These results on freeze-dried and frozen stored meat indicate that more collagen is dissolved in 0·1N-sodium hydroxide solution after blending for 30 seconds than after stirring with the alkali.

As to the results obtained on the defatted freeze-dried meat by the hydroxyproline method, it is remarkable that after preliminary extraction by blending with acid or alkali they were consistently higher than those for freeze-dried meat (not defatted), e.g. after extraction with 0·1N-sodium hydroxide solution, 4·32% compared with 3·78%. This result suggests either that the collagen in the defatted powder is less soluble during blending with acid or alkali or that it is more completely solubilized during the aqueous autoclave treatment. On the other hand, the results obtained for both types of powder after extraction by stirring with 0·1N-sodium

Table V

Comparison of results on freeze-dried meat, before and after defatting, with acid and alkaline reagents for preliminary extraction; collagen contents are expressed as percentages of non-fatty solids

	Aqueous autoclave extract of residue from treatment with extractant				
	Acid at pH 2·0		With 0·1N-NaOH		
			Blending		Stirring
	H-P	Wt. diff.	H-P	Wt. diff.	H-P
Freeze-dried meat	3·25	4·07	3·76	3·78	4·49
	3·22	3·89	3·81	3·89	4·52
					4·34
					4·59
Average	3·23	3·98	3·78	3·83	$4·48^{+0·11}_{-0·14}$
Defatted, freeze-dried meat	4·20	11·0	4·35	6·66	4·45
	4·18	15·6	4·30	7·25	4·61
					4·15
					4·32
					4·59
					4·69
Average	4·19	13·3	4·32	6·96	$4·47^{+0·22}_{-0·32}$

hydroxide are virtually the same, and higher than those obtained after blending. This suggests that the important factor is loss of collagen during blending.

Finally, the results obtained by weight difference are consistently higher than those obtained by the hydroxyproline method, indicating that some non-collagenous material is not extracted by the acid or alkaline reagents but is solubilized on autoclaving with water. The results for defatted meat powder are very much higher than any of the others, suggesting that the defatting treatment renders some of the muscle protein insoluble in dilute acid or alkali.

(*iv*) *The effect of defatting on the collagen found in freeze-dried meat*

In the previous section the figures quoted show that the results obtained for freeze-dried meat after defatting were higher than those obtained for freeze-dried meat before defatting, no matter which method of determining collagen was employed. However, the results referred to had all been obtained by method (iii), in which the meat powder was submitted to preliminary extraction with acid or alkali. It was considered desirable to test this point again and to include determinations by methods (i) and (ii). The experiment was carried out with powders prepared from a silverside roll of total solids content 27·4% and non-fatty solids 23·1%. The results obtained are recorded in Table VI.

The results obtained by weight difference show clearly that although some non-collagenous material is insoluble in the reagents used for extracting freeze-dried meat, resulting in higher results for collagen than by the hydroxyproline method, the defatting process renders still more non-collagenous protein insoluble in the extractants, giving still higher results.

The results obtained by the hydroxyproline method are mostly lower than those obtained by weight difference; only two results on the defatted meat powder are markedly higher than those obtained on the non-defatted meat powder, namely the result obtained after blending with 0·1N-sodium hydroxide solution and also after stirring with 0·1N-sodium hydroxide solution.

Summary

(1) The collagen in a sample of meat was determined by the following methods:

(i) direct acid hydrolysis and determination of hydroxyproline;

(ii) acid hydrolysis of the aqueous autoclave extract and determination of hydroxyproline;

(iii) acid hydrolysis of the aqueous autoclave extract of the residue left after extraction of the meat (*a*) by treatment in a blender for 30 sec. with 0·1N-sodium hydroxide solution, (*b*) by treatment in a blender for 30 sec. with water, followed by addition of more concentrated sodium hydroxide to a final concentration of 0·1N, (*c*) by addition of 0·1N-sodium hydroxide solution slowly with stirring to give a homogeneous paste, (*d*) by treatment in a blender for 30 sec. with hydrochloric acid at pH 2, and (*e*) by treatment in a blender for 30 sec. with 0·1N-hydrochloric

Table VI

Further comparison of results obtained on freeze-dried meat before and after defatting; collagen contents are expressed as percentages of non-fatty solids

