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ROTHAMSTED RESEARCH

Report for 1955

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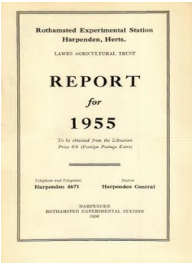

Biochemistry Department

N. W. Pirie

N. W. Pirie (1956) *Biochemistry Department ;* Report For 1955, pp 80 - 88 **- DOI: https://doi.org/10.23637/ERADOC-1-77**

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BIOCHEMISTRY DEPARTMENT

N. W. PIRIE

Marjorie Byers and A. J. Clarke joined the department, and M. V. Tracey has been given leave of absence to accept a Royal Society and Nuffield Foundation Commonwealth Bursary. He will be working in the Wool Textile Laboratory of the Commonwealth Industrial and Scientific Research Organization at Melbourne. Five members of the department attended the Third International Congress of Biochemistry in Brussels, and N. W. Pirie attended the World Symposium on Applied Solar Energy in Arizona.

PLANT ENZYMES

Ribonuclease (W. S. Pierpoint)

Work on the characterization of leaf ribonuclease has been continued. It has proved possible to obtain, by a process of gradient elution chromatography on a cation-exchange resin, a dilute but purified preparation of the enzyme free from phosphatase activity against ATP, metaphosphate and β-glycerophosphate. This confirms the previous suggestion that the enzyme, like the pancreatic ribonuclease, is a specific phosphodiesterase.

A number of properties of the enzyme have been described and a number of compounds tested against it as possible inhibitors. A specific inhibitor of ribonuclease could possibly be of great help in the isolation of unaltered nucleoproteins from plant leaves and in elucidating the physiological role of the enzyme. The compounds tested included some antiviral compounds which are structural analogues of the naturally occurring purines and pyrimidines, and a number of antibiotics which inhibit protein synthesis. However, no specific inhibition was observed.

Metaphosphatase (W. S. Pierpoint)

One of the enzyme activities present in the pea-leaf extracts which is difficult to remove from the ribonuclease is metaphosphatase. Metaphosphate itself has not been detected in the higher plants, although it seems to form a very large percentage of the total phosphorus of some moulds and fungi, and to be very active metabolically. A number of observations have therefore been made on the metaphosphatase activity of the pea extracts.

Some evidence has been obtained which suggests that the metaphosphatase activity is associated with an unspecific acid phosphomonoesterase. Thus the ratio of activity towards metaphosphate and β-glycerophosphate was found to be constant in a wide range of pea-leaf extracts, and is not significantly altered by various protein fractionation treatments, including precipitation, heat denaturation and adsorption. Attempts were made to prepare a purified sample of this enzyme by gradient elution chromatography on an ion-exchange resin. The enzyme is eluted, however, in two distinct peaks, both with the same ratio of metaphosphatase to glycerophosphatase activity. Whether these peaks represent

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different enzymes or are different forms of the same enzyme has not been determined.

Amine oxidase and auxin formation (P. J. G. Mann)

There is much evidence that 3-indolylacetic acid (IAA) is formed in the plant from tryptophan, but the metabolic pathway is not yet established. It has been suggested that the route is by way of tryptamine and 3-indolylacetaldehyde (IAc) or 3-indolylpyruvic acid and IAc. We have already reported preliminary evidence suggesting that the oxidation of tryptamine to IAc is catalysed by plant amine oxidase. This work has been continued, and it has now been established (by A. J. Clarke) that the primary oxidation product is IAc. It has also been shown that the enzyme catalyses the oxidation of 5-hydroxytryptamine. There is evidence that this compound, as well as tryptamine itself, occurs in plants. A method has been worked out for the estimation of IAc. This depends on the oxidation of IAc to IAA with silver oxide. The IAA formed is then estimated. Using this method, the conditions favouring the accumulation of IAc in the enzyme-catalysed oxidation of tryptamine have been investigated. At pH 7 the IAc found is about 80 per cent of theory, provided the reaction time is short and the tryptamine concentration is low. Under alkaline conditions the yield is lower, possibly owing to polymerization of the aldehyde. At high tryptamine concentrations the low yield appears to be due to the combination of the aldehyde with unchanged tryptamine.

