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Report for 1956

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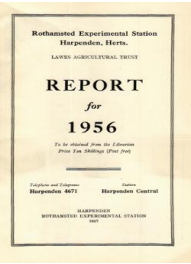
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Biochemistry Department

N. W. Pirie

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BIOCHEMISTRY DEPARTMENT

N. W. PIRIE

The Biochemical Society paid its first visit to Rothamsted and held an all-day meeting here on 25 May. The farm and several departments arranged demonstrations of aspects of their work. N. W. Pirie attended a Conference on Chromosomes at Wageningen and a symposium arranged by the New York Academy of Sciences on Modern Ideas on Spontaneous Generation.

PLANT ENZYMES

Enzymes of bracken (R. H. Kenten)

Thiaminase is present in bracken and other Pteridophytes but absent from a large number of species of higher plants, and it is possible that this difference might be exploited for the development of specific bracken-killing agents.

Thiaminases catalyse the fission of the methylene–quaternary nitrogen bond of thiamine. The fission may be hydrolytic (equation 1), or transfer of the pyrimidine moiety of thiamine to an amine acceptor (equation 2) may take place.

$$P{-}CH_2{-}^{+}T + H_2O \longrightarrow P{-}CH_2OH + T + H^{+} \quad . \quad . \quad (1)$$

$$P{-}CH_2{-}^{+}T + R{\cdot}NH_2 \longrightarrow P{-}CH_2{-}NH{\cdot}R + T + H^{+} \quad . \quad (2)$$

P and T stand for the pyrimidine and thiazole components of thiamine respectively. Thiaminases are known which catalyse both the hydrolytic and transfer reactions or the transfer reaction alone. With extracts of bracken the destruction of thiamine was negligible compared to that found when certain amines were added. Such an effect was most likely due to the added amine taking part in the transfer reaction rather than accelerating hydrolytic fission, but it was clear that this could only be decided unequivocally if a method was available for determining the pyrimidine–amine product. Tests with a variety of amines suggested that bracken thiaminase catalysed the transfer reaction between thiamine and pyridine most readily. This reaction leads to the formation of 1 mol *N*-(-2-methyl-4-aminopyrimidyl-(5))-methylpyridine (heteropyrithiamine) per mol of thiamine destroyed, and can be followed by measuring the rate of formation of heteropyrithiamine. A method of estimating small amounts of heteropyrithiamine in the presence of thiamine has therefore been worked out. It depends on the destruction of thiamine by incubation with strong alkali and subsequent oxidation of heteropyrithiamine to 2-methylpyrichromine by ferricyanide. The 2-methylpyrichromine is estimated spectrophotometrically at 386 mμ. Using this method of determining heteropyrithiamine, conditions suitable for the measurement of bracken thiaminase transfer activity have been established. Under these conditions, with crude bracken extracts in the presence of pyridine, the conversion of thiamine to heteropyrithiamine is virtually quantitative,

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showing that no significant destruction of thiamine by side reactions takes place. The method of determining thiaminase transfer activity has been used successfully with two other ferns, and may prove generally useful with plant and animal extracts. No activity has been found so far in the few species of higher plants examined.

Concentrated and partially purified preparations of thiaminase have been made from water extracts of air-dried bracken fronds by fractionation with ammonium sulphate and calcium phosphate gel. Such preparations are capable of catalysing, in the presence of pyridine, the destruction of 400–1100 mg. thiamine per hour per mg. N. Tests with relatively large amounts of these concentrated preparations in the absence of pyridine, over a wide range of pH, have failed to indicate the presence of a thermolabile factor capable of destroying thiamine. Mn^{2+} has been reported to stimulate the action of certain animal thiaminase preparations, but after incubation with small amounts of Mn^{2+} the bracken thiaminase preparations still gave negative results for hydrolytic activity and the transfer activity was not affected. It can be seen from equations 1 and 2 that when thiaminase catalyses either the hydrolytic fission or the transfer reaction with primary and secondary amines a H^+ is released. With tertiary amines the pyrimidine–amine product retains the quaternary nitrogen configuration and no release of H^+ takes place. The hydrolytic or transfer reaction of thiaminase with primary and secondary amines can be studied manometrically in bicarbonate buffer and N_2–CO_2 atmosphere by measuring the CO_2 output which accompanies the release of H^+. By this technique no evidence of the hydrolytic fission of thiamine by concentrated bracken thiaminase preparations in the absence of added amines was found, but a number of primary and secondary amines have been studied, and the results suggest that among others the amines ethanolamine, tyramine, taurine and piperidine, all of which are known to occur in plant material, can take part in the thiaminase-catalysed transfer reaction. The manometric technique suffers from the disadvantages that only a narrow range of pH can be studied and that the presence of amines can cause CO_2 retention, the extent of which varies with the dissociation constant and amount of the particular amine present. When the amine is strong, e.g., piperidine, or very weak, e.g., aniline, even when large amounts (0·1*M*) are present, CO_2 retention is small and 85–100 per cent of the thiamine destroyed can be accounted for by the evolution of CO_2. In spite of the disadvantages, the method has been studied because it should be possible to test with it the action of thiaminase on compounds of similar structure to thiamine but for which analytical methods are not available. Hence it may prove useful in the studies of the substrate specificity and structural inhibitors of bracken thiaminase which are in hand.

