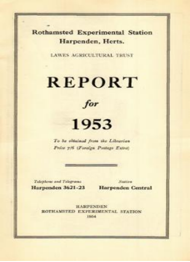
Report for 1953

Full Table of Content



Biochemistry Department

N. W. Pirie

N. W. Pirie (1954) *Biochemistry Department ;* Report For 1953, pp 75 - 82 **- DOI: https://doi.org/10.23637/ERADOC-1-75**

BIOCHEMISTRY DEPARTMENT

N. W. PIRIE

W. R. Smithies has been awarded a National Research Council of Canada Fellowship tenable for one year in the Department of Applied Biology of the National Research Council Laboratories, Ottawa. He left for Canada in August.

N. W. Pirie attended the 6th International Microbiological Congress in Rome and was a participant in the Symposium on " Interaction of Viruses and Cells " that was held in the Institute Superiore di Sanita at the same time.

D. Fairclough has joined the department to take charge of the work on large-scale preparation of leaf protein and on such other aspects of Biochemical Engineering as we are equipped to undertake.

THE OXIDATION OF INDOLEACETIC AND INDOLEPROPIONIC ACID BY PEROXIDASE SYSTEMS

(R. H. Kenten)

It is known that indoleacetic acid (IAA) is a principal native growth hormone of higher plants. A knowledge of the enzyme systems which control the proportions of IAA present in the plant is therefore important. Previous evidence suggested that manganese and peroxidase might be concerned in the oxidative inactivation of IAA, and in the hope that some evidence of the physiological role of manganese as well as the metabolism of IAA might be got, the oxidation of IAA and indolepropionic acid (IPA) by certain plant saps and other peroxidase systems has been studied.

The system in waxpod bean (*Phaseolus vulgaris*) root sap which catalyses the oxidation of IAA has been shown to consist of a thermolabile fraction and a thermostable fraction. The thermolabile fraction appears to be a peroxidase, and can be replaced by horseradish peroxidase. While both the thermolabile fraction and highly purified horseradish peroxidase preparations catalyse the oxidation of IAA by O_2, in the presence of the thermostable fraction the rate of oxidation is much faster. Evidence has been obtained which suggests that although the thermostable fraction contains several components, a large part of its activity is due to the presence of peroxidase substrates. Certain peroxidase substrates, e.g., monophenols, cause a large increase in the rate of oxidation of IAA by peroxidase, but if other peroxidase substrates, e.g., di- and trihydric phenols are also present in small amounts (10^{-5} or 10^{-4} M.) the oxidation is completely inhibited. The inhibition by these polyphenols can be reversed by the addition of an amount of H_2O_2 slightly greater than that required for the complete oxidation of the polyphenol by peroxidase. In this connection it is of interest that horseradish root sap, although rich in peroxidase, will not oxidase IAA unless it is first treated with a suitable amount of H_2O_2, suggesting the presence of inhibiting peroxidase substrates.

The mechanism of the oxidation is not clear, but at pH 6·5 or 7, which is around the optimum for catalase action, catalase inhibits strongly, suggesting that the reaction is accompanied by, and depends on, the formation of H_2O_2. The monophenols are active in catalytic amounts, and it seems likely from previous experience with such systems that they act via an oxidation–reduction cycle, being oxidized by peroxidase and reduced by IAA. Such a mechanism would account for the inhibiting effect of polyphenols, since these are more readily oxidized by peroxidase than monophenols, and would successfully compete for the H_2O_2 formed. It presupposes that polyphenols cannot act as oxidation–reduction carriers in the oxidation of IAA, but a similar situation is known with manganese oxidation by peroxidase and H_2O_2 where mono- but not polyphenols are active as carriers.

The oxidation of IPA differs from that of IAA in that it is not catalysed by peroxidase alone or by peroxidase plus monophenols. However, in the presence of small amounts of Mn^{2+} (10^{-5} M.) or the thermostable fraction of waxpod bean roots the oxidation of IPA by peroxidase proceeds readily. The oxidation of IAA by peroxidase is also stimulated by Mn^{2+}(10^{-5} M.) but the effect of Mn^{2+} is much less than that of the monophenols.

