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absorbed as badly or worse. Some 'dienone' may be absorbed in chylomicrons but, if so, the liver must destroy it quickly, in which case it is difficult to account for the storage of dienone in the liver by the cholesterol-fed birds.

The occurrence of cholesta-3:5-dien-7-one in atherosclerotic aortas could have been the result of a metabolic anomaly peculiar to the aorta and secondary to cholesterol accumulation. This is made less likely by the clear proof that the 'dienone' (or possibly a precursor) is present in liver and intestinal tissue of the cholesterol-fed birds. Its accumulation in detectable amounts suggests that the exogenous cholesterol is overloading a disposal mechanism. In man, the endogenous cholesterol is in negative balance to the extent of 0·3 g./day (Frazer, 1953). Whether cholesta-3:5-dien-7-one is on a normal pathway of synthesis or degradation cannot be stated; too little is known about cholesterol biosynthesis or metabolism.

More work on the origin and biological properties of cholesta-3:5-dien-7-one is necessary.

SUMMARY

1. Cockerels maintained from the 8th to the 16th week of age on diets containing cholesterol (2 g./bird/day) or cholesta-3:5-dien-7-one (0·33 g./bird/day) have been compared with control birds on the unsupplemented diet.

2. The blood cholesterol remained between 105 and 152 mg./100 ml. whole blood for the controls and the 'dienone'-fed birds, but reached 526–860 mg./100 ml. for those given cholesterol.

3. The cholesta-3:5-dien-7-one was poorly absorbed and there was no significant change, compared with the controls, in liver unsaponifiable matter, liver cholesterol or liver vitamin A. The intestines were, however, much heavier than those of the controls, and contained a larger amount of total unsaponifiable matter and of cholesterol.

4. The cholesterol-fed birds had enlarged fatty livers with a more than tenfold rise in unsaponifiable matter (nearly all due to cholesterol) compared with the controls. Nearly 40% of the liver vitamin A store had been lost. The liver and intestine unsaponifiable matter contained respectively 16–18 mg. and 6–7 mg. of cholesta-3:5-dien-7-one. This material is regarded as a metabolite and not a laboratory artifact.

5. The presence of a substance showing $\lambda_{max.}$ 272 mμ. (in light petroleum) in the livers of all three groups of cockerels provides additional evidence that the material is a normal constituent. It is accompanied by a substance of similar chromatographic properties which gives rise to an absorption band near 315 mμ. in concentrated sulphuric acid.

We wish to express our gratitude to Messrs J. Bibby and Sons Ltd., for their co-operation, and to the Medical Research Council for financial support. We are indebted to Professor H. L. Sheehan for the histological tests.

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The Partial Purification of Leaf Ribonuclease

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Nucleic acids have been found in every virus preparation that has so far been made and they are widely held to play a part in protein synthesis. For these reasons, and also because of their intrinsic interest, the nucleases have been extensively studied. They are generally thought to split nucleic acids into oligo- or mono-nucleotides and this may well be part of their function *in vivo*; but it is also possible that they take part in the synthesis of nucleic acid and in the rearrangement of components in it or in polynucleotide fragments.

Ribonucleases (RNase), that is enzymes that degrade ribonucleic acid so that it no longer shows its typical macromolecular properties, are widely distributed. They have, for example, been studied in pancreas and other tissues, snake venom, bacteria,

and the leaves, shoots and seeds of several plants. Most of the published work has dealt with the pancreatic enzyme, and in some papers the unqualified word 'ribonuclease' is used as if it could only refer to this enzyme. This restriction is unreasonable and, because enzymes from different sources have significantly different properties, the enzyme source should always be stated.

There is now substantial unanimity (cf. Brown, Heppel & Hilmoe 1954; Markham & Smith, 1954) that in ribonucleic acid the nucleotides are connected predominantly, even if not exclusively, by phosphate bridges connecting position 5 in the ribose of one nucleotide to position 3 in the ribose of the next. Every RNase is a phosphodiesterase because it is able to split this phosphodiester link and the distinction sometimes made between RNase and a phosphodiesterase is misleading. Differences arise because some preparations of RNase break the link connecting phosphate and position 5 in a ribose, while others break it on the other side between the phosphate and position 3. There is no evidence to suggest that a RNase can break it in both positions nor that any can act as phosphomonoesterases as well and release free PO_4^{3-}.