Plant enzyme reactions leading to the formation of heterocyclic compounds (A. J. Clarke and P. J. G. Mann)

We have already reported the formation of unsaturated pyrrolidine and piperidine compounds by oxidation of putrescine, cadaverine and lysine. We now find that the oxidation of agmatine and ornithine, catalysed by the enzyme, results in the formation

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of unsaturated pyrrolidine compounds. The product of ornithine oxidation is probably Δ′-pyrroline-2-carboxylic acid; it is converted to proline by catalytic hydrogenation.

The oxidation of the diamino acids ornithine and lysine is catalysed much less readily by the enzyme than that of the diamines of the same hydrocarbon chain length. It is likely that the carboxyl group inhibits the formation of the enzyme substrate complex probably by repelling another acidic group on the surface of the enzyme. If the carboxyl groups of these amino acids are masked by forming the methyl esters, the rates of oxidation are increased about sevenfold. The products of the oxidation of these esters undergo spontaneous cyclization, yielding the methyl esters of Δ′-pyrroline-2-carboxylic acid and Δ′-piperideine-2-carboxylic acid.

We have already reported evidence suggesting that when the oxidation of putrescine and cadaverine, catalysed by plant amine oxidase, is carried out in the presence of reactive methylene compounds condensation takes place between the reactive methylene compounds and the unsaturated ring compounds formed as a result of the oxidation of the amines. These reactions are of interest, since, with appropriate reactive methylene compounds, the condensations should produce pyrrolidine and piperidine alkaloids. We have now been able to prove that this is the case by the isolation and identification of the products of two such reactions. The two alkaloids so far prepared are norhygrine (2-acetonylpyrrolidine) and *iso*pelletierine (2-acetonylpiperidine), which are formed by the oxidation of putrescine and cadaverine respectively, in the presence of acetoacetate. The results support the suggestion that the formation of pyrrolidine and piperidine alkaloids depends on the spontaneous condensation of plant metabolites with the unsaturated ring compounds.

The oxidation of pyridoxine and allied compounds by peroxidase systems (A. J. Clarke and P. J. G. Mann)

The fact that plant amine oxidase is inhibited by carbonyl reagents has led to the suggestion that a pyridoxal compound may be the prosthetic group of the enzyme. Evidence apparently supporting this suggestion has been obtained by other workers, who have shown that when plant saps containing the amine oxidase are dialysed for several days with the object of removing the prosthetic group, the subsequent rates of oxygen uptake of the dialysed saps in the presence of added amines are increased by the addition of pyridoxal hydrochloride. During attempts to establish the nature of the prosthetic group we have been able to confirm these observations with pyridoxal hydrochloride and have obtained similar effects with pyridoxal phosphate, pyridoxamine dihydrochloride and pyridoxine hydrochloride. Our results suggest that the increased oxygen uptakes are due to oxidation of these compounds rather than to activation of the apo-enzyme of the amine oxidase. This oxidation is brought about by the hydrogen peroxide produced during the oxidation of the amines, and is catalysed by the peroxidase of the plant saps. It results in increased oxygen uptakes, since in absence of the pyridoxine compounds the hydrogen peroxide is decomposed by the catalase of the plant saps. The effect is best

