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A Comparison of Leaf and Pancreatic Ribonuclease

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The ribonucleases are relatively specific phosphodiesterases and their action can be followed by methods that depend either on the appearance of an acid or hydroxyl group when the ester link is broken or else by methods that detect changes in the particle size of the nucleic acid. It may be profitable to discuss the merits of the various methods that have been used, paying particular attention to their applicability to nucleic acid and nucleoprotein solutions in which the concentrations of nucleic acid or nucleoprotein fall in the physiological range, e.g. equivalent to about 10 mg. P/l. Methods that depend mainly on the first steps in the attack on the macromolecule are of more interest than those that place equal weight on all its stages.

The appearance of a new acid group has been followed by measuring the increase in buffering power in the region pH 6·8–7·9 (Allen & Eiler, 1941) or manometrically in bicarbonate buffer (Bain & Rusch, 1944). The latter method has been used in several laboratories but it calls for nucleic acid concentrations as high as 5 g. P/l.

Diminution in the molecular weight of the nucleic acid has been followed directly by Carter & Greenstein (1946), who carried out the reaction in dialysis sacs and measured spectrophotometrically the split products that diffused out. This method depends mainly on the first stages of the reaction but it is tedious if many determinations are being made and, as Markham & Smith (1952) have emphasized, variations in the salt concentration cause variation in the diffusibility of partly hydrolysed nucleic acid. For these reasons, diminution in molecular weight is generally inferred from a change in the acid precipitability of the nucleic acid. Many different conditions have been used and, in the experimental section of this paper, we compare some of them.

On hydrolysis there is a diminution in the absorption of light at 300 mμ. (Kunitz, 1946), but the effect is not large and is mainly a consequence of the later stages of the action. There is an increase in absorption at 260 mμ., which is discussed more fully in another paper (Holden & Pirie, 1955*c*); this increase depends on the extent of hydrolysis but is not proportional to it. The volume of the solution increases and then diminishes (Vandendriessche, 1951) and the ability to bind trimethyl *p*-(*p*-hydroxybenzeneazo)phenyl ammonium chloride decreases (Cavalieri, 1952). Inorganic phosphate is a product of the action of some enzyme preparations but there is reason to think that this action is due to contamination with a phosphatase so that any measurements depending on it deal with two enzymes simultaneously.

It is important to remember that the different methods of following the reaction may measure different stages of it. Thus Kunitz (1940) found that acid appeared more slowly than acid-soluble P and

that when the action of pancreatic ribonuclease had apparently gone to completion, approximately one acid group appeared for every two atoms of P. Complete hydrolysis of an indefinitely large polynucleotide to mononucleotides would give one new acid group for each atom of P; on the other hand, the fission of an acid-precipitable tetranucleotide to two dinucleotides, neither of which is acid precipitable, would give four atoms of acid soluble P for each new acid group. The actual state of affairs appears, as would be expected, to be intermediate. Measurement of the appearance of acid groups includes in the same estimation the initial conversion of acid-precipitable nucleic acid into acid-soluble oligonucleotides and the further splitting of these to mononucleotides. It is not, therefore, well adapted for investigating the successive phases of the action, and it is particularly ill-adapted for following the first stage.

MATERIALS AND METHODS

The nucleic acids were made by the methods described in the accompanying paper (Holden & Pirie, 1955*b*).

Four preparations of pancreatic ribonuclease (PRNase) have been used. One made by Dr A. Klezkowski by Kunitz's (1940) method, the two fractions separated by Martin & Porter (1951) from crystalline material made in the same way, and a commercial preparation (Armour and Co.). No differences were observed between them.

Many different preparations of enzyme from pea leaves (LRNase), made by the method described in the accompanying paper (Holden & Pirie, 1955*a*), and a few preparations from tobacco leaves were used. As stated in that paper the pea enzyme is the more highly purified but no qualitative difference between them was noticed.