Five, or possibly six, different bases are known as components of ribonucleic acids and the susceptibility of an internucleotide link to attack depends on which bases are present in adjacent nucleotides. There is, therefore, ample foundation for some specificity in RNase action, and there is no reason to think that there are fewer RNases than there are, for example, proteases. The predominant RNase of pancreas (PRNase) appears to be more specific than the enzyme from other sources; perhaps because its purification has been carried the furthest. PRNase cannot, for example, split the link between purine nucleotides nor those between a purine nucleotide and position 5′ in a pyrimidine nucleotide. PRNase does not therefore convert nucleic acid completely to mononucleotides but a residue remains which still precipitates with many of the reagents used to precipitate nucleic acid, and some material also remains indiffusible through cellophan. These are probably, but not necessarily, properties of the same substance (commonly called 'core'). Leaf enzyme (LRNase), on the other hand, carries the hydrolysis so far that no 'core' is left (Pirie, 1950; Shuster & Kaplan, 1953). Neither enzyme can, however, act on deoxyribonucleic acid or its breakdown products.

This paper deals with the purification of the enzyme from pea seedlings and with the specificity of its action. Many of the enzymes present in the original extract along with ribonuclease are no longer present in the final preparation but no claim is made that all the activities in the final preparation are due to the same enzyme. Some observations on tobacco leaves are also included, but these leaves are less satisfactory for the purification of the enzyme because they give extracts which are of lower activity and contain other proteins that are more difficult to remove than those in pea-leaf extracts.

METHODS

Phosphorus. This was determined by the method described by Holden & Pirie (1955*b*). Inorganic P was estimated by developing colour in samples without previous incineration.

Nitrogen. This was determined by a micro-Kjeldahl method and the NH_3 measured either by titration with HCl or by nesslerization. To determine the amount of trichloroacetic acid (TCA)-precipitable N in enzyme solutions, an equal vol. of 100 g./l. TCA was added, the precipitate centrifuged down at 5000 *g*, washed with 50 g./l. TCA, and then dissolved in a small volume of water with the addition of a few drops of NaOH.

Dry matter. Samples were dried in an oven at 100° overnight.

pH *measurements.* These were made with a glass electrode.

Measurement of enzyme activities

Thymol was used as an antiseptic throughout and the fluids were incubated at 37°.

Ribonuclease. In an accompanying paper (Holden & Pirie, 1955*b*) we discuss the merits of the different methods for following the action of a nuclease and define the conditions under which precipitation by uranyl nitrate in trichloroacetic acid (UrTCA) is satisfactory. The experiments described in that paper were made with the nucleic acid concentration in the physiological range (20 mg. P/l.) because we were interested in processes that might play a part *in vivo*; for the same reason we worked at 25°. In the assay of ribonuclease preparations it is convenient to use more concentrated solutions of nucleic acid (0·4 g. P/l.) and to work at 37°. Purified commercial yeast nucleic acid (YNA) and material made from yeast in the laboratory, both prepared by the methods described elsewhere (Holden & Pirie, 1955*a*), were used as substrates and no large differences between them were noticed. For the tests a total volume of 4 ml. contained the sodium salt of YNA to give 0·4 g. P/l., sodium citrate buffer 25 mM (pH 6) and enzyme solution. In buffers other than citrate there are some differences between different substrate preparations (cf. Holden & Pirie, 1955*b*).

Phosphomonoesterase. The amount of inorganic P liberated from sodium β-glycerophosphate was used as a measure of enzyme activity. A vol. of 4 ml. contained glycerophosphate at pH 5 to give 0·6 g. P/l., sodium citrate buffer 25 mM (pH 5) and enzyme solution.

When the enzyme activity of unfractionated sap is being measured it is necessary to remove protein from the samples by precipitation with TCA before analysis, but after the first step in the purification so little protein is present that this is unnecessary. The sample is pipetted directly into the mixture of H_2SO_4 and molybdate, and the colour is developed with $SnCl_2$ as in the method for total P.

A few measurements only have been made with other P-containing substrates such as metaphosphate, glucose 6-phosphate and adenosine triphosphate. These were done at pH 6 in 10 mM citrate with the substrates at 100 mg. P/l.