	Direct acid hydrolysis	Aqueous autoclave extract of residue after extraction								
		No preliminary extraction	Blended with HCl at pH 2 H-P	0·1N-NaOH			Blending with HCl at pH 2	0·1N NaOH		
				Blending H-P	Water blending H-P	Stirring H-P		Blending	Water blending	Stirring
							Wt. diff.	Wt. diff.	Wt. diff.	Wt. diff.
Freeze-dried		3·29				3·15				4·65
		3·32				3·38				5·02
						3·23				4·98
Average		3·30				3·25				4·88
Defatted freeze-dried						3·66				5·14
						3·42				4·96
						3·40				4·97
Average						3·49				5·02
Freeze-dried*	3·31	3·56	3·42	3·43	3·38	3·44	5·13	4·10	4·28	4·06
	3·68	3·16	3·13	3·42	3·57	3·32	5·30	3·93	4·30	3·75
Average	3·49	3·36	3·26	3·42	3·48	3·38	5·25	4·01	4·29	3·90
Defatted freeze-dried*	3·24	3·64	2·88	4·26	3·57	3·46	8·56	5·47	4·19	4·68
	3·28	3·46	3·43	4·10	3·18	3·97	9·10	5·04	4·06	5·19
Average	3·26	3·55	3·15	4·18	3·38	3·71	8·83	5·25	4·12	4·93

* Ground in Raymond mill ($\frac{1}{4}$-in. mesh)

acid. In all the variations of this method the collagen was determined as hydroxyproline in the acid hydrolysate and also by the difference in weight of the residue left before and after autoclaving with water.

(2) The results obtained for fresh meat (and for meat stored frozen) are considered first. The extent of deviation from the average results on replicate samples is generally not greater than $\pm$ 13%. To what extent this degree of variation is due to sampling errors and to what extent it is due to defects in the methods of determination it is not possible to say, but the degree of variation does make it difficult to draw conclusions from the results presented. Results not significantly different from one another were obtained by determination of hydroxyproline in methods (iii*a*), (iii*d*) and (iii*e*), and by the determination of weight difference in method (iii*a*); significantly higher results were obtained by methods (iii*d*) and (iii*e*), when collagen was determined by weight difference, presumably owing to failure of the acid to extract all the muscle protein and to the dissolution of some of the muscle protein on autoclaving with water. The most satisfactory method for the determination of collagen in meat is considered to be (iii*a*) and determination of hydroxyproline.

(3) The results obtained for freeze-dried and defatted freeze-dried meat indicate that these materials require further study, although it is clear that methods (iii*a*), (iii*b*), (iii*c*) and (iii*d*) and determination of weight difference give high results, particularly for the defatted material.

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The Lyons Laboratories
149 Hammersmith Road
London, W.14

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THE UTILIZATION OF CARBOHYDRATES OF SEAWEED BY RUMEN MICROFLORA *IN VITRO*

By M. L. McNAUGHT,* J. A. B. SMITH and W. A. P. BLACK

Tests have been made to determine whether some of the carbohydrates of the brown algae and of dried milled samples of *Laminaria cloustoni* and *Ascophyllum nodosum* can be utilized by the mixed microflora of the bovine rumen when incubated in rumen liquid for 4 h. at 39°. The conversion of non-protein nitrogen into protein and changes in pH were used as indications of utilization.

Of the various products that were tested, only laminarin and the frond of *L. cloustoni*, which contains laminarin, promoted the type of vigorous activity obtained under the same conditions with maltose.

It was shown by Pearson & Smith[1, 2] that when soluble starch, D-(+)-galactose, sucrose, maltose, lactose and D-(+)-glucose were added to bovine-rumen liquid during incubation *in vitro* vigorous bacterial proliferation occurred and resulted in the predomination of protein synthesis over protein breakdown. The amount of non-protein nitrogen (N.P.N.) converted into protein was used as a measure of the bacterial activity in the presence of the various carbohydrates that were tested. Later, McNaught[3] extended the work to include tests on some other naturally occurring carbohydrates and their derivatives. The polysaccharides starch and inulin, the trisaccharide raffinose, and several disaccharides, hexoses and pentoses were found to be readily used by the rumen bacteria, but since oxidation products of glucose, such as gluconic and glycuronic acids, and reduction products, such as mannitol and alginic acid, produced no measurable activity, it appeared that both a potential aldehyde and a primary alcohol group were essential. Molecular configuration was also important, since fructose was readily fermented whereas sorbose showed no activity. Similarly, L-(+)-arabinose gave marked bacterial growth, whereas with D-(−)-arabinose activity was slight.