A SIMPLE METHOD FOR THE PREPARATION OF HORSERADISH PEROXIDASE

(R. H. Kenten and P. J. G. Mann)

Of the two available methods for the preparation of highly purified horseradish peroxidase one requires the use of an electrophoresis apparatus, while the other involves many fractionation procedures. A simpler method has been devised which avoids the use of electrophoresis and considerably reduces the number of fractionation procedures. This method depends on the removal of much inactive material from the crude extract by precipitation with a mixture of chloroform and ethanol (Tsuchihashi's reagent). Following this treatment, highly purified peroxidase preparations have been obtained by fractional precipitations with ammonium sulphate and ethanol. The product is obtained as a stable dry preparation which can be crystallized from ammonium sulphate solution. The activity of the dry preparation varies from P.Z. 900 to 1,000. This compares with values of 930 to 1,220 given by other workers for " pure " horseradish peroxidase.

PLANT ENZYME REACTIONS LEADING TO THE FORMATION OF HETEROCYCLIC COMPOUNDS

(P. J. G. Mann and W. R. Smithies)

Work has been continued on plant amine oxidase and has mainly been concerned with a study of the formation of heterocyclic compounds resulting from oxidations catalysed by the enzyme. With aliphatic diamines of the $\alpha\omega$ series as substrates the enzyme-catalysed reaction has, in presence of catalase, generally been represented by the equation :

$$NH_2{\cdot}CH_2{\cdot}(CH_2)_n{\cdot}CH_2{\cdot}NH_2 + \tfrac{1}{2}O_2 \longrightarrow NH_2{\cdot}CH_2{\cdot}(CH_2)_n{\cdot}CHO + NH_3 \quad . \quad . \quad (1)$$

The reaction is an oxidative deamination, in which only one of the amino groups of the substrate is attacked, with the formation of an amine aldehyde. We have previously suggested that with 1 : 4-diaminobutane (putrescine) and 1 : 5-diaminopentane (cadaverine) as substrates the amine aldehydes formed undergo spontaneous cyclization to give unsaturated ring compounds by interaction between the aldehyde group and the residual amino group with elimination of water. This has now been established. By hydrogenation of the oxidation products using platinum black as catalyst, the corresponding saturated ring compounds, pyrrolidine and piperidine, have been formed and derivatives of these compounds isolated. If the product of the enzyme-catalysed oxidation of L-lysine undergoes similar ring closure L-pipecolinic acid should be formed on hydrogenation. Attempts to isolate this compound from the hydrogenated reaction mixtures have not yet been successful, though evidence of its presence has been obtained by paper chromatography.

With the $\alpha\omega$ series of diamines ring compound formation, as would be expected, depends on the length of the hydrocarbon chain. With 1 : 6-diaminohexane as substrate, evidence has been obtained of the formation of an unstable ring compound, while with 1 : 10-diaminodecane ring formation does not occur. The oxidation of the latter substrate takes place in two stages. With small amounts of enzyme it proceeds according to equation (1). With sufficiently large amounts of enzyme the O_2 uptake increases to 1 mol./mol. substrate with the formation of 2 mols. NH_3/mol. substrate. The results suggest that the amine aldehydes formed according to equation (1) are themselves substrates for plant amine oxidase but that when these compounds undergo spontaneous cyclization they are protected from further oxidation by the enzyme.

The possibility of ring compound formation following oxidation catalysed by plant amine oxidase is not confined to the oxidation products of the diamines and of L-lysine. We have previously shown that the oxidation of phenylalkylamine is catalysed by the enzyme and that with β-phenylethylamine as substrate phenylacetaldehyde is formed. It has now been found that the oxidation of *o*-amino-β-phenylethylamine catalysed by the enzyme leads to formation of indole. Presumably the product of the enzyme-catalysed reaction is *o*-amino-phenylacetaldehyde. It is known that *in vitro* this compound readily forms indole.