Deoxyribonuclease. There are two possible substrates for

this measurement; intact or at least not deliberately degraded deoxyribonucleic acid (DNA), and oligonucleotides made from it. We have used both because the enzyme (DNase) from some sources acts preferentially on one of the substrates but we have no evidence that this is the case with leaf extracts. The DNA was a commercial product from thymus (British Drug Houses Ltd.) and the oligonucleotide was made from it by incubation with 'Streptokinase–Streptodornase' of bacterial origin (Burroughs Wellcome Ltd.) at pH 7 in the presence of Mg^{2+}. Incubation was continued until there was no precipitation with HCl but the oligonucleotides were still almost completely precipitated by UrTCA. This is a clearly defined point because the bacterial enzyme only carries the action so far as to make UrTCA-soluble products if large amounts are used and the incubation is prolonged. The oligonucleotide solution was used without further fractionation.

For the measurements a volume of 4 ml. contained one or other substrate at 0·4 g. P/l., sodium diethylbarbiturate + sodium acetate buffer, 14·3 mM with respect to each anion (pH 6), $MgSO_4$, 12·5 mM, and enzyme solution. Samples were removed at intervals, and precipitated either by adding an equal volume of 0·5N-HCl or of the UrTCA reagent according to which substrate is being used. The precipitates were centrifuged down and P was determined in the usual way.

Definition of an enzyme unit

For these four main measurements of activity the reaction mixture was assembled at 0° and a sample taken. Further samples were taken after 0·5, 1 and 2 hr. at 37°. The amount of enzyme needed to make in 1 hr. 31 mg. P/l. soluble in the nucleic acid precipitant, or to increase the amount of PO_4^{3-}-P by 31 mg./l., is taken as one unit and the assay is repeated with different amounts of enzyme preparations until 0·5–1·5 units are present in the test mixture.

For the phosphatases other than glycerophosphatase we use no unit system.

Phosphodiesterase. The liberation of phenol from diphenyl phosphate (Bios Laboratories Inc., New York) was used as a measure of activity. A volume of 4 ml. contained substrate to give 0·6 g. P/l., sodium acetate buffer 40 mM (pH 4·5) and enzyme solution. A sample was removed after mixing enzyme and substrate and another after incubating for 1 hr. at 37° and phenol determined using the Folin–Ciocalteu (1927) reagent.

One unit of phosphodiesterase activity is the amount of enzyme which will liberate 94 mg. phenol/l. under the above conditions.

Peroxidase was determined by the method of Keilin & Hartree (1951). An amount of preparation containing 1 enzyme unit (EU) would form 1 g. purpurogallin under the standard conditions of the method.

Catalase was determined by the method of Euler & Josephson (1927).

EXPERIMENTAL AND RESULTS

Distribution of RNase. RNase activity was found in the sap from leaves of all the species tested which included broad bean (*Vicia faba* L.), french bean (*Phaseolus vulgaris* L.), tomato (*Lycopersicum esculentum* Mill.), potato (*Solanum tuberosum* L.), dead nettle (*Lamium album* L.), groundsel (*Senecio vulgaris* L.), narcissus sp., bracken (*Pteridium aquilinum* Kuhn) and bryony (*Bryonia dioica* Jacq.).

A few measurements have been made of the distribution of RNase in leaf fractions of both pea and tobacco. Extracts of the washed fibre made with 0·2M sodium citrate at pH 7·5 contain about 0·33 as much enzyme as the original sap and washings. The amount of enzyme in the precipitates that can be centrifuged from the sap at between 2000 and 10000 *g*. in 30 min. can be accounted for by the sap included in the precipitate but 0·2–0·1 of the activity is associated with the material sedimenting at 100000 *g*. Some properties of this enzyme association have already been described (Pirie, 1950) and more will be in a later paper.

Change in levels of RNase and phosphatase during growth of pea seedlings. Dried peas were soaked in water overnight and a weighed sample ground with 0·1M sodium citrate buffer (pH 5) in a high-speed macerator (Measuring and Scientific Equipment Ltd.). Both RNase and phosphatase were at a very low level in the extract. Soaked peas were sown in compost in boxes, harvested at intervals from 2 to 17 days, and ground with citrate solution, 25 ml./20 g. wet wt. At each sampling, duplicate lots were taken and sometimes a third which was divided into shoots, roots and cotyledons. After being weighed the three portions were ground separately and enzyme activities determined in the extracts.