Having thus established that the first stage in the postulated pathway from tryptamine to IAA is catalysed by plant amine oxidase, we have begun a study of the second stage—the oxidation of IAc to IAA. Much of the previous work on this reaction has been done with a heterogeneous material containing only 2 per cent IAc made by treating tryptophan with ninhydrin or isatin or by the use of plant extracts containing IAc. While a chemical method of synthesis is now available by which pure IAc can be obtained, the procedure is difficult, and for this reason, and also because of the instability of the compound, the enzymic method of preparation has obvious advantages. Work in this department has already shown that the oxidation of phenylacetaldehyde is catalysed by plant saps. The system in pea-seedling sap catalysing this reaction was found to consist of a peroxidase together with a thermostable factor which can be partially replaced by manganous ions. It has now been shown that the oxidation of IAc is catalysed by plant saps and by peroxidase, particularly in presence of Mn^{2+}. By analogy with the phenylacetaldehyde reaction 3-indolylaldehyde would be expected as the product of such oxidation. However, small amounts of IAA accumulate during the oxidation, despite the fact that the plant saps catalyse the oxidation of IAA. It is possible therefore that a significant part of the IAc is oxidized to IAA either by the peroxidase system or by another system as yet unidentified.

Amine oxidase and alkaloid formation (A. J. Clarke and P. J. G. Mann)

We have reported that the oxidation of putrescine and cadaverine, catalysed by plant-amine oxidase, results in the formation of Δ′-pyrroline and Δ′-piperideine compounds. It was suggested that

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these ring compounds are formed by spontaneous cyclization of the aldehydes (γ-aminobutyraldehyde and δ-aminovaleraldehyde), or of the aldimines, which are the probable products of the enzyme-catalysed reactions. The suggestion has been made that, *in vivo*, the only enzymatic reactions directly concerned in the formation of certain pyrrolidine and piperidine alkaloids are those producing γ-aminobutyraldehyde and δ-aminovaleraldehyde and that the alkaloids result from the spontaneous condensation of these aldehydes with plant metabolites. If alkaloids are waste products of metabolism it seems likely that they might be formed by such a non-specific mechanism. We have therefore begun a series of experiments to test whether alkaloids are formed when the amine oxidase-catalysed oxidation of putrescine or cadaverine is carried out in the presence of the appropriate reactive methylene compounds.

The enzyme-catalysed oxidation of putrescine and cadaverine takes place without CO_2 formation; however, with acetoacetate or acetonedicarboxylate present in the reaction mixture the O_2 uptake is accompanied by a CO_2 output. The CO_2 arises from the reactive methylene compound, the concentration of which decreases during the reaction. This is not a simple decarboxylation of these compounds. Thus acetoacetate gives acetone on decarboxylation, but only traces of acetone are found in the reaction mixture, and there is little or no CO_2 output if the amine is omitted from the reaction mixtures or if boiled enzyme is used in the complete reaction mixtures. These results suggest that the CO_2 arises from a reaction between the reactive methylene compounds and the products of the amine oxidase reaction. In reaction mixtures containing both amines and reactive methylene compounds the Δ'-pyrroline and Δ'-piperideine compounds, which are the products of the oxidation of the amines, do not accumulate. New compounds are present which resemble pyrrolidine and piperidine. The results therefore suggest that condensation has taken place between the oxidation products of the amines and the reactive methylene compounds. Such condensations should result in the formation of norhygrine and norcuscohygrine from putrescine and *iso*pelletierine from cadaverine. Further chemical evidence is required to establish conclusively the nature of the products.

Manganese oxidation in the pea plant (Pisum Sativum, L.) (R. H. Kenten and P. J. G. Mann)

It has been suggested that the physiological effects of manganese may, in part, be due to its capacity for valency change. We have previously shown that Mn^{2+} is oxidized by peroxidase systems and by illuminated chloroplasts and have reported the results of preliminary experiments demonstrating the accumulation of higher oxides of manganese in the stems of pea plants grown in water culture at high Mn^{2+} concentrations. This work on *in vivo* manganese oxidation has been continued. A method of extracting the higher oxides of manganese from the plant stems has been worked out. The tissue was washed first with orthophosphate to remove interfering substances, then the manganese higher oxides were dissolved in pyrophosphate and estimated spectrophotometrically. It was only possible to demonstrate accumulation of higher oxides of manganese in the plant stems under conditions of manganese