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shown in those plant saps where the ratio of peroxidase to amine oxidase is high. It is not obtained with purified amine oxidase preparations of low peroxidase content, but can be demonstrated with such preparations, in presence of catalase, if peroxidase is added. It is only dependent on the amine oxidase reactions in so far as these constitute a source of hydrogen peroxide. The oxidation of glucose catalysed by glucose oxidase also produces hydrogen peroxide, and with this system, in presence of peroxidase and catalase, the addition of pyridoxine compounds causes increased oxygen uptakes. Finally, in reaction mixtures of the pyridoxine compounds and peroxidase with direct addition of hydrogen peroxide the oxidation can be demonstrated by spectrophotometric methods. These observations diminish the relevance of this piece of evidence that plant amine oxidase is a pyridoxal enzyme. We have so far been unable to establish the nature of the prosthetic group, but we have obtained other indirect evidence suggesting that it contains pyridoxal phosphate. This depends on the fact that the rate of inhibition of the enzyme by *iso*nicotinyl hydrazide is the same as that of glutamic decarboxylase, a known pyridoxal enzyme. Other workers have suggested that the inhibition of pyridoxal enzymes by *iso*nicotinyl hydrazide is due to the formation of pyridoxal phosphate *iso*nicotinyl hydrazone and that the rate of inhibition should be constant for all pyridoxal-requiring enzymes. We still adhere to the view expressed in the Report for 1954 that there is also a metal in the prosthetic group of the amine oxidase.

Polyphosphatase (W. S. Pierpoint)

Gradient elution chromatography on a cation-exchange resin resolves the polyphosphatase from pea leaves into two fractions. Both these fractions are present in dialysed extracts of pea leaves, and therefore are not products of the " purification " procedure to which the extracts are subjected. Nor are they products of the chromatographic procedure itself, for each behaves as a single homogeneous substance when rechromatographed separately. The two fractions have the same pH optimum, are not affected by some metal ions or chelating agents, have similar heat stabilities and differ only qualitatively in their specificities. This suggests that they are not different enzymes, but rather slightly different forms of the same enzyme. The phosphatase is not unique in this respect, since several other biologically active proteins consist of two or more active components. We do not know whether the different forms of these proteins exist *in vivo* or are autolytic artifacts produced during the disintegration of the tissues.

Some attention has been paid to the specificity of the phosphatase in view of its possible use as an analytical tool in the analysis of polyphosphate compounds. It will liberate orthophosphate from tripolyphosphate, adenosine-5′-phosphate (AMP), adenosine-5′-triphosphate (ATP), ribonucleic acid from various sources, a mixture of adenosine-2′- and 3′-phosphates, and a mixture of uridine-2′- and 3′-phosphates. Although it breaks the diester bonds in trimetaphosphate, it will not break those in tetrametaphosphate, diphenylphosphate or the nucleic acids. The enzyme therefore has unspecific phosphomonoesterase activity and a more specific

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diesterase activity, and will be of only limited use as an analytical tool. It is very unlikely that the diesterase activity is due to a contaminating protein, since it cannot be separated from the monoesterase activity by a wide range of protein separation techniques.

Chemical Studies on Materials of Biological Importance

Polyphosphates (W. S. Pierpoint)

A number of phosphorus-containing materials of biological importance, including such diverse materials as pea leaves, bee intestines, phosphate rocks and the excreta of certain insects, have been tested for the presence of inorganic polyphosphates. The method used was a chromatographic one which will separate those phosphates, both linear and cyclic, containing less than six phosphate groups.

The excreta of the Greater and Lesser Wax-Moths, *Galleria mellonella* and *Achroia grisella*, were tested, as they are the only materials derived from the tissues of higher animals in which appreciable amounts of polyphosphate are known to occur. Large amounts of these materials were available from the Bee Department.