The RNase and phosphatase activity expressed as units/g. wet wt. of tissue rose rapidly between the 2nd and 6th days after sowing and then remained fairly constant until the 10th day. The RNase level was then about 40 units/g. wet wt. and phosphatase 70 units/g. wet wt. After this the RNase activity fell gradually, whereas the phosphatase remained at nearly the same level. The total units of phosphatase per pea plant continued to rise during the whole experiment and the total units of RNase rose until the 13th day and then fell slightly by the 17th day. At 6 days 70 % of the total phosphatase and RNase activity of the plant was in the cotyledons; at 10 days about 45 % and at 17 days 30 %. The amount in the shoot increased from 20 % of the total at 6 days to 45 % at 10 days and to 60 % at 17 days. The activity of both enzymes per g. wet wt. of the root tissue was lower than in the shoot and the root contained only a small proportion of the total activity.

Preparation of LRNase

As a compromise between getting too small a yield and getting too large a proportion of other protein in the initial extract, pea plants grown for 2–3 weeks in a glasshouse or 3–4 weeks out of doors were generally used. They were cut off above the cotyledons because, although there is RNase in the cotyledons, the activity per mg. N, of preparations made from them, is only one-tenth of that of pre-

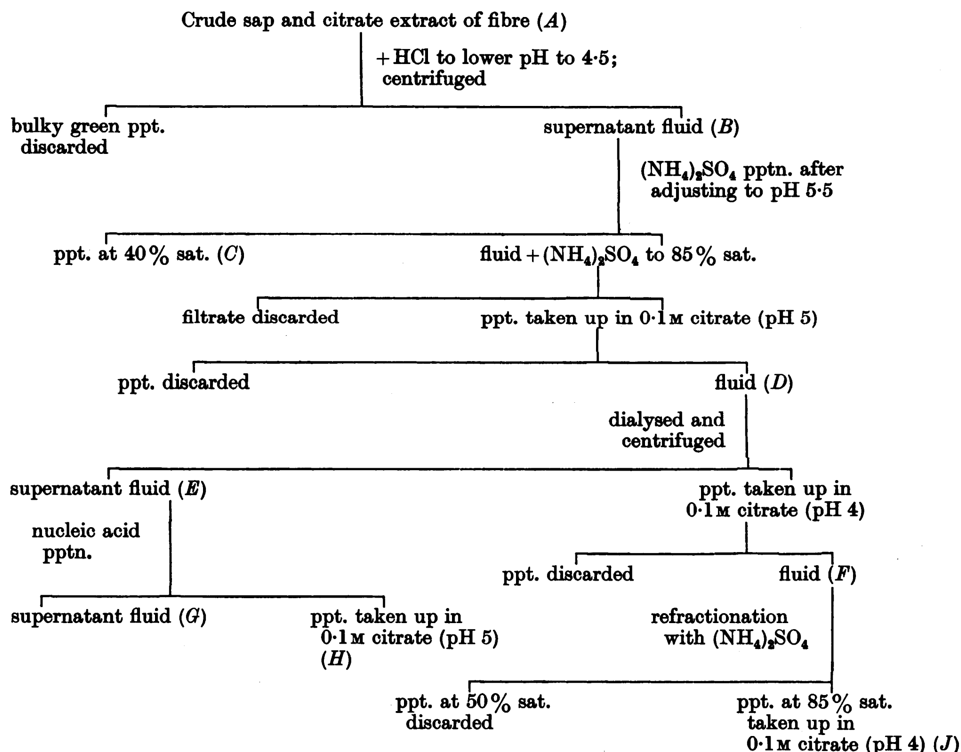
parations made from leaves alone. The separation of enzyme from residual seed protein has not yet been carried so far as the separation from inert leaf protein. Tobacco leaves (*Nicotiana tabacum* var. White Burley) grown in a glasshouse were used in some experiments but the sap has only one-quarter the enzyme activity of pea sap.

The course of fractionation is shown in Scheme 1 and the different stages are described below. On each fraction the TCA-precipitable N was determined and on dialysed fractions the dry matter content was also measured. RNase activity of the various fractions was compared with the activity on other substrates. The purity of enzyme preparations (U/mg. N) is expressed as RNase units/mg. TCA-precipitable N.

some other leaf enzymes, e.g. phosphomonoesterase, we have not found it advantageous to use heat at any stage in the purification.

