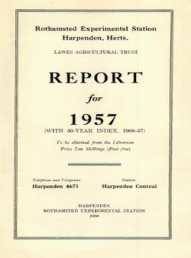
ROTHAMSTED RESEARCH

Report for 1957

Full Table of Content



Biochemistry Department

N. W. Pirie

N. W. Pirie (1958) *Biochemistry Department ;* Report For 1957, pp 96 - 103 **- DOI: https://doi.org/10.23637/ERADOC-1-90**

BIOCHEMISTRY DEPARTMENT

N. W. PIRIE

Mr. W. E. Whitman has joined the department to apply the methods of food technology to the leaf-protein preparations that are being made. N. W. Pirie attended conferences on "Cellular biology, nucleic acids and viruses" organized by the New York Academy of Sciences and the Poliomyelitis Foundation in January, and on "The origin of life on the earth" organized by the Moscow Academy of Sciences and the International Union of Biochemistry in August. He was the guest of the Czechoslovak Academy of Sciences and the World Federation of Scientific Workers for a week visiting institutes in Czechoslovakia on the way to Moscow. M. V. Tracey returned from leave of absence in Australia on a Royal Society and Nuffield Foundation Commonwealth Bursary. At the invitation of the U.S. Navy he attended a symposium on "Marine biology" in Seattle in September. A. J. Clarke attended the XVI International Congress of Pure and Applied Chemistry in Paris in July.

Plant leaf mitochondria (W. S. Pierpoint)

The enzymes of the tricarboxylic acid cycle and its associated phosphorylations are located on the mitochondria of both animal cells and of non-photosynthetic plant cells, and probably, although there is less evidence for this, on the mitochondria of photosynthetic plant cells. The mitochondria therefore play important roles in cell metabolism, and alterations in their functioning will be reflected in the organism as a whole. Some of the phenomenon of fruit ripening and of plant infections, for example, may be due primarily to mitochondrial disfunction, and a useful understanding of these phenomena requires information on the state of mitochondria in various plant conditions.

Work previously done at Rothamsted has suggested that during the extraction of Rothamsted tobacco necrosis virus from leaves of *Nicotiana tabacum*, the mitochondria produce a virus inhibitor. This inhibitor is responsible in part at least for the variation in infectivity of different virus preparations. In order to characterize further this inhibitor production, and to assess its importance in the intact plant, as well as to provide a basis for the study of healthy and abnormal leaves, work has begun on the preparation from leaves of *N. tabacum* of actively respiring mitochondria.

The most active preparations obtained so far were made by a method which was essentially that which gave active mitochondria from animal and etiolated plant tissues. The leaves were ground in a medium containing 0·4*M*-sucrose, 0·2*M*-tris-buffer, 0·005*M*-ethylenediamine tetracetic acid and 0·02*M*-citrate. The mitochondrial fraction was then separated by centrifugation between 1,000 and 10,000 g. Although omission of any one of the constituents of the extraction medium lowered the oxidative capacity

https://doi.org/10.23637/ERADOC-1-90

of the final preparation, citrate was the most essential single constituent.

Such preparations will oxidize, to varying degrees, all the intermediates of the tricarboxylic acid cycle. Highest activity was obtained with succinic acid, and it is with this substrate that optimal conditions for measuring activity of the extracts have been worked out. Addition of the cofactors cytochrome c, diphosphopyridine nucleotide, co-enzyme A and thiamine pyrophosphate stimulates activity, suggesting that the oxidative mechanism is essentially the same as in animal tissues.

The mitochondria in the preparation are probably damaged during their isolation. This is suggested by their requirement for added cytochrome c, and also by their pitiably small oxidation of pyruvate, even in the presence of "sparker" amounts of other acids. The preparations are also impure, for they are bright green in colour and contain a little less than a third of the total chlorophyll in the extracts. Microscopic examination reveals the presence in them of much material resembling disrupted chloroplasts.