It has long been the custom in some countries to include small proportions of seaweed in cattle foods, and much work is now being done in Britain on the chemical constitution and nutritive value of seaweeds and their constituents.[4] One of the most plentiful seaweeds in British waters is the brown alga, *Laminaria cloustoni*. Seasonal variations in the growth and composition of this weed were discussed recently by Black.[5] On a dry-matter basis the frond may contain 5 to 37% of mannitol and as much as 36% of laminarin, which is a polysaccharide composed of about twenty β-D-glycopyranose units linked through carbon atoms 1 and 3. It also contains the cell-wall constituents, alginic acid, fucoidin (which is believed to be a polyfucose monosulphate) and cellulose. In the earlier work, McNaught[3] found that the rumen microflora did not appear to utilize mannitol or alginic acid when these substances were incubated in rumen liquid for 4 h. at 39°. It was therefore decided to test laminarin, fucoidin and L-fucose itself, and to determine whether the mixed flora of the rumen can utilize these substances to such an extent that protein synthesis from N.P.N. predominates over protein breakdown when they are incubated with rumen liquid at 39°. An attempt was also made to determine whether the carbohydrates can be utilized when they are added to the rumen liquid in the form of the dried milled weed. For this purpose *Ascophyllum nodosum* and both the fronds and the stipes of

* Present address: National Institute for Research in Dairying, University of Reading

Table I

The composition of the three seaweed products used for the incubation tests

	Moisture, %	Total ash	Mannitol (% of dry matter)	Laminarin (% of dry matter)	Crude protein
L. cloustoni, frond	9·3	20·1	15·5	27·1	7·0
L. cloustoni, stipe	9·3	34·9	4·1	Nil	8·9
A. nodosum	11·1	27·5	7·2	4·4	8·4

L. cloustoni were used. The figures in Table I show that the *A. nodosum* contained about 4% of laminarin, whereas the fronds of *L. cloustoni* contained about 27% and the stipes no measurable amount.

Experimental

The method of obtaining and incubating samples of fresh rumen liquid has already been described.[1-3, 6] Most of the protozoa were removed by light centrifugation before incubation began. Urea (0·05%) was added to all samples of rumen liquid to ensure that lack of N.P.N. did not limit bacterial activity. The carbohydrates or the dried milled seaweeds (Table I) were added at a level of 1%, and incubation lasted for 4 h. at 39°. Samples from the incubation flasks were preserved before and after incubation for chemical analysis. The amount of protein synthesis that occurred was taken as a measure of bacterial activity, the protein nitrogen and N.P.N. being partitioned by the sodium tungstate procedure of Pearson & Smith.[1] Records were also kept of pH, since increase in acidity has been found to be a reliable indication of rapid carbohydrate utilization in this particular type of incubation.

Samples of laminarin, fucoidin and L-fucose were prepared by the methods already reported[7-9] and the appropriate amounts dissolved in water and added to the rumen liquid. When the dried milled seaweeds themselves were used, the desired amount was put in an incubation flask, some cold water added and the mixture allowed to stand for 15–45 min. to ensure that the weed was thoroughly wetted. On some occasions duplicate flasks that had been prepared in this way were heated for a similar period on a boiling-water bath in an endeavour to extract and make as available as possible any utilizable carbohydrate that the seaweed might contain. The requisite amount of rumen liquid with 0·05% urea was then added to the cooled flasks and the contents were thoroughly mixed. In all experiments a control flask with rumen liquid containing 1% of maltose was included, and in some experiments a further control flask containing rumen liquid to which no carbohydrate had been added was also included. It was therefore possible to compare the effects produced by the carbohydrates of the seaweed with the well-established maltose effect. It was also possible to determine whether substances that did not stimulate protein synthesis, increased protein hydrolysis by comparison with the control in which no carbohydrate had been added.