Removal of inert protein at pH 4·5. The sap and fibre extract (*A*) were adjusted to pH 4·5 with HCl. After standing for a few hours in the cold room the suspension was centrifuged at 1400 *g* and the green deposit discarded. The removal of this precipitate brought about an approximately tenfold increase in the U/mg. N of the RNase (and also of the phosphatase) and caused a loss of 10–15 % in the total activity; this was entirely due to loss of fluid in the bulky precipitate. Catalase activity is removed at this stage. Some increase in U/mg. N can be got by storing extracts at pH 4·5 and 0° for several weeks, and removing the precipitate formed, but this is

Scheme 1. *Fractionation of pea-leaf sap*



The leaves and stems were minced in a domestic meat mincer and the sap squeezed out by hand through strong cotton cloth. The fibre was extracted with 0·2 M trisodium citrate and the extract added to the sap. In making preparations of PRNase it is usual at some stage to heat for a short period to boiling because that enzyme is unusually thermostable. LRNase is less thermostable (Holden & Pirie, 1955*b*) and, although it is more stable than

not a necessary step and does not give a better final product. The clear brown supernatant (*B*) was adjusted to pH 5·5 by addition of NaOH before fractional precipitation with ammonium sulphate; there is loss of enzyme activity if the precipitation is done at pH 4·5.

Fractionation with ammonium sulphate. Ammonium sulphate (30 g./100 ml.) was added and after standing at 0° for several hours the precipitate

was centrifuged down at 1400 *g* and taken up in 0·2 M sodium citrate buffer pH 5 (*C*). Up to one-quarter of the total RNase activity was in this fraction when extracts of pea leaves or mature tobacco leaves are used but with young tobacco leaves it contains most of the activity. To the supernatant fluid from the first ppt. more ammonium sulphate was added so that it was about 85% saturated. The precipitate was filtered off on to Hyflo Supercel (Johns Manville and Co. Ltd.), and taken up in pH 5 citrate buffer (*D*), any insoluble material was discarded. The enzyme in fraction *C* can be purified further but the main preparation proceeds from *D*.

Dialysis. The solution *D* was dialysed in cellophan sacs against distilled water, which was changed frequently, for 24 hr. at 0°. During dialysis a ppt. formed and this was taken up in pH 4 citrate buffer (*F*) and the insoluble portion discarded. More than one-third of the total RNase activity but less than 3% of the phosphatase remaining after dialysis was associated with the precipitate. *F* was refractionated with ammonium sulphate and the 50–85% precipitate (*J*) which contained the RNase had no activity detectable under the conditions of assay on DNA, DNA 'oligonucleotides', and diphenyl phosphate. It was also substantially free from peroxidase activity and the activity on glycerophosphate was only just detectable. If *J* is dialysed a precipitate appears in the dialysis sac but little of the RNase activity is associated with it, and the enzyme, which until this stage had been stable, becomes much less so.

When the 40–85% sat. ammonium sulphate ppt. from tobacco-leaf extracts is dialysed there is little adsorption of the RNase on the precipitate in the sac. This method of purification is, therefore, convenient for making highly specific enzyme preparations from pea seedlings but it is not general and it recovers only part of the enzyme.

Removal of peroxidase activity. Peroxidase is precipitated by ammonium sulphate under the same conditions as the RNase and is a major contaminant of crude preparations. It could be removed by precipitation of the RNase (and phosphatase) by YNA. To 50 ml. of a dialysed enzyme solution (*E*), containing 10 000 RNase units, 100 mg. of the sodium salt of YNA were added at 0°. The pH was then adjusted to 3·8 with dil. HCl, the suspension allowed to stand for about 1 hr. at 0°, and the precipitate centrifuged down. Some precipitate appears immediately the YNA is added and more comes out on acidification. The precipitate was extracted with 0·1 M sodium citrate buffer (pH 5) and the insoluble material discarded. This extract then contained the bulk of the RNase and phosphatase activity, but only about 10% of the original peroxidase activity. A second cycle of YNA precipitation will remove all but traces of peroxidase activity.

Much of the DNase is apparently destroyed by this treatment, but what remains is mainly associated with the precipitate. There is, therefore, a useful but incomplete removal of DNase.

Other procedures investigated as possible methods of purification

Adsorption on calcium phosphate. Calcium phosphate (prepared according to Singer & Kearney, 1950) adsorbed less RNase than either phosphatase or peroxidase from dialysed enzyme solutions but the difference was not sufficiently great to be of use.

Adsorption on Celite. Erratic and variable results were got when adsorption from dialysed enzyme solutions on Celite in the pH range 3–6 was tested and this procedure was not investigated further.

Fractional precipitation with organic solvents. Acetone, ethanol and methanol precipitate the enzyme along with other proteins and we have not achieved any useful fractionation with them.