Determination of the extent of enzyme action. Acid-precipitation followed by nephelometry is often used to measure the amount of unhydrolysed nucleic acid; alternatively the P content or light absorption at 260 mμ. of fluid from which the precipitate has been separated is used as an index of the amount hydrolysed. Thus Dubos & Thompson (1938) added HCl to give a final concentration of 0·2N, Schneider & Hogeboom (1952) used 0·2N-$HClO_4$, and Kunitz (1940) 0·5N-HCl or 10 vol. acetic acid. Acid-precipitation remains incomplete even with nucleic acid that is not, by other criteria, degraded. It is not improved simply by increasing the strength of the acid; 0·5N-$HClO_4$ precipitates less than 0·2N, and 1·3N precipitates very much less. It can be improved by various additions. Roth & Milstein (1952) used ethanol along with HCl, Caspersson, Hammarsten & Hammarsten (1935) added lanthanum salts, and Davidson & Waymouth (1944) added ethanol in addition to lanthanum salts. We find that it is not always possible to get clear supernatant fluids with these agents and, since they offer no particular advantages, have not examined them in detail. MacFadyen (1934) used uranyl acetate with trichloroacetic acid (TCA), and uranyl salts were also used by Kunitz (1940). In Table 1 the result of a comparison of these different conditions of precipitation with yeast nucleic acid (YNA) at different concentrations, is set out. Clearly, with these dilute solutions, it is only when ethanol or uranyl salts are present that precipitation is complete. Zittle (1946*b*) pointed out that unless strongly acid conditions were maintained during precipitation in the presence of uranyl salts, mononucleotides would precipitate also. The amount of P that is precipitated from a hydrolysate, and so the apparent extent of hydrolysis, will therefore depend not only on the initial concentration of the YNA but also on the concentration of the TCA. This is shown in Table 2. The final concentrations of P and uranyl nitrate in each precipitating mixture were 200 mg./l. and 2·5 g./l., respectively, but the concentration of TCA varied

Table 1. *Precipitation of YNA by different reagents*

Different amounts of a solution of YNA were diluted to the same volume so as to give twice the P concentration stated in col. 2. To this solution at 0° an equal vol. of reagent at twice the concentration stated in col. 1 was added. The final concentrations were therefore those stated. After 30 min. the mixtures were centrifuged and the supernatant fluids analysed.

	P mg./l. in	
Precipitant	Initial precipitating mixture	Supernatant after precipitation
2·5 g./l. uranyl nitrate and 12·5 g./l. TCA	15	<1
	7·5	<1
	4	<1
	2·5	<1
0·01N-HCl in 25% (v/v) ethanol	15	<1
	7·5	<1
	4	<1
	2·5	<1
0·1N-HCl	15	5·0
	7·5	2·8
	4	1·9
	2·5	1·3
0·1N-$HClO_4$	15	5·8
	7·5	3·2
	4	2·1
	2·5	1·4

Table 2. *Effect of different concentrations of TCA on the precipitability by uranyl nitrate of partly hydrolysed YNA*

A YNA solution containing 400 mg. P/l. was incubated in 0·025M citrate buffer, pH 6·0, at 37° with suitable amounts of the two enzymes. Samples of the reaction mixture were precipitated with equal volumes of the four reagents. For simplicity the results on only one pair of samples are set out. Reagent used as a precipitant contains 5 g./l. uranyl nitrate throughout.

	Percentage of P soluble after incubation with	
TCA concn. (g./l.)	LRNase	PRNase
100	17	23
40	16	18
25	13	16
10	12	12·5

from 5 to 50 g./l. It is clear that with both LRNase and PRNase there is less precipitation with the more concentrated acid and that the effect of varying the acid concentration is greater with PRNase.

Table 3 shows the dependence of the apparent extent of hydrolysis on the concentration of YNA and its split products in the precipitating mixture, the concentration of the uranyl reagent being kept constant. The more dilute the YNA the greater is the apparent hydrolysis. The result is similar when HCl is used instead of the uranyl–TCA reagent for precipitation.

Table 3. *Effect of varying the concentration of YNA in the precipitation mixture on the apparent percentage hydrolysis*

Solutions containing purified commercial YNA at 0·4 g. P/l. in 0·025 M citrate buffer, pH 6·0, were incubated at 37° with the enzymes specified. At intervals 0·5 and 0·05 ml. samples were taken from each and diluted with 0·5 and 0·95 ml. of water, respectively. 1 ml. lots of the UrTCA reagent were added to the four fluids and P was determined on suitable samples from the supernatant fluids.