Results

The results are summarized in Table II.

Discussion

The first test (Table II) showed that laminarin was utilized by the rumen microflora, though it did not cause as great a synthesis of protein as maltose. Neither fucoidin (test 1 in Table II) nor its constituent L-fucose (test 2) was utilized. This lack of ability of the rumen microflora to use fucoidin and L-fucose might have been due to the presence of some substance that was toxic to the rumen bacteria. When, however, fucose was tested in the presence of maltose in test 3 the utilization of maltose was not diminished. It would appear therefore that something in the structure of fucose mitigates against its utilization. In tests 4 and 5 the frond of *L. cloustoni* supported bacterial activity to an extent about as marked as that of maltose, whereas the stipe of the same seaweed did not give rise to protein synthesis. The presence of the stipe in test 6, however, did not prevent the utilization of maltose.

A. nodosum (test 4) gave very little activity, and when it was incubated in the presence of maltose (test 6) it reduced the activity compared with that obtained with maltose alone, so that it may have contained some constituent with inhibitory properties or possibly a small amount of its own protein may have been hydrolysed by the rumen bacteria.

Table II

The conversion of N.P.N. into protein and the change in pH when seaweeds and carbohydrates from seaweeds were incubated with rumen liquid for 4 h. at 39°

Test no.	Carbohydrate or seaweed added (1% unless otherwise stated)	Protein synthesis (+) or hydrolysis (−) (mg. of N/100 g. of rumen liquid)	Increase (+) or decrease (−) in pH units
1	No addition	+ 0·3	+ 1·0
	Maltose	+ 9·5	− 0·3
	Laminarin	+ 6·2	0·0
	Fucoidin	− 0·1	+ 0·8
2	No addition	− 0·5	+ 0·2
	Maltose	+ 8·8	− 1·2
	Fucose	− 2·3	—
3	Maltose, 0·5%	+ 4·5	− 0·4
	Maltose, 1·0%	+ 5·8	− 0·5
	A mixture of maltose, 0·5%, and fucose, 0·5%	+ 5·0	− 0·75
4	No addition	− 0·5	+ 0·2
	Maltose	+ 8·8	− 1·2
	L. cloustoni frond (cold)*	+ 7·8	− 0·6
	L. cloustoni frond (after heating)*	+ 10·8	− 0·7
	L. cloustoni stipe (cold)	− 2·7	+ 0·1
	L. cloustoni stipe (after heating)	− 2·5	+ 0·2
	A. nodosum (cold)	+ 1·3	+ 0·1
	A. nodosum (after heating)	+ 0·9	+ 0·3
5	Maltose	+ 5·8	− 0·5
	L. cloustoni frond (cold)	+ 3·7	− 0·4
	L. cloustoni frond (after heating)	+ 4·1	− 0·4
	L. cloustoni stipe (cold)	− 2·1	+ 0·8
	L. cloustoni stipe (after heating)	− 3·3	+ 0·7
6	Maltose	+ 12·5	− 1·2
	L. cloustoni stipe (cold)	− 2·6	+ 0·7
	A mixture of maltose and *L. cloustoni* stipe (cold)	+ 12·1	− 1·3
	A. nodosum (cold)	− 0·4	+ 0·3
	A mixture of maltose and *A. nodosum* (cold)	+ 3·6	− 0·6

* For explanation of 'cold' and 'after heating' see Experimental section

It appears from these results that laminarin, which is present in the frond of *L. cloustoni* but absent from the stipe and which is present in only very low concentrations in *A. nodosum*, is the only constituent of these two species of the grown algae so far tested that is readily utilized by the rumen bacteria. Mannitol and alginic acid in earlier work[3] and fucoidin and L-fucose in the present work did not appear to promote protein synthesis in rumen liquid under the conditions used in these tests. This does not mean that these substances cannot be digested over long periods in the rumen in much the same way as cellulose is digested—to study that point would require a different approach. It does mean, however, that of these seaweeds and seaweed products only laminarin has been found to be readily and rapidly utilized under the same conditions that starch and maltose are utilized.

The Hannah Dairy Research Institute
Kirkhill, Ayr

and

Institute of Seaweed Research
Inveresk, Midlothian

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