Comparison of LRNase with PRNase

In the fractionation set out in Table 1, the final preparation (*J*) contained 4100 units/mg. of TCA-precipitable N and other preparations made in the same way have given activities similar to this. One unit of RNase activity is, therefore, associated with about 1·5 μg. of protein with the average N content. These preparations are by no means homogeneous; for example, they contain some other enzymes besides RNase and they can still be separated into fractions with different precipitabilities with $(NH_4)_2SO_4$ but all carrying RNase activity. It is therefore interesting that their activity is the same as that of PRNase. Under the conditions of assay used 1 unit of RNase is carried by 1–2 μg. of commercial PRNase or of crystallized preparations made in the laboratory.

The specificity of action of the enzyme preparation

Little comment is called for on the separation from our preparation of many of the enzyme activities that we have tested for. The nature of the substrate is clear and it is intrinsically improbable that the same substance should be both peroxidase, for example, and nuclease; the absence of enzyme activity is therefore readily accepted as evidence for the absence of the enzyme. The position with the various phosphoric esterases is less simple. As Table 1 shows, the final product does not act on glycerophosphate or diphenyl phosphate. Boroughs (1954), using *p*-nitrophenyl phosphate as substrate, has suggested that metals such as Cu are essential cofactors for the action of leaf phosphatase and this suggests the possibility that our preparation is inactive because of the loss of the cofactor. There is

Table 1. *The activity towards different substrates of material obtained at successive stages in the fractionation of 2l. of extract*

	Units/mg. N							
				DNase measured on				
Fraction	RNase	Phosphatase	Diphenyl-phosphatase	DNA	Oligonu-cleotides	Peroxidase	Total RNase units	Yield of RNase (%)
A	18	—	—	—	—	—	67 300	100
B	138	256	16	75	113	0·262	57 200	85
C	39	208	15	32	32	—	5 460	8
D	660	1020	34	74	126	—	50 000	74
E	318	1080	28	95	127	0·400	21 200	32
F	2400	174	5	3	16	0·038	18 000	27
J	4100	80	<1	<1	<1	0·005	14 800	22

no need to look for such a phenomenon in our experiments because the total phosphatase in the original sap is satisfactorily accounted for by the phosphatase found in the fractions other than the final LRNase. We have, however, added Cu^{2+} at 1–0·1 mM to several preparations without eliciting any glycerophosphatase activity in them, and though, using pea sap, we confirm Boroughs's (1954) statement that phosphatase activity is destroyed by dialysis at pH 2·0, we have been unable to restore activity by adding Cu^{2+}.

Sap from pea and tobacco leaves contains enzymes able to hydrolyse metaphosphate, glucose 6-phosphate and adenosine triphosphate and these activities are, in part, carried out on the microsome fraction (NP) that can be centrifuged from the sap (Pirie, 1950). We have not been able to remove these activities so completely from the final LRNase preparation, but the ratio of RNase activity to phosphatase activity has been sufficiently enhanced to make it unlikely that both are activities of the same enzyme. Comparisons were made of the rate of initial action on these three substrates with purified LRNase and with pea sap from which most of the protein had been removed by acid precipitation (stage *B* in the scheme), and most of the phosphate and phosphoric esters by dialysis against several changes of water for 3–4 days. To get equal rates, amounts of the purified preparation were needed which contained 8–12 times as much RNase as was present in the dialysed sap.

A low level of DNase activity was observed in tobacco-leaf sap, and in fibre extracts, using commercial thymus nucleic acid as substrate (Holden, 1952). The action was measured by following the disappearance of precipitability with UrTCA. This measures two sequential actions: DNA has first to be converted into 'oligonucleotides' and these are then broken down further. It is more convenient to follow the two stages separately; first by using DNA as substrate and HCl as precipitant, and then by using DNA 'oligonucleotide' as substrate and UrTCA as precipitant. The study of this enzyme system is also made easier by the use of pea extracts for they are much stronger than extracts of tobacco.

The figures in Table 1 show that activity towards both substrates is removed from RNase preparations during purification and we do not think that the differences which appear in the table, in the ratio of the activities towards the two substrates with different enzyme fractions, are large enough to be used as a basis for postulating two different enzymes. Nor are they sufficiently consistent in different preparations. The behaviour when pH is varied is, however, different. With DNA as substrate there is a broad optimum at about 5·4, whereas with oligonucleotides it is sharp and at pH 5·0. Frisch-Niggemeyer, Keck, Kaljunen & Hofmann-Ostenhof (1951) described a DNase of garlic, but its optimum pH was at 6·5 so it appears to be distinct from the enzyme from peas.