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toxicity. Thus the lowest concentration of manganese in the culture solution with which such accumulation could be clearly demonstrated was 22 p.p.m. Under conditions of severe manganese toxicity (110 p.p.m. Mn^{2+} in the culture solution) relatively large amounts of higher manganese oxides accumulated in the tissue equivalent to 100–200 μg. Mn^{3+}/g. fresh weight. We have shown that with peroxidase systems the oxidation *in vitro* takes place with physiological concentrations of Mn^{2+}. It is probable therefore, that *in vivo*, under normal conditions of manganese supply, the oxidized manganese is reduced by plant metabolites as fast as it is formed. This would involve the manganese in an oxidation–reduction cycle. Under conditions of manganese toxicity, part of the oxidized manganese escapes reduction and accumulates in the plant tissues.

Enzymes of bracken (R. H. Kenten)

Work has continued on the enzymes of bracken, and attention has been largely confined to the enzyme thiaminase. This enzyme is present in bracken, but has been reported absent from a large number of species of higher plants. A study of the inhibitors and substrate specificity of this enzyme may lead to the development of specific bracken-killing agents. For this work thiaminase preparations have to be made, and a simpler method for estimating thiaminase activity than that used hitherto would be valuable. Some progress along these lines has been made. The estimation of thiaminase activity involves the determination of small quantities of thiamine. As the known methods are subject to interference from a variety of substances, it is usual to separate the thiamine from other materials by absorption and elution from a column of suitable absorbent. Attempts to modify an existing method to avoid this time-consuming step have been partially successful, and we can now obtain reasonable results with some partially purified thiaminase preparations. It is of interest that this modified method was satisfactory with crude thiaminase preparations obtained from young bracken fronds, but failed with similar preparations from older fronds. While it is known that methods found to be satisfactory with one species of plant may not be suitable with another, it is not generally recognized that a similar situation may exist with tissues from the same species of plant in different physiological states.

The oxidation of indolepropionic and indolebutyric acids by peroxidase systems (R. H. Kenten)

A knowledge of the systems which are responsible for the metabolism of substances with growth-hormone properties may throw light on their mechanism of action in the plant. Work on the oxidation of indoleacetic acid (IAA) by plant extracts and peroxidase systems has already been reported. Two homologues of IAA, namely indolepropionic acid (IPA) and indolebutyric acid (IBA), are also oxidized by peroxidase systems. Both IPA and IBA have growth-hormone properties, and a study of their oxidation has been made, partly on account of interest in systems which may be responsible for their metabolism, and partly because the results might help to elucidate the mechanism of oxidation of IAA.

In contrast to the results obtained with the peroxidase-catalysed oxidation of IAA by O_2, where the presence of monophenols, maleic

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hydrazide or Mn^{2+} increased the rate of oxidation, it was found that peroxidase catalysed the direct oxidation of IPA or IBA only when Mn^{2+} was present. The oxidation of IPA or IBA by peroxidase plus Mn^{2+} was usually preceded by a lag period which could be overcome by the addition of small amounts of H_2O_2. The addition of catalase stopped the reaction, suggesting that it was accompanied by, and depended on, the formation of H_2O_2.

The oxidation of IPA takes place without the formation of CO_2, and proceeds at concentrations of peroxidase and Mn^{2+} commonly encountered in plant extracts. These facts, together with other evidence, suggest that the oxidation of IPA by plant extracts observed by other workers was due to the presence of peroxidase and Mn^{2+} in the extracts.

Although the oxidation products of IAA, IPA and IBA were not characterized, from the results obtained in these and other investigations it is likely that the indole nucleus is the site of peroxidase attack.

Studies on some Leaf Components

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

The studies of the change in concentration of organic acids in leaves, included in the Reports for 1953 and 1954, were extended and completed. In addition to citric, malic and ascorbic acid, French bean leaves contain *iso*citric, glycolic, malonic, fumaric, succinic and one as yet unidentified acid. The content of each of these acids can be altered by darkening the whole plant or leaves detached from it, by infiltration with the potassium salts of these and other acids or by placing the plant in an atmosphere containing little carbon dioxide. Changes in composition have been compared with changes in susceptibility to infection. The largest changes in acid composition were produced by darkening (increase in citric and decrease of malic and other acids), whereas the largest alteration in susceptibility (a decrease) was produced by detaching the leaf. There was no correlation between concentration of any one acid and susceptibility to tobacco necrosis virus, and none of the acids infiltrated into detached leaves altered susceptibility as much as did changes in the environment. It was confirmed that plants darkened or placed in an atmosphere containing little carbon dioxide for 2 days before inoculation are sometimes more susceptible than control plants, but at other times they are not. Some progress was made towards defining conditions under which the response is reproducible.