With suitable phenylalkylamines as substrates it should be possible to prepare quinoline, and possibly *iso*quinoline, ring compounds. This would complete the first part of the work, which aims at demonstrating that plant amine oxidase may be used as a tool in the synthesis of most of those ring compounds which form the framework of the plant alkaloids. The work should throw light on the synthesis mechanisms *in vivo* and may, in some cases, represent the actual mechanism. Thus it is now known that L-pipecolinic acid can be formed in the plant from L-lysine. The amine-oxidase-catalysed oxidation of L-lysine may well be the first stage of this reaction. If so, the plant should be able to reduce the unsaturated ring compounds formed from the amine aldehydes of equation (1). This is under investigation. By suitable oxidation of these unsaturated ring compounds pyrrole and pyridine rings would be formed. We have already found that peroxidase catalyses the oxidation of these compounds, but the nature of the oxidation products has not yet been established.

LEAF RIBONUCLEASE AND NUCLEOPROTEIN

(M. Holden and N. W. Pirie)

Work has been continued on the purification and properties of leaf ribonuclease. All preparations of the nuclease which have been made so far also have phosphatase activity. Some of the properties of the phosphatase have been studied so that use can be made of differences in the two enzymes in finding a method of separating them. Ribonuclease and phosphatase are stable over the same pH range, and the nuclease is only slightly more stable to heat than the phosphastase. Precipitations with ammonium sulphate, ethanol, and acetone do not give a useful separation. The phosphatase is adsorbed from a dialysed solution on to calcium phosphate, but the nuclease is adsorbed to a much smaller extent. It is hoped to make use of this difference in making purer nuclease preparations. In the early stages of the purification of the nuclease, peroxidase is present. This enzyme can be removed by adding ribonucleic acid to a dialysed solution at pH 4; the nuclease and phosphatase are coprecipitated, leaving the peroxidase in solution.

Changes in nuclease and phosphatase levels during the early stages of development of pea seedlings have been followed. In ungerminated peas both enzymes are at a very low level, and there is a steep rise in the activity of both enzymes from the second to sixth day after germination. The activity per gram wet weight then remains constant until the tenth day, after which the nuclease falls slightly, though the phosphatase remains about the same. The total activity per plant continues to rise throughout the whole period of observation. At six days 70 per cent of the total phosphatase and nuclease activity is in the cotyledons, and at seventeen days about 30 per cent, whereas the activity in the shoot increases from 20 to 60 per cent of the total. The root has low activity of both enzymes, and contains only a small proportion of the total.

Various methods that have been used by other workers for measuring ribonuclease activity have been compared. The rate of enzyme action has frequently been measured by precipitation of unattacked ribonuclease acid (RNA). A number of precipitating agents have been used, ranging from acid alone (HCl, $HClO_4$, acetic acid) and acid + alcohol, to lanthanum and uranium salts. A reagent made up from uranyl nitrate in trichloracetic acid (TCA) has been widely used, and we have investigated in some detail the precipitation of RNA by it. The amount of P precipitated from a partly hydrolysed mixture, and therefore the apparent extent of hydrolysis, depends both on the concentration of the TCA used and on the concentration of RNA in the hydrolysate. There is less precipitation with more concentrated acid, and the lower the concentration of RNA, the greater is the apparent hydrolysis.