Enzyme	Concentration of P in the precipitation mixture (mg./l.)	Apparent percentage hydrolysis after (hr.)		
		1	2	4
LRNase,	100	11	21	40
0·3 U/ml.	10	25	45	78
PRNase,	100	9·5	14	18
0·3 mg./l.	10	28	40	47

As a result of these measurements we have chosen as final concentrations 2·5 g./l. uranyl nitrate and 12·5 g./l. TCA and generally add a stock solution, called UrTCA, with twice these concentrations to an equal volume of digestion fluid when that contains 20 mg. P/l. When the digestion fluid contains 0·4 g. P/l. it is diluted with an equal volume of water immediately before precipitation. After standing for 30 min. at 0° the precipitate is centrifuged down for 10 min. at 1000 *g*. The amount of P precipitated from fluids in which hydrolysis has gone approximately half-way increases by 10–20% if the mixture is allowed to stand overnight at 0°.

Determination of phosphorus. Because of the presence of varying amounts of light-absorbing material in some of the enzyme and substrate preparations, and because buffers containing maleate, picoline and diethylbarbiturate were sometimes used, the extent of precipitation of nucleic acid has been measured by determining the P content of the fluid rather than its absorption at 260 mμ. When the sample of enzyme digest taken contained only 20–30 μg. P the amount of P in the precipitate was generally determined too.

Our method is based on that of Kuttner & Lichtenstein (1932) and it has been used during the last 20 years because more colour is given by the same amount of phosphomolybdate when it is reduced by $SnCl_2$ than by the other commonly used agents. With a visual colorimeter the method is suitable for 3–10 μg. P with an uncertainty of less than 0·5 μg. With a photoelectric colorimeter the convenient range is 0·1–2·0 μg. Because of its sensitivity and simplicity, and because all the P in the sample taken is used to produce the colour finally observed, it is well adapted to work on materials not available in quantity. We do not, however, claim any other merit for it over the standard methods.

To a sample of UrTCA supernatant fluid that is expected to contain about 10 μg. P, 1 ml. of 2 N-H_2SO_4 is added and the mixture is evaporated in a 15 × 150 mm. test tube, on an incineration rack or in an oven at 105°. The tube is heated on the rack until there are fumes of H_2SO_4, cooled, 1 drop of '60%' perchloric acid is added and heating is continued, gently at first and then sufficiently vigorously to fill the tube with acid fog. Clearing and decolorization are almost immediate. The operation from the time that the fluid is evaporated down takes 10 min. and large batches of tubes can be dealt with at once. 4 ml. of 2 N-H_2SO_4 are now added and each tube is brought to the boil, while being shaken over a bunsen burner, and then left to cool. Any metaphosphate that has been formed is thus hydrolysed. 1 ml. of 75 g./l. ammonium molybdate solution and 4 ml. of water are added and mixed well. Finally 1 ml. of a solution (approximately 2 g./l.) of $SnCl_2$ is added. It is important that this addition and mixing should be quick and complete; the $SnCl_2$ is, therefore, blown into the fluid from a narrow-tipped 1 ml. pipette and each tube is shaken immediately after the addition. The $SnCl_2$ solution is made up freshly for each set of determinations by diluting 1:200 a solution of 40 g. $SnCl_2$ in 100 ml. conc. HCl. Blanks are put up with each set and should give a colour corresponding to less than 0·1 μg. P. The colour is measured after a few minutes against a suitable standard that has been put through the same incineration procedure.

When the P content of the UrTCA precipitate is being determined, 1 ml. of 2 N-H_2SO_4 is added to the precipitate in the tube in which it was centrifuged down. It is digested in an oven at 105° for some hours and is then made up to a suitable volume and a sample taken for the determination, a complementary volume of 2 N-H_2SO_4 being added so that the usual 1 ml. is used for the incineration.