The excreta from *G. mellonella* contains about 0·6 per cent of its dry weight as phosphorus, and about $\frac{2}{3}$ of this is water soluble and composed mainly of inorganic polyphosphates. A typical analysis suggests a composition of 14 per cent orthophosphate, 23 per cent pyrophosphate, 44 per cent tripolyphosphate, and the residue of more highly polymerized polyphosphates and unidentified organic phosphates. Although the material from *A. grisella* contained more phosphate on a dry-weight basis (about 0·8 per cent), the composition of the inorganic phosphate fraction was very similar to that of the other insect. This similarity of composition argues against these low-molecular-weight compounds having been formed as a result of bacterial or enzymic decomposition after the excreta were voided. Moreover, the excreta are not a suitable medium for enzymic activity, as they contain only about 1 per cent of water. Direct attempts to observe a polyphosphatase activity in aqueous extracts gave negative results.

It has so far proved impossible to detect any of the smaller polyphosphates in pea-leaf extracts. If they are present at all they must account for less than 2 per cent of the total phosphate present. Similarly, none could be detected in extracts of the ventriculi of honeybees, although these organs are known to contain large numbers of intracellular particles which are very rich in phosphorus.

The organic acid content of sugar-beet leaves (G. H. Wiltshire)

Oxalic acid was found to be present in large quantities in sugar-beet leaves, together with smaller amounts of citric, malic and fumaric acids. Leaves which had been wilted for 1, 3 or 10 days contained the same acids in much the same proportions, but, because of the loss of water by evaporation and of solids by oxidation, wilted leaves contained 2·1 g. oxalic acid per 100 g. fresh weight, as compared with 0·3 per cent in unwilted leaves. Thus an animal fed on

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wilted sugar-beet leaves eats as much or more oxalic acid as one feeding on fresh leaves, and in so far as conclusions can be drawn from the experiment, it would appear unlikely that oxalic acid is the toxin in unwilted sugar-beet tops. It would appear, therefore, that there is no foundation for the widespread belief that oxalic acid is the cause of scouring when sugar-beet leaves are fed to cattle and that the advantage of wilting depends on oxalic acid destruction.

The potato-root eelworm hatching factor (G. H. Wiltshire)

The Nematology Department has provided us with three or four hundred litres of Root Diffusate (R.D.) this year. Most of this was from plants grown in pots of sand watered with nutrient solution, some from plants in soil and some from plants grown in nutrient without sand or soil. The latter was the most active. The average L.A. value (log concentration in arbitrary units) was about 3, and the dry-matter content about 0·46 g./l. If activity is expressed in the same arbitrary units, 1 litre of R.D. of L.A. 3 contains 1,000 activity units, and its specific activity on a dry-matter basis is 2·18 units/mg.

The R.D. was acidified and shaken with 1 g. partly deactivated charcoal/l. The charcoal was recovered on a basket centrifuge, washed, dried and eluted with acetone. An average of 55 per cent of the active material was recovered by concentration of the eluate to dryness, but recovery was occasionally as low as 20 per cent. The proportion of factor adsorbed could be increased from about 90 to 99 per cent by increasing the amount of charcoal to 10 g./l., and was reduced by decreasing the amount to 0·1 g./l. The optimum recovery was got at a rate of 1–2 g./l.

Dried acetone eluate could be stored at room temperature in the desiccator without loss of activity. The specific activity of several preparations ranged from 100 to 1,100 units/mg. Further purification was achieved by solution in dilute hydrochloric acid, transfer to *n*-butanol and return to aqueous sodium bicarbonate. The best preparation had a specific activity of 8,300 units/mg., and produced the same hatch as untreated R.D. at a concentration of $0{\cdot}5 \times 10^{-6}$. This preparation, which is probably not homogeneous, has an apparent pK of 5·1 and equivalent weight about 200. It is soluble in water, and can be transferred from acidified solution into immiscible organic solvents and recovered by extraction into bicarbonate. It migrates towards the anode on paper electrophoresis, and on paper chromatograms moves at the front in several solvents. It can be recovered from chromatographic partition columns, and the activity is associated with a titratable acid in the effluent. Every preparation so far made shows a blue fluorescence under ultra-violet light, but most of the fluorescent material in the acetone eluate is not associated with hatching activity. It appears to have no absorption band between 200 and 300 mμ.

The hatching activity of acid aqueous solutions kept at +2° or at room temperature is reasonably stable, and activity is not much reduced at any pH up to 9, but in 0·1*N*-sodium hydroxide activity is lost within seconds at room temperature and cannot be restored by neutralization. It is probably for this reason that recovery from a weakly-basic anion-exchange resin is better than from strongly

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basic resins. The factor is not adsorbed on cation-exchange resins.