Latent phenolase of broad bean (Vicia faba, *L.*) *leaves* (R. H. Kenten)

Water extracts of broad-bean leaves contain much latent phenolase (*Rep. Rothamst. exp. Sta. for* **1954**). It has now been found that active phenolase is released by brief exposure of the extracts to alkaline (pOH 2·5–5·3) as well as acid (pH 3–3·5) conditions. Non-ionic or cationic wetting agents are without effect, but all of the anionic wetting agents tested brought about some activation of the latent phenolase. With the homologous series of sodium *n*-alkyl sulphates the concentration at which activation takes place decreases with increase in chain length. Furthermore, at pH 8 the rate of activation with sodium dodecyl sulphate and sodium dioctylsulphosuccinate is very slow or negligible compared with that at pH 6. Since it is known that the affinity of anions for proteins increases with the size of the anion and that an acid pH favours combination of the anion with protein, the results suggest that the wetting agents activate by virtue of their capacity to combine with proteins. Such combination alters the net charge on the protein, and would permit the dissociation of a phenolase–inhibitor complex held together by salt linkages. Alternatively, configurational changes which sometimes accompany the binding of large anions by proteins could unmask the catalytic centre of a prophenolase. Sufficient evidence is not yet available to decide which of the two alternatives is involved in the activation mechanism.

Enzymes of bracken

This year's and previous work is reviewed in an accompanying article.

Work has continued on the manometric method of following bracken thiaminase action. The general equation for the thiaminase catalysed transfer reaction is

$$P\text{–}CH_2\text{–}T^+ + RNH_2 \longrightarrow P\text{–}CH_2\text{–}NHR + T + H^+ \quad . \quad . \quad (1)$$

where P and T stand for the pyrimidine and thiazole components of thiamine respectively. Since the reactants are bases, and the

https://doi.org/10.23637/ERADOC-1-90

reaction gives rise to other bases, which at about neutral pH may differ considerably from the reactants in the extent of their ionization, acid may be released. The amount of acid produced will be a measure of the thiaminase action, and can be estimated manometrically in bicarbonate-CO_2 buffer by following the evolution of CO_2. The following examples of systems at pH 7·5 make this clear. If the acceptor amine is a strong, or moderately strong, base (pKa 10 or greater), then the two extreme possibilities may be written

$$P\text{–}CH_2\text{–}T^+ + RNH_3^+ \longrightarrow P\text{–}CH_2\text{–}NH_2^+R + T + H^+ \quad . \quad (2)$$

$$P\text{–}CH_2\text{–}T^+ + RNH_3^+ \longrightarrow P\text{–}CH_2\text{–}NHR + T + 2H^+ \quad . \quad (3)$$

and between 1 and 2 mol. of acid would be released according to the extent of ionization of the pyrimidylmethyl-amine product. Where the acceptor amine is a very weak base (pKa 5 or less) and the pyrimidylmethyl-amine product is a strong base, then the reaction can be formulated,

$$P\text{–}CH_2\text{–}T^+ + RNH_2 \longrightarrow P\text{–}CH_2\text{–}NH_2^+R + T \quad . \quad . \quad . \quad (4)$$

and no release of acid, and hence no evolution of CO_2, would take place. The manometric method is therefore not suitable for studying such systems.

Using aniline, piperidine, pyridine and trimethylamine as acceptor amines, either on account of their difference in ionization at pH 7·5 or because of the degree of ionization of the product they give by the thiaminase reaction, it was found that there was a reasonable agreement (within 10 per cent) between the calculated and experimental outputs of CO_2, although with thiamine limiting, some 5–10 per cent of the thiamine may be lost in side reactions. The effect of varying such factors as the concentration of thiamine and the concentration and type of acceptor amine have been studied. Conditions have been found under which thiaminase activity can be estimated manometrically. It has been shown that the thiamine analogues heteropyrithiamine and quinilinothiamine, in which pyridine and quinoline replace the thiazole heterocycle of thiamine respectively, and possibly oxythiamine, are thiaminase substrates. Studies of other thiamine analogues have shown, in particular, that 3-(*o*-aminobenzyl)-4-methylthiazolium chloride (ABMT) is an extremely powerful inhibitor of thiaminase action. It is known that ABMT inhibits fish thiaminase competitively, but with bracken thiaminase it was found to act non-competitively; the inhibition was not relieved by increasing the thiamine concentration, and the extent of inhibition increased over the first 30–40 minutes of the reaction period. Concentrations of $2 \times 10^{-6}M$ ABMT inhibited bracken thiaminase by 15–20 per cent, and at $5 \times 10^{-5}M$ ABMT inhibition was virtually complete.