The final volume of fluid in which the colour is developed and measured is 10 ml. and it is obviously important that the various volumes that go to make this up should be pipetted with sufficient accuracy to achieve the precision of estimation that is being aimed at. The final volume is accurate to within 5% and when greater precision is needed the volume is adjusted in a graduated flask. The amount of colour developed depends on the concentration of H_2SO_4 in the final fluid; for this reason only 1/5 is added for the incineration so that even if most of it should be used up, the remaining 4/5 will maintain sufficient constancy. 1 ml. is sufficient for the incineration of 5 mg. of organic matter, which is more than the amount generally present in these experiments. If more is present, so that there is a charred mass rather than a pool of liquid after the initial evaporation, a further quantity of 2 N-H_2SO_4 is added, and heating is continued till the char is in solution, before adding the $HClO_4$.

RESULTS

Stability of ribonuclease. Jones (1920) recognized the unusual thermostability of the PRNase, and Kunitz (1940) showed that this property was retained by the purified enzyme, which could be heated at 100° for 30 min. in the pH range 2–4 with only a 20% loss of activity. A short period of boiling is therefore an important step in most of the

methods used for freeing PRNase from other enzymes and for getting crystalline preparations.

LRNase is much less stable but it is more stable than many other leaf enzymes. It is most stable between pH 4 and 5, and at pH 4·7 can be kept for many weeks at 0°. Fig. 1 shows the effect of pH on the stability of the enzyme at 20° and 37°. No activity was lost with 3 min. heating at 54°, but at higher temperatures the loss of activity was substantial and at 88° less than 2% was retained.

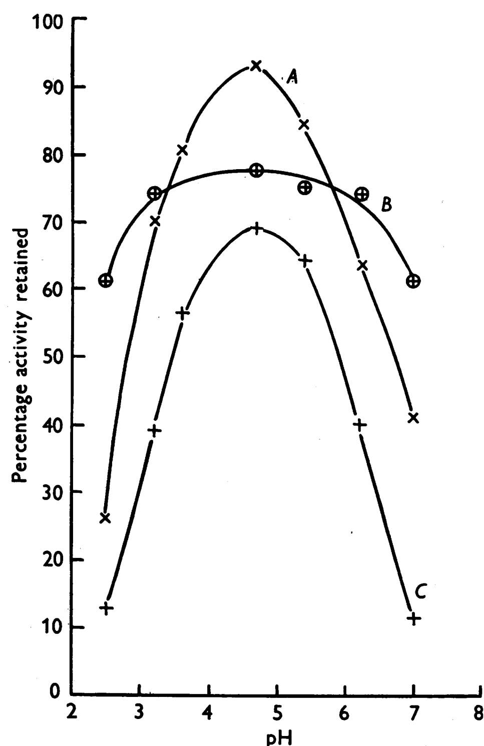

Fig. 1. Effect of pH and temperature on the stability of leaf RNase. Portions of a dialysed solution of partially purified pea RNase were diluted with equal volumes of Michaelis sodium diethylbarbiturate–sodium acetate buffers (0·0143 M with respect to both substances) of pH 2·5–7. These fluids were then either incubated at 37° and samples removed after 4 hr. and 17 hr. or kept at room temperature (*ca.* 20°) for 17 hr. The enzyme activities were then compared (Holden & Pirie, 1955*a*) with that of a sample diluted with pH 4·7 buffer which had been kept at 0°. *A*, 37° for 4 hr. *B*, 20° for 17 hr. *C*, 37° for 17 hr.

pH *optimum.* Fig. 2 shows the effect of pH on the activity of enzymes from the two sources. This comparison was made in buffer solutions which were 14·3 mM with respect to both sodium acetate and sodium diethylbarbiturate and adjusted to the required pH by the addition of HCl. There is clearly a significant difference in the position of the two pH optima though the optimum with LRNase is 0·2–0·3 of a pH unit nearer to that of PRNase if the values for 1 hr. rather than 7 hr. incubations are considered. This apparent shift is probably caused by the greater stability of LRNase at 37° at the lower pH. In a later section, evidence will be given that inhibition by metals such as Cu and Fe gets stronger as the solution is made more alkaline. The precise position of the optimum will, therefore, depend on the extent to which the substrate is contaminated by inhibitors such as these. The optimum for LRNase action was also at about pH 5·5 in experiments at 25° and in 10 mM maleate buffer with 20 mg. P/l.