Activity is reduced to one-tenth by boiling in 1*N*-hydrochloric acid for 5 minutes. It is not reduced by warming with sulphurous acid.

Plant Viruses

Factors affecting the infectivity of plant viruses (N. W. Pirie)

The mitochondrial fraction made from tobacco leaves infected with tobacco necrosis virus contains a system that, *in vitro*, robs purified virus of its infectivity. The material that is actually responsible for the disinfection diffuses through cellophane, and it withstands drying and boiling. It is present in the successive supernatant fluids made by sedimenting the mitochondria repeatedly from water. This could happen because the material is slowly released, but it is more probable that it is made continuously by an enzymic process. There is no inactivation if air is excluded or if the mitochondria have been heated. Extracts are ineffective if the mitochondria have been frozen or exposed to chloroform. In the presence of leaf sap there is no inactivation; it is therefore unlikely that the system controls infectivity *in vivo*, but it gives a satisfactory explanation for many of the variations in infectivity that we have already described with this virus.

Mitochondria from uninfected leaves also inactivate but much less strongly; those from leaves infected with tobacco ringspot virus, on the other hand, inactivate both necrosis virus and ringspot virus. None of these preparations seems to affect tobacco mosaic virus. The mechanism of these inactivations is obscure, but they can be simulated by low concentrations of several substances (ascorbic acid, citrate and some aldehydes), and the effect may well not be specific.

From many different laboratories evanescent infectivity has been reported in fragments from tobacco mosaic virus; there is therefore increased interest in the infectivity of particles shorter than the canonical 300 mμ. We confirm the infectivity of some TMV fractions that contain predominantly nucleic acid and find that this infectivity is lost on exposure to leaf ribonuclease. This may mean that nucleic acid can be infective in the absence of other virus components; this has been claimed, but has not been firmly established. Ribonuclease is detectable in all TMV preparations; some TMV fragments inhibit the infectivity of both intact TMV and the infective nucleic acid fractions; the stability of the infectivity of fractions depends greatly on the other components of the system. There are therefore ample factors to create a situation in which the unequivocal interpretation of infectivity results is difficult. It may be wise to regard claims that TMV has been resynthesized from non-infective split products as plausible rather than substantiated. (With F. C. Bawden.)

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The susceptibility of French beans to infection with tobacco necrosis virus (G. H. Wiltshire)

In 69 tests of the effect of darkening on susceptibility over the past three years, there was an increase in 48 and a reduction in 21. This variability has made it difficult to explore the possible connection between the effects of darkening on composition and on susceptibility. Some of it may be ascribed to differences in season and weather, especially to differences in illumination and temperature. An integrating photometer has been constructed, and tests with it are in progress. Temperature differences between darkened and control plants have been reduced by use of a much larger dark hood with adequate ventilation, and where possible, one leaf of each plant has been darkened by covering with aluminium foil and the other leaf used as a control.

There is generally an inverse relation between carbohydrate content and susceptibility in comparisons of older and younger plants on the same date, summer-grown and winter-grown plants, and control *vs.* darkened plants. Over a short period, however, as, for instance, in plants inoculated at different times on the same day and plants kept at elevated temperatures for a few hours, susceptibility may vary in the same sense as carbohydrate content or be apparently unrelated to it. Covering the underside of the leaf with a thin film of gum, which reduced carbohydrate content by blocking the stomata, always reduced susceptibility, but the water content was also increased by this treatment.

It was not possible to increase the carbohydrate of leaves on the plant by more than 20 per cent by infiltration of sugars, probably because the extra sugar was transferred to the stem and roots. Sucrose infiltrated together with potassium phosphate for 3 days before inoculation reduced susceptibility of darkened leaves, but did not alter that of illuminated leaves. Sucrose alone had no effect, whereas glucose produced a small increase in susceptibility. The effect of sucrose with phosphate, together with the effects of darkening and of restriction of carbon dioxide supply, make it likely that susceptibility depends, in part, on the content of some photosynthetic product. It is, however, not directly related to the content of carbohydrate because infiltration of 2-4-dichlorophenoxyacetic acid reduces susceptibility of darkened leaves and at the same time has little effect on carbohydrate content.