We are primarily interested in the kinetics of the action of leaf ribonuclease on leaf nucleoprotein and nucleic acid, but many experiments have been made with pancreatic enzyme on these two substrates and on yeast nucleic acid, and also with leaf enzyme on yeast nucleic acid. This has led to a study of the components in commercial yeast nucleic acid, and some methods of getting uniform fractions from it have been developed. As a result, we now find that variations in the ionic environment affect the hydrolysis of some fractions from commercial yeast nucleic acid in substantially the same way as they affect that of nucleic acid made from the normal leaf or from tobacco mosaic virus. Other fractions more closely resemble leaf nucleoprotein. It is still not clear to what extent these differences are the consequence of the presence of varying amounts of enzyme inhibitors such as Fe, Cu and Ca. The point is of some importance, because they are able to act at physiological concentrations and so may play a part in the control of nucleoprotein metabolism, *in vivo*. We realize, however, that this whole investigation is taking us too far away from the original object of the research, and propose to finish it as soon as possible.

THE PROPERTIES OF FRACTIONS FROM VIRUS INFECTED LEAVES

(N. W. Pirie)

In recent years more widespread attention has been paid to the fact that virus infection leads to the formation of several anomalous proteins besides the one that is generally accepted as the virus. In particular, there are claims that tobacco leaves infected with tobacco mosaic virus contain a non-infective protein that is free from nucleic acid but still carries the serological activity characteristic of the virus. There is nothing intrinsically improbable in this claim, but we do not think that it has as yet been properly substantiated, and so have been re-investigating slowly sedimenting fractions similar to those that we made in 1944. No conclusion has yet been reached, but we are in the odd position that, although several other laboratories are now finding a large proportion of the anomalous protein in the slowly sedimenting fraction, we no longer find this. The cause of this change since 1944 is being actively looked for.

The factors affecting the loss of infectivity by tobacco necrosis virus have been discussed in recent Annual Reports. Our results are now relatively systematic, and during the past year none have contradicted the conclusion that virus preparations made by gentle methods may carry with them an enzyme system which, in the presence of a suitable substrate, robs the virus of its infectivity. We do not know what the substrate is normally, but it can be replaced by glucose and some other substances. Other explanations of the phenomena are, of course, possible; we hope soon to get evidence compatible with only one explanation. (In collaboration with F. C. Bawden.)

METABOLISM OF TRYPTOPHAN IN PLANTS

(G. H. Wiltshire)

The reaction of tryptophan with hydrogen peroxide mentioned in the last report was further defined as a reaction of two molecules of peroxide with one of tryptophan. The product was tentatively identified as 3-hydroxykynurenine. It has previously been suggested (Galston, A. V., *Plant Physiol.*, 24, 577, 1949) that tryptophan is converted in pea seedlings epicotyls to nicotinic acid through the intermediates kynurenine and 3-hydroxyanthranilic acid, and to indoleacetic acid. Neither kynurenine nor indoleacetic acid was detected in the products of tryptophan oxidation by the enzyme system studied here. The rate of production of hydroxykynurenine was faster, and the proportion of tryptophan converted to it greater than in the production of indoleacetic acid studied by Galston. Hydroxykynurenine was already known to be a product of tryptophan oxidation in rats and some insects, but had not been found in plants.

THE INFLUENCE OF LIGHT ON THE RESISTANCE OF LEAVES TO INFECTION WITH VIRUSES

(G. H. Wiltshire)

Both the number of local lesions and the yield of some viruses are increased by shading or darkening the host plant before inoculation. Some changes produced by darkening in the nitrogenous constituents of bean and tobacco leaves were described by Humphries and Kassanis in the last report from the Botany Department. The relative and absolute concentration of organic acids is also known to be altered by darkening, and experiments have been started to explore the relation between these changes and susceptibility. Ascorbic is one of these acids, and it is already known to inactivate viruses under some conditions and protect them from inactivation under others.

Leaves of French beans in normal greenhouse lighting in the spring contain about 0·6 m.equiv. ascorbic acid per 100 g. fresh weight. Young leaves contain more than old, and the content increases during the day and falls at night. The precise level depends also on the amount of sunlight received during the day or two before sampling. One-quarter of the acid is lost from plants shaded under muslin for two days, and one-half from plants in darkness. The loss from tobacco leaves similarly darkened is from 0·3 to 0·1 m.equiv. per 100 g. This loss occurs during a period of increasing susceptibility, but it continues until the concentration falls to zero, while susceptibility decreases if darkness is prolonged. When only one-half of a bean leaf is darkened, the ascorbic acid content of the darkened part falls by about one-tenth in two days and two-thirds in six days. At two days the darkened half produces three times as many lesions as the control half, while at six days both halves produce the same number.