Among the plants, the thiaminase system is restricted to Pteridophytes, and its role *in vivo* is not understood. Evidence about its function could be got if it could be inhibited *in vivo*, but injections of up to 5 mg. ABMT into the stems of bracken fronds caused no obvious damage to develop in a period of 3–4 weeks. The failure

https://doi.org/10.23637/ERADOC-1-90

to get an effect may have been due to the ABMT not reaching the site of thiaminase action.

Further purification of plant amine oxidase preparations (A. J. Clarke and P. J. G. Mann)

We have already described a method giving a 300-fold purification of the amine oxidase of pea-seedling extracts. Further improvements have brought the purity of the final preparations to 50 per cent. The enzyme is absorbed on calcium phosphate and eluted by stepwise increases in the concentration of the eluant (phosphate buffer, pH 6·8). To concentrate the purified enzyme in the combined active eluates, the phosphate is removed by dialysis, and the enzyme is reabsorbed on calcium phosphate and eluted with 0·2*M*-phosphate buffer, pH 6·8. Since the purified enzyme is pink, its movement down this column can be seen; at this stage the greater part of the activity in 50 ml. of dilute enzyme has been recovered in less than 1 ml. of eluate. Examined spectrophotometrically, the strongly pink enzyme preparations show an absorption band at 470–550 mμ.

When substrate (putrescine) is added under anaerobic conditions, the pink colour is discharged and the absorption at 470–550 mμ falls. The difference spectrum shows a broad absorption band with a maximum at 490–520 mμ. The fact that this absorption is reduced to less than half by the addition of putrescine is the basis for the suggestion that the purity of the preparation exceeds 50 per cent. Since hydrogen peroxide is one of the products of the reactions catalysed by the enzyme, it is likely that a flavine nucleotide forms part of the prosthetic group. Other workers have obtained indirect evidence of the presence of flavine adenine dinucleotide in the prosthetic group. While the magnitude of the observed absorption is consistent with this suggestion, the position of the absorption maximum is not. Flavine adenine dinucleotide has an absorption maximum at about 450 mμ, and while it is known that combination with proteins may displace its absorption band towards longer wavelengths, the extent of such displacements, so far recorded, are not as great as that required in the present case.

Plant amine oxidase and the formation of heterocyclic compounds (A. J. Clarke and P. J. G. Mann)

We have already reported the formation of norhygrine, a pyrrolidine alkaloid, and of *iso*pelletierine, a piperidine alkaloid, when the amine oxidase-catalysed oxidation of putrescine and cadaverine, respectively, is carried out in presence of acetoacetate. The study of this type of reaction has now been extended, using a range of amine oxidase substrates and four β-keto acids as reactive methylene compounds. CO_2 formation is a measure of those condensation reactions which depend on the presence of the cyclic forms of the products of the amine oxidase reactions. Sometimes the CO_2 outputs approached the theoretical values even at low concentrations of the reactants. Evidence was obtained by paper chromatography that the evolution of CO_2 was accompanied by the formation of a series of saturated heterocyclic compounds whose R_F values differed both with the nature of the substrate and of the reactive

https://doi.org/10.23637/ERADOC-1-90

methylene compound. With cadaverine as the substrate and indole as the reactive methylene compound, β-(2-piperidyl)-indole is formed. Reactions of this type are of particular interest, since, in some cases, the products are alkaloids. They are unlikely to occur to any great extent in tissues where rapid specific pathways exist for the metabolism of the unsaturated ring compounds. A likely alternative pathway would be direct reduction leading, with putrescine as substrate, to the formation of pyrrolidine. We have recently obtained a fraction from pea-seedling extracts which catalyses the oxidation of reduced diphosphopyridine nucleotide by Δ′-pyrroline, the unsaturated ring compound formed as a result of of the amine oxidase-catalysed oxidation of putrescine. So far we have been unable to identify pyrrolidine as a product of this reaction.

Peptides as substrates of plant amine oxidase (A. J. Clarke and P. J. G. Mann)