DNA, heated in a boiling-water bath for 1 hr. with N-NaOH, to cause depolymerization to non-dialysable polynucleotides (Zamenhof & Chargaff, 1950), was tested as substrate for LRNase preparations. Both this, and DNA only slightly degraded by 'Streptodornase', were also attacked by crude but not by purified preparations of LRNase.

'Thymic acid', another breakdown product of DNA, has been tested as substrate for leaf-enzyme preparations because of the remarkable statement of Durand & Thomas (1953) that this material was attacked by PRNase. (The preparations of PRNase that we have used, unlike those used by Durand & Thomas, do not make 'thymic acid' acid soluble.) To prepare 'thymic acid', DNA was heated at 37° for 3 hr. with N-HCl, the insoluble material centrifuged down, washed with ethanol and ether and dried. This substance was converted into acid-soluble material by crude LRNase preparations, but not by those that had been highly purified.

For these reasons we look on LRNase as a ribonuclease rather than an unspecific phosphodiesterase, but as an enzyme with a wider range of specificity than PRNase. This is most clearly shown by its ability to breakdown YNA 'core'. Prepara-

tions of 'core' were made by incubating YNA with PRNase for several days at 37° and then dialysing for a week. To the solution from the dialysis sac 7 vol. of glacial acetic acid were added; to the mixture an equal volume of ethanol was added and the precipitate was centrifuged down, washed with ethanol and ether, and dried. These preparations are completely resistant to PRNase.

Several different preparations of 'core' have been compared with YNA and there is no consistent difference in the rates of enzyme action and we attribute the small differences that have sometimes been found to variations in the contamination of our YNA control by metals. This is treated more fully in a later paper (Holden & Pirie, 1955*b*). These comparisons have been made with both crude and highly purified LRNase with the same result. There is, therefore, no reason to postulate an enzyme in the leaf with a specificity similar to that of PRNase which is being separated from the main LRNase preparation during the fractionation.

Purified LRNase preparations do not, however, attack all the phosphate bridges in YNA with equal readiness but attack preferentially those not susceptible to PRNase. This phenomenon is illustrated in Fig. 1. In this experiment YNA was incubated with different amounts of LRNase. PRNase was then added to part of each digest and the incubation was continued. By determining at intervals the P not precipitated by UrTCA the extra hydrolysis brought about by the PRNase can thus be ascertained. It is clear that the fission of some bonds by LRNase does not diminish the number available for PRNase attack. There is indeed an apparent increase. Thus the intercepts between the ends of curves *A* and *E*, or *B* and *F* represent a larger amount of UrTCA-soluble P, than the 30% fission that the same amount of PRNase produced when acting alone (*D*). Even when LRNase has carried the action more than half-way, there are still nearly the normal number of bonds apparently available to PRNase (*C*–*G*).

One possible interpretation of this phenomenon is that there may be bonds in YNA potentially susceptible to PRNase but not accessible to it until they have been uncovered by LRNase. If this were so the effect with 'core' should be even more striking. There is, however, no such effect at all. PRNase, whether added at the beginning along with LRNase or added at different stages of LRNase digestion does not increase the rate of appearance of UrTCA-soluble P from 'core'. There is, therefore, no reason to think that any bonds in YNA are inaccessible to PRNase. The most probable explanation of the phenomena shown in Fig. 1 is that each enzyme acts more rapidly on partly degraded YNA so that more bonds are broken before the enzyme inactivation, which is a prominent feature of these experiments with minimal amounts of enzyme, becomes serious. This greater initial rate of attack by PRNase on YNA partly hydrolysed by LRNase is clearly seen in Fig. 1, and it is probable that during the PRNase action the effectiveness of the residual LRNase is also enhanced.