Detached leaves standing in water in the dark lose ascorbic acid rather more slowly than the intact plant. The level can be restored to normal or even higher by standing the leaves in 0·005–0·05M-ascorbic acid solution, instead of water. Detached leaves become more easily infected if placed in the dark, just as does the intact plant. Leaves which had been darkened for two days, and in some of which the ascorbic acid level had been restored, were inoculated with virus. Tobacco leaves containing 3·0 m.equiv. per 100 g. produced more lesions with aucuba mosaic virus than leaves containing 0·2 m.equiv. Bean leaves containing 1·2 m.equiv. produced as many lesions as those containing only 0·3 m.equiv. per 100 g.

The results show that the number of lesions produced on a leaf is not a direct function of the ascorbic acid concentration in that leaf. There is a coincidence of loss of ascorbic acid and increase of susceptibility when plants are darkened, but the loss at the time of greatest susceptibility is relatively small, and making up that loss does not confer resistance.

TECHNOLOGICAL PRODUCTION OF LEAF PROTEIN

(D. Fairclough and N. W. Pirie)

Last year the Annual Report contained an article, " Large-scale production of Edible Protein from Fresh Leaves ", which summarized work that has been done during the last thirteen years. It also suggested that the time was ripe to get the first part of this work to a definitive stage with machinery for the reliable production of protein in bulk for feeding trials. During the past year there have been no experimental advances, but a building for the work has been erected and machinery is being made and designed. Production should start in the spring of 1954.

THE PROPERTIES OF THE RESISTANT PARTS OF FUNGAL MYCELIUM WHICH WOULD ACCUMULATE IN SOILS

(M. V. Tracey)

An intensive investigation of conditions under which the " non-chitin glucosamine polymer ", referred to in last year's report, can be brought into solution has been undertaken, together with an examination of methods by which it can be hydrolysed with minimal loss of the products of hydrolysis. The use of sodium chlorite solutions seems to be the most promising method of bringing the material into solution, but the method is not completely satisfactory. Phenol, monochloroacetic acid and alkaline solutions are not successful. Many enzyme solutions from a variety of sources have been shown to have no effect on the material. Hydrolysis with hydrochloric acid results in little decomposition of the glucosamine formed, but has the disadvantage that subsequent determination of volatile acids, such as acetic, is not possible. Sulphuric acid results in more extensive decomposition of glucosamine, but its use under defined conditions appears hopeful. The results of work done in previous years have been amplified, and a number of papers are being prepared for publication.

During the year under review the preparatory work for and initial editing of a new comprehensive text-book on plant analysis has been undertaken with Professor Dr. K. Paech of the Botanical Institute, Tübingen. It is hoped to publish it in four volumes during the course of 1954–55; each volume is expected to be of about 600 pages. The contributors, who are drawn from seven countries, include the following members of this Station :

N. W. Pirie (and R. L. M. Synge) (Biochemistry Dept.)	General Methods of Separation (IIA) Proteins, Peptides, Amino Acids, and Amides (IXA)
M. J. R. Healy (Statistics Dept.)	Principles of Bioassays (IID)
R. H. Kenten (Biochemistry Dept.)	Gas Analysis in Plant Investigation (IIH)
E. C. Humphries (Botany Dept.)	Mineral Components and Ash Analysis (III)
M. V. Tracey (Biochemistry Dept.)	Chitin (IVH)
F. A. Skinner (Soil Microbiology)	Antibiotics (XII)

Most of the material submitted is now in the press, but it is expected that the final editing and preparation of indexes will be a commitment in the coming year.