We have already reported that the slow rate of oxidation of the diamino acids, ornithine and lysine, by plant amine oxidase, compared with that of aliphatic diamines of the same hydrocarbon chain length, is due to inhibition by the carboxyl groups of these amino acids. When the carboxyl groups are masked, as in the methyl esters, the rates of oxidation are increased about sevenfold. Lysine amide has now been tested as a substrate for the amine oxidase. It is oxidized at about the same rate as lysine methyl ester. These results suggested the possibility that ornithine and lysine peptides, particularly those in which the amine groups of these acids are not involved in the peptide linkage, might serve as substrates for the enzyme. Lysyl–glycine, however, is oxidized at about the same rate as lysine. In experiments to test whether such oxidation was dependent on the presence of the two free amino groups of the lysine residue, it was found that the methyl ester of glycylglycine was slowly oxidized, and those of di- and tri-glycylglycine more rapidly oxidized by the enzyme. While we have shown that aliphatic monoamines are oxidized by the enzyme, the rate of such oxidation is very slow compared with that of aliphatic diamines. In compounds of this type the presence of two suitably spaced amino groups is necessary for the formation of an active enzyme substrate compound. The fact that the methyl esters of di- and tri-glycylglycine are oxidized much more readily than aliphatic monoamines suggests that an imino group of one of the peptide bonds may take part in the formation of the enzyme substrate compound. These preliminary results therefore suggest the possibility that a new group of substrates of plant amine oxidase may be found among the peptides.

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

Pyridine, α- and β-hydroxy pyridine, pyridyl-β-carbinol and pyridyl-γ-carbinol have been tested as substrates for peroxidase. Only β-hydroxy pyridine was oxidized. This suggests that the oxidation of pyridoxine and allied compounds by peroxidase, pre-

https://doi.org/10.23637/ERADOC-1-90

viously reported, depends on the presence of the hydroxyl group in the β position in these compounds.

Blackening of potatoes after boiling (A. J. Clarke and P. J. G. Mann)

Samples of potatoes have been taken from the Rothamsted and Woburn Six-Course Rotation and Dung NPK experiments, to study the effects of the different fertilizer treatments employed on the intensity of the blackening of the potatoes after boiling. In tests made during the first few weeks after harvesting, the differences observed were only small. Further tests will be made after storage. An investigation has also been started of the nature of the pigment responsible for the blackening and of the mechanism of its formation.

The infectivity of fragments of tobacco mosaic virus (N. W. Pirie)

Tobacco mosaic virus retains its infectivity in spite of exposure to treatments that would destroy the activity of most other viruses and enzymes. The infectivity of fragments made from it by disruption with phenol or detergents is, on the other hand, unusually vulnerable. Because the most infective fractions consist predominantly of nucleic acid, various sources of ribonuclease have been tried and found to destroy infectivity with a readiness that approximately parallels their enzyme activity. Saliva is interesting here, for its ribonuclease activity is often overlooked and even when diluted 1 in 1,000 it inactivates TMV fragments. The behaviour of these enzymic inactivators depends greatly on the nature of the buffer in which the tests are carried out, and there is partial protection in the presence of a few known inhibitors of ribonuclease, e.g., heparin, polygalacturonic acid sulphonate and Zn^{++}. In suitable environments these agents also diminish the spontaneous inactivation, but no environment has yet been found in which the infectivity of virus fragments is stable for more than a few hours at room temperature. Other mechanisms of inactivation are probably also playing a part. Thus Ca^{++}, Mg^{++}, glutathione and ascorbic acid are powerful inactivators, as are washings of intact skin and leaves. These effects go some way towards explaining the low infectivity of virus fragments compared to a corresponding amount of the original virus. (With F. C. Bawden.)

Large-scale production of leaf protein (M. Byers, D. Fairclough, N. W. Pirie and W. E. Whitman)

The machinery for making and processing leaf juices has remained essentially unaltered during the year, but it is now all accommodated in a new shed and extension so that the original building can be kept for the clean and small-scale work of handling the finished protein and getting it into the various forms in which it is used. The amount of labour needed to make protein has been greatly diminished by more convenient lay-out and more liberal use of pumps. It would now be possible, if there were demand, to make 100 lb. of protein a week, and more than 500 lb. was in fact made during the summer. Our arrangements for pressing protein into blocks and for wet and dry grinding, canning, solvent extracting, drying and sieving improve steadily.

Young cereals were the main crop used, and this year we included

https://doi.org/10.23637/ERADOC-1-90

maize, which comes in conveniently from the beginning of August when there are few other crops suitable for processing. All give unflavoured or pleasantly flavoured products containing 10–12 per cent N before defatting. Bracken was the only other addition to the list of species processed, and from it we could extract 40 per cent of the protein.