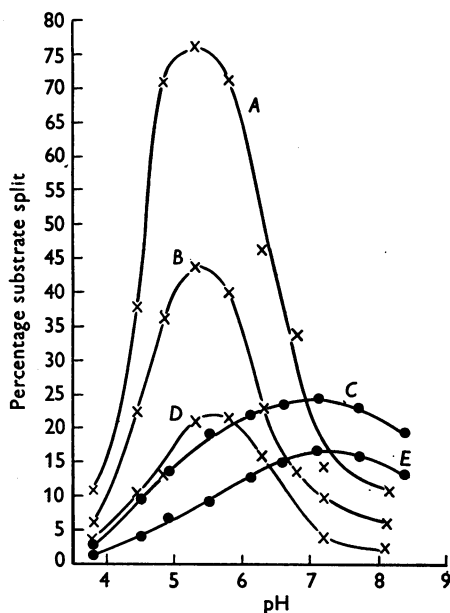

Fig. 2. Effect of pH on activity of leaf and pancreatic ribonucleases on YNA. For each pH value, 4 ml. of soln. contained YNA to give 400 mg. P/l., sodium diethylbarbiturate–sodium acetate buffer (0·0143 M with respect to both substances and adjusted to the required pH with HCl) and either PRNase, 1 mg./l., or a purified preparation of pea LRNase. The fluids were incubated at 37° and samples removed after 1 hr. and 3 hr. from the PRNase incubate and after 1, 3 and 7 hr. from the LRNase incubate. Total P was determined on the supernatant fluids after precipitation with UrTCA. ×, Leaf RNase; ●, pancreatic RNase. *A*, 7 hr. *B*, 3 hr. *C*, 3 hr. *D*, 1 hr. *E*, 1 hr.

Other plant species have not been examined systematically, but the pH optimum of preparations of tobacco (*Nicotiana tabacum*) LRNase is also about 5·5, whereas that of bryony (*Bryonia dioica*) is

near 6·5. It may be germane that the pH of tobacco sap is usually in the range 5·6–5·9, whereas that of bryony is 7·0 (Holden, 1948).

Effect of different ions on the action of ribonuclease on yeast nucleic acid. In the two preceding sections the amount of enzyme was determined by the assay method described in the accompanying paper (Holden & Pirie, 1955*a*) at 37° and with YNA solutions containing 0·4 g. P/l. These conditions are convenient but unphysiological for a leaf enzyme. When comparing the action of agents which might control the action of these enzymes *in vivo* therefore, it is preferable to work with 20 mg. P/l. at 25° and these are the conditions used in all the experiments in the remainder of this paper. Thymol was used throughout as an antiseptic.

The experiment set out in Table 3 showed that a diminution in the concentration at which partially hydrolysed YNA is precipitated by UrTCA can more than double the apparent percentage hydrolysis. It is clear therefore, that, for this reason alone no strict comparison can be made between experiments run under the two conditions; furthermore, the temperatures are different and it is not convenient, with dilute substrate, to make comparisons during such an early phase of the reaction as with more concentrated substrate. It is not surprising therefore that whereas with YNA at 0·4 g. P/l., we found (Holden & Pirie, 1955*a*) that 1 unit of LRNase was equivalent to 1–2 μg. of commercial PRNase, we find with dilute substrate that 1 unit is more nearly equivalent to 0·5 μg. PRNase. In spite of these differences we retain the quite arbitrary unit already defined (Holden & Pirie, 1955*a*). The experiments described here were made during a period of 5 years with many different enzyme and substrate preparations; there are, therefore, some variations in the rates of action under conditions that are ostensibly the same.