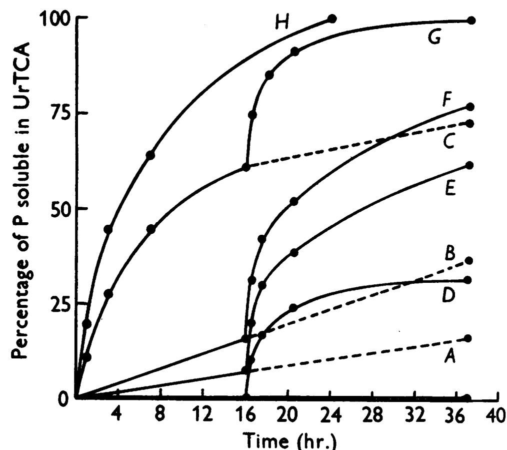


Fig. 1. The effect of preliminary incubation with LRNase on the action of PRNase on YNA. Solutions of YNA at 0·44 g. P/l. in 0·025M sodium citrate (pH 6) were incubated at 37° with LRNase (*A*) 0·03 U/ml., (*B*) 0·06 U/ml., (*C*) 0·3 U/ml., (*H*) 0·6 U/ml. and another solution was incubated without added enzyme. At the end of 16 hr. (and at intervals during the incubation for samples *C* and *H*) samples were removed for determination of UrTCA-soluble P. Each solution, except *H*, was then divided into two; to one lot PRNase was added (one-tenth volume of the solution) at the rate of 1 μg./ml. and to the other the same vol. of water was added and the P content was then 0·4 mg./l. Incubation at 37° was continued and samples withdrawn at intervals for determination of UrTCA-soluble P.

DISCUSSION

Ribonuclease was recognized in the leaves, roots and seeds of plants by Jono (1930) and he examined in particular the products of the action of extracts from soybean shoots on YNA. Schlamowitz & Garner (1946) extended this work and reported the nearly complete removal of phosphomonoesterase from the enzyme preparation. Bheemeswar & Sreenivasaya (1953) have partially purified the enzyme from sprouted castor beans and Shuster & Kaplan (1953) used sprouted barley or rye grass. Axelrod (1947) commented on the heat stability of the ribonuclease present in phosphatase preparations made from citrus fruit. No one has claimed that the enzyme from a plant source is homogeneous and the preparations have generally been shown to carry other enzyme activities.

The action catalysed by extracts from leaves (Pirie, 1950) and sprouted barley (Shuster & Kaplan, 1953) resembles that catalysed by the enzyme from spleen and some snake venoms in being more extensive than the action catalysed by PRNase but the nature of the end products has not yet been established. Parker (1952), on the basis of the solubility of the Ba salts, recognized mononucleotides in LRNase hydrolysates but did not characterise them, and Shuster & Kaplan (1953) mention preliminary evidence that 5′-nucleotides are a product of the action of the barley enzyme, which suggests that this enzyme, unlike PRNase, splits the molecule between the phosphate and carbon-3 on the ribose. There is no published evidence that mononucleotides are the sole, or even the principal, products of the action and there may be oligonucleotides that differ from those remaining after PRNase action by being soluble in UrTCA. Finally there is no evidence that only one type of RNase is present in leaf extracts; it is only for convenience that we have referred to LRNase as if it were one enzyme. All that is clear is that LRNase is not an unspecific phosphodiesterase because it is only crude preparations that carry activity towards diesters unrelated to RNA.

This fractionation was undertaken to ascertain the range of substrate specificity of LRNase and it has only been carried far enough to satisfy us that phosphate esters and polynucleotides other than those containing ribose are not attacked. Incidentally, the activity of the enzyme per mg. of N has been increased 230-fold and the final product is as active on suitable substrates as are crystallized preparations of the pancreatic enzyme. At this stage there is a marked diminution in the stability of LRNase so that, for our purpose, there is no advantage in carrying the fractionation further. The final preparations are obviously inhomogeneous and none of the usual criteria of purity have yet been applied to them. Further work on the fractionation is in progress.

SUMMARY

1. From pea seedlings ribonuclease preparations have been made which attack P-containing substrates other than ribonucleic acid so slowly as to make it unlikely that the enzyme has an unspecific action.

2. The enzyme differs from pancreatic ribonuclease in that it hydrolyses nucleic acid so completely that no acid-precipitable 'core' is left.

3. Less thoroughly fractionated preparations have been made from tobacco leaves and some properties of the enzyme in other leaves are described.

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The Preparation of Ribonucleic Acid from Yeast, Tobacco Leaves and Tobacco Mosaic Virus

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There is no conclusive evidence that nucleic acid ever exists *in vivo* in the free state. Markham (1953) has argued that in some situations, e.g. turnip yellow mosaic virus, it is free and simply held as a clathrate complex inside a protein cage so that it is liberated when the cage is opened. But the treatments needed for the opening, though indubitably unusually gentle, denature some proteins and denaturation is often all that is needed to release a prosthetic group. In other nucleoproteins the