We have begun a series of measurements of the yield of protein per acre, and three results are set out in Table 1. The sugar beet

TABLE 1

Some yields of extracted protein from different crops

Date	Crop	Description	Ex-traction	Lb. protein per acre	% N on DM on protein
22.7.57	Tares	1st cut	1st	99·8	11·77
			2nd	20·3	11·53
19.8.57		2nd cut	1st	26·2	11·44
			2nd	6·6	11·53
31.7.57	Maize	1st cut	1st	50·8	11·28
			2nd	15·8	
29.8.57		1st cut	1st	110·5	11·03
3.9.57	Sugar-beet leaves	High watering	1st	730·0	10·16
		Low watering	1st	477·0	9·66
		Intermediate watering	1st	471·0	9·36

was grown under optimal conditions by the Botany Department, and is included to show the yields at which we aim. At present the two by-products—fibre and low-molecular-weight leaf components—are discarded. The yields quoted are therefore only part, perhaps less than half, of the total product. Furthermore, with crops such as the usual cereals we are off the land and it is ready to take another crop by the beginning of June.

The standard end product is a deep-frozen 5-lb. block containing about 50 per cent of water. Most of this year's production has gone or is going to feed pigs in an experiment at the National Institute for Research in Dairying. Some protein after removal of the lipids with acetone has been used in chicken-feeding experiments at the Rowett Research Institute. Both experiments show that the protein has the high nutritive value that was expected from its amino-acid composition.

Although cooking is no permanent part of the programme of the department, we have been forced to try, both at home and in the laboratory, various recipes so as to have something other than the raw material to show to sceptics. Protein that has simply been washed with water and then canned or kept in deep freeze has so far proved preferable to dried or solvent-extracted material. Cakes and biscuits baked with it are palatable, but the green colour is at first unattractive. In soups, curries, stuffed vegetables or spreads, with some suitable flavours such as herbs, onion, cheese or fish, the colour is more immediately acceptable. If the protein is put into ravioli or a pastry casing with a suitable spice, the colour is less obtrusive; these are probably the most effective vehicles during

https://doi.org/10.23637/ERADOC-1-90

the phase in which a green main food seems odd. We have had a great deal of help and advice on cooking, both from members of other departments and from outside Rothamsted, and have had such a steady demand for information and protein, particularly from vegetarians, that we have mimeographed a leaflet of cookery hints. It is clear that in any region or at any time, if there were a shortage of conventional protein, there would be no difficulty in making acceptable and useful foods from this one.

The properties of resistant parts of fungal mycelium which might accumulate in soils (M. V. Tracey)

During the tenure of a Royal Society and Nuffield Foundation Commonwealth Bursary at the Commonwealth Scientific and Industrial Research Organization Wool Textile Research Laboratories in Melbourne, aspects of the solubilization of wool protein by proteolytic enzymes were examined. Conditions under which proteolysis could be activated by the addition of other substances were sought with some success. The effects found, however, were attributable, not to any direct effect on the process of proteolysis as had been hoped, but to reduction of surface denaturation of the enzymes used.

The object of this visit was to learn methods that could be applied to the resistant parts of fungal mycelium in the hope of making them amenable to enzymic attack. This aspect of the work is now being undertaken. Much of the nitrogen in the mycelium is in the form of chitin, so the opportunity was also taken to study the chitinase of termites.

The potato-root eelworm hatching factor (A. J. Clarke)

The Nematology Department has supplied $1\frac{3}{4}$ tons of potato-root diffusate during the past season. Under the conditions mentioned in the previous Report, 90 per cent of the hatching factor is removed from solution by charcoal. In the absence of sufficient water during subsequent elution with acetone, the recovery of the factor is reduced. Contrary to reports by other workers, the charcoal can be re-used after extraction of adsorbed material.

The product obtained by concentrating the acetone solution is an acidic resinous mixture (0·6 g./40 litres root diffusate). Purification by fractional precipitation of the salts formed with various organic bases has not been found satisfactory. A useful initial purification is effected by partition between a buffered aqueous phase and ethyl acetate. By paper and column chromatography, several fluorescent substances present in the starting material are separated. Hatching activity is associated with a discrete spot having a blue fluorescence and phenolic properties. The various properties suggest a coumarin-like structure, and a number of naturally occurring coumarins and related hydroxy-cinnamic acids have been screened for hatching-factor activity. Chlorogenic, caffeic and ferulic acids, as well as aesculin, rutin and 4-hydroxycoumarin have been found inactive.

https://doi.org/10.23637/ERADOC-1-90