Plants kept at constant 24°, 30° or 36° before inoculation were more susceptible and contained less carbohydrate than plants in the uncontrolled glasshouse, which fluctuated between 8° and 38°. Raising the temperature to 36° for as little as 2 hours doubled the number of lesions produced. It might be expected that the temperature and illumination factors would interact, resulting in a daily fluctuation of susceptibility. Such a diurnal cycle, with maxima at 4 a.m. and 4 p.m., was in fact found in the glasshouse without temperature control, but at constant 36° susceptibility declined steadily, and at 24° and 30° the diurnal cycle was less marked. A diurnal cycle of carbohydrate content with a single maximum was not apparently related to the times of maximum susceptibility.

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Large-scale Production of Leaf Protein

(M. Byers, D. Fairclough and N. W. Pirie)

No radical changes in the equipment or in the way in which it is used were made during the summer, but with the end of the cropping season a complete reorganization has been begun so as to make full use of a new shed and new equipment. Rye, wheat, oats, tares, lupins, serradella and birdsfoot trefoil have been added to the list of crops processed. The most interesting feature of the results is the high quality and ready extractibility of the protein from young cereals, even when the crop has a low N content. Thus barley containing only 2·5 per cent N (on the dry matter) gave a yield of protein, containing 11 per cent N, accounting for half the crop N. Work on other species presaged poor yields of mediocre protein from leaf with such a low protein content. Yields as high as this cannot be attained if the water initially in the leaf is relied on to bring out the liberated protein. The effects have therefore been studied of different ratios of crop to added water, and of the addition of alkali and sodium sulphite to the water, on the yield and efficiency of the process. A few figures are collected in the table showing the increased extraction got by adding water.

Table 1

Effect of pulping, with and without added water

					Without Water		With Water		
	Date	Dry matter, %	N on dry matter, %	Pulper speed, r.p.m.	Protein N in the extract crop N, %	Protein N per kw.hr. pulper, g.	Water added per 112 lb. crop, lb.	Protein N in the extract crop N, %	Protein N per kw.hr. pulper, g.
Cocksfoot	15 May	23·8	2·7	950	4·6	11·6	66	33·8	46·0
Grass	31 July	21·5	3·37	950	10·15	10·7	140	49·0	57·2
Lucerne	24 Oct.	25·7	2·16	950	29·55	64·1	60	40·6	78·4
Rye	29 May	21·7	1·5	950	27·6	18·6	65	47·5	55·9

The occasional use of alkaline extraction fluids and the wide range of crops have forced us to control the pH of the extract when it is coagulated and filtered. For easy filtration it should not be more alkaline than pH 5·8 (sugar beet can be 6·5 without added alkali), so that an adjustment before coagulation is often needed. As a routine the protein is pressed until the DM rises to 40 per cent, resuspended in 20 times its weight of water, the pH is adjusted to 4·0 before filtering and pressing again. After this amount of washing less than 1 per cent of the DM of the protein cake can be extracted with cold water.

The protein is compressed into hard blocks that are perishable and have to be kept sterile or under refrigeration. Much of the material has therefore been dried and defatted; it is convenient to combine the processes by using acetone as extractant. This solvent has the advantages of easy separation from water and low boiling point, but losses with our present discontinuous arrangements are high. The extracted lipid is being kept in case anyone has a use for it.

After extraction, the protein contains about its own weight of acetone, and the texture of the final product depends on the condi-

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tions under which this is removed. The best of the many arrangements tried is to pack the protein loosely into a tube that can be warmed and that is connected to a receiver at —20°. At 60–100 mm. pressure the acetone comes off without great shrinkage and hardening of the protein. The final traces are removed at a lower pressure or by ventilation.

In this way the protein appears as a soft powder, dark grey when made from grass, pale from cereals and faintly green from kale or tares. With care the N content can be kept consistently above 10 per cent; 12 per cent is about the best attainable at present. *In vitro* it is 40–55 per cent digestible by enzymes such as commercial trypsin and pepsin, and so resembles many other proteins in feeding-stuffs. The reasons for the lower digestibility of bulk leaf protein compared with some leaf-protein fractions and the commonly handled proteins, such as casein, is being studied, for it is of both theoretical and practical interest.

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