Properties of fractions from leaves infected with tobacco mosaic virus (N. W. Pirie)

From tobacco plants infected with tobacco mosaic virus many preparations have now been made of a non-infective antigen related to the virus but with a much smaller particle size. This resembles the virus in many ways; thus it contains firmly bound ribonucleic acid, it is not digested by the common proteolytic enzymes and it aggregates readily *in vitro* to give rods very similar to those of the virus. It contains only a third to a tenth as much nucleic acid as the virus, and much effort has gone into verifying that some nucleic

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acid is invariably present regardless of the strain of virus used or the conditions under which the host plants are grown. During the past few years we have found much less slowly sedimentable material in extracts from infected leaves than we found ten years ago, and have no explanation of this. We confirm our old observation that the fraction of virus that is only liberated from the leaf fibre by fine grinding is less infective than the fraction that comes away more easily, but in other respects the two fractions are similar. (With F. C. Bawden.)

Work on the non-infective antigen produced an abundant supply of fresh centrifugally isolated virus, and the opportunity was therefore taken to co-ordinate the scattered observations made during the last ten years. All preparations, made from fresh sap, that have not been incubated contain labile nitrogen and phosphorus. This is mainly split off by incubation with citrate or phosphate, especially in the presence of trypsin or nuclease, but its removal is accompanied by changes in the virus, particularly in its behaviour on precipitation with antiserum or ammonium acetate. This behaviour is probably due to the presence of normal leaf nucleoprotein in virus preparations made by gentle methods. These preparations also contain ribonuclease, and the experience gained with this enzyme (described in earlier Annual Reports) has been of great value in controlling its assay and removal.

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

The dry summer did not seriously affect work on protein extraction. Even during the drought period, when the dry-matter content of some of the harvested crops rose to 36 per cent, protein precipitates contained 60 per cent protein with an extraction of 30–40 per cent of the original crop nitrogen. This was obtained by adding water or dilute alkali to the leaves during pulping and adjusting the degree of pulping by varying the speed and pitch of the pulper hammers. The results have emphasized the findings of earlier work in that the degree of pulping, controlled by means of variable speed and pitch of the pulper hammers, affects the quantity and quality of the extracted protein from suitable raw materials. A typical example is given in the table on page 86.

Here samples of the same batch of kale were pulped at four different speeds, and at each speed the same two settings of the hammers were used. The coarse setting gives rapid movement through the pulper, and the fine setting slower movement, so that pulping is more complete. Obviously this increases the extraction without necessarily increasing the power consumption. Other experiments have shown that the precise distribution of the two types of hammer in the pulper affects the power consumption when other factors are kept the same. The ultimate economics of the process depend on our being able to find a set of optimum speeds and settings for each type of crop.

The new conveyor press installed in 1954 works well, both for protein extraction and for dewatering steamed crops. The water ratio of grass and other crops can be reduced from 6 : 1 to 2 : 1 quite easily after steaming, for the expenditure of 1 h.p. on the press motor, at a throughput of 10 cwt./hour.

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A continuous process of protein precipitation has been developed to replace the batch method used hitherto. Live steam is injected along the bottom of a vertical U-tube through which juice flows. The steam mixes with the cold juices, giving an almost instantaneous rise in juice temperature. A thermostat bulb near the discharge point of the hot juice at the top of the "up" leg controls a flow valve for the cold juices entering the "down" leg of the U, and so keeps the temperature of the discharge at 80° C. This steaming unit simplifies the whole operation, and the coagulum filters much more easily than that produced by slower batch heating.

The effect of varying the speed and pitch of the pulper hammers

Pulper speed r.p.m.	Hammer settings	Through-put per hour, cwt./hr.	Power required per ton pulp kw.hr./ton	Extraction of N in Juices. 1st Extract. Total N as a percentage of crop N	Protein N liberated from fibre expressed as g. N from 1 ton pulp (4·675 kg.N) gms.	Protein N liberated per kw.hr. used on pulper gms.
523	Coarse	17·5	4·6	4·12	128	27·8
	Fine	17·5	7·0	4·69	138·5	19·8
653	Coarse	20	8·0	10·2	325	40
	Fine	17·5	5·8	16·6	541	93
760	Coarse	21·8	9·4	17·8	595	63
	Fine	15·6	7·8	24·3	829	106
950	Coarse	14·5	14·8	19·2	511	34·5
	Fine	12·0	16·0	21·5	565	35·4

Raw material used—Kale. 15·4 per cent D.M.
5 October 1955. 2·91 per cent N on D.M.

After steaming, the juices are poured into vertical calico or nylon tubes 5 feet long and 5 inches in diameter. Quick-fitting clamps seal one end, and snap holders secure the other end to a filter funnel. 15–20 gal. are handled in a few minutes. More juice is pressed out of the protein paste by twisting the tubes in an automatic unit that keeps the torque on them constant as the juice comes away. Finally, the tubes are put under a beam press which gets the water content down to 50 per cent. In order to purify the protein still further, the coagulum cake is shredded, washed in water and filtered once more.

A wide variety of raw materials have been used to produce protein from a range of crops on the one hand, and to study the performance of the machinery on materials of different texture and composition. These crops include grass from leys and permanent pasture, lucerne, cereals, beans, kale, mustard, potato tops, sugar-beet tops, artichoke tops, nettles, "fat hen", etc. The final product, after partial removal of the fat, is pale green or fawn in colour, tasteless and contains 10–13 per cent N (60–80 per cent protein).

The protein is got into a state in which it can be stored without disinfectants by extracting the water and part of the lipid from it

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with methanol or acetone. We are not yet satisfied with our arrangements for extracting the lipids and recovering the solvents, but progress is being made. Different batches vary somewhat in their digestibility by enzymes *in vitro*. This is being investigated thoroughly because it may give a partial explanation of the low feeding value of some samples of leaf protein made in other laboratories.

Zinc distribution in leaves (W. S. Pierpoint)

Work on the decomposition of normal leaf nucleoprotein by leaf or pancreatic ribonuclease had suggested that some component of the nucleoprotein was inhibiting the action of the leaf enzyme more than that of the pancreatic one. This substance appeared to be zinc, and consequently a number of estimations have been performed to see if the nucleoprotein, or any other of the fractions of tobacco leaves obtained during its preparation, is particularly rich in this metal. There did not appear to be a concentration in any of the fractions. This may represent the even distribution of the metal in these fractions *in vivo*, or the disruption of some zinc-containing organelles during the fractionation procedure. It was noticed, incidentally, that old yellow tobacco leaves have a much higher zinc content than younger green ones when expressed on a dry-weight basis, but very little more on a wet-weight basis. This probably means that tobacco leaves accumulate zinc during their growth, but do not lose it as rapidly as other materials during their decay. In this respect they behave in a similar manner to certain cereals (Wood and Sibly (1949), *Aust. J. Sci. Res.*, **3**, 14).

Soil

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

The mature puff-ball mycelium has so far resisted all attempts to induce its breakdown with enzymes. It was reported last year that a number of bacteria were being isolated (in collaboration with F. A. Skinner of the Soil Microbiology Department) from a suspension of mature puff-ball mycelium in water that, after standing some months, appeared to be undergoing fermentation. Two species of clostridia were isolated, one of which produced gas within a few days in anaerobic culture, with the mycelium as sole carbon and nitrogen source. At the same time the mycelium lost some of its dark-brown colour. But neither species, alone or in the presence of any of three aerobes isolated from the material, was able to effect any measurable chemical change in the mycelium.

Further work on the chemical fractionation of the mature puff-ball mycelium shows that dichloroacetic acid may be of promise as a solvent. It " dissolves " chitin, but it is possible to dialyse it away from extracts using cellulose membranes. Dispersed materials of high molecular weight can be recovered from the solution, and the dichloroacetic acid in the dialysate may be removed by ether extraction. Some degradation of chitin to soluble non-dialysable forms appears to occur under the conditions so far used, and the conditions of use of dichloroacetic acid need further definition.

A number of papers have been prepared that relate to topics

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discussed in earlier reports, and these are now either published or in the press. The editing of *Modern Methods of Plant Analysis* has been completed, and the last volume should appear early in 1956.

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

Some preliminary experiments have been done, in collaboration with the Nematology Department, on the isolation of the hatching factor from potato-root diffusate. They confirm that the factor is probably an acid which can be adsorbed on charcoal or on anion-exchange resins.

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