The use of dilute substrate has the advantage that it minimizes the effect of the various other components of nucleic acid preparations. The most obvious of these is the cation used to neutralize the acid. Levene & Simms (1926) found that two preparations of YNA required 0·62 and 1·0 molecule of alkali, respectively, for each atom of P to bring the pH to 6·0; five preparations used by Fletcher, Gulland & Jordan (1944) gave values between 0·56 and 0·82. The amount of alkali needed to bring those of our preparations that have been precipitated with acid, into solution at pH 6, comes in this range also and we assume that a similar amount of cation is associated with the preparations that have been precipitated by NaCl instead of acid. A solution of nucleic acid containing 20 mg. P/l. is 0·5 mM with respect to Na^+ if the nucleic acid needs for neutralization 0·78 molecule of NaOH for each atom of P, and in it the activating effects of certain ions would show clearly. In the presence of the 10 mM-Na^+ that accompanies a 0·4 g./l. solution they show less clearly and in the very much stronger solutions that are sometimes used they would not show at all.

Solutions of YNA in the different environments being studied were assembled and cooled in ice. A suitable amount of enzyme was added, 3 ml. of the solution were withdrawn and precipitated with UrTCA, and the remainder was incubated at 25°. Further samples were withdrawn and precipitated at suitable intervals. After precipitation and centrifuging in the manner already described, P was determined on samples from both the solid and the fluid. In calculating the percentage hydrolysis that is set down in the tables, the P value found on the fluid was used in the early stages of the digestion and that on the solid in the late stages; in the middle both were used. As a safeguard, P was always determined in the fluid after precipitating the initial 3 ml. sample, and controls without enzyme were incubated but in no experiment quoted was there any blank to be subtracted.

A buffer is needed to minimize the pH shift brought about by the hydrolysis of the ester link. Most of the experiments have been made in citrate, maleate or picoline; the first two with the pH adjusted with NaOH and the last with acetic acid. In picoline, therefore, the concentration of Na^+ or other metal ion may be very small and can be made even smaller if the substrate is made up by neutralizing acid-precipitated YNA with picoline. With commercial YNA, and purified preparations made from it, there are considerable differences between the rates of action in the different buffers; the rate is always greatest in citrate and least in picoline but the difference varies from a factor of 20 to only 1·5. In Tables 4 and 5 the phenomenon is illustrated with each enzyme. It is clear from these tables that when citrate is present as well as picoline or maleate a rate of action equal to, or at least similar to, the rate in citrate alone is established. This suggests that it is more likely that citrate acts by removing an inhibition than that the other buffers are inhibitory. Inhibition by the metals present in commercial YNA is the probable explanation and it is supported by the fact that activations similar to those brought about by citrate are given by 1–5 mM glutathione or ethylenediaminetetraacetic acid. When YNA prepared in the laboratory is used as substrate the effect is never so large as that shown in Tables 4 and 5, but there is invariably a 20–40% difference between the rates in picoline or maleate, and in citrate; this also is probably due to residual metal. We have already commented (Holden & Pirie, 1955*b*) on the presence of Fe in all the samples of commercial YNA that we have handled, and attribute most of the effect to it.

Tables 4 and 5 also show that the rate of action increases with an increase in the concentration of the buffer. Other salts bring about a similar increase and the effect of NaCl, $NaNO_3$ and $(NH_4)_2SO_4$ on LRNase in 3 mM citrate are shown in Table 6. Many other ionized substances increase the rate of action to approximately the same extent; for example, $MgSO_4$, $CaCl_2$, $Ba(NO_3)_2$, LiCl and the hydrochlorides or sulphates of bases such as hydrazine, benzylamine, heptylamine, diethylamine, guanidine and aminotrishydroxymethylmethane. All these act both in citrate and picoline buffer. Un-ionized substances such as ethanol, glucose and urea do not affect the rate when tested at 10 mM nor does the ampholyte, alanine. These salts and un-ionized substances have a similar effect, or lack of effect, on PRNase in citrate or picoline buffer. The activating effect of $MgSO_4$ in experiments where the total salt concentration is low is interesting because Mg^{2+} has a slight inhibitory effect on LRNase in the presence of stronger maleate or citrate. Even at 10 mM-Mg^{2+} the inhibition is only 30 %. With PRNase there is no significant inhibition by 10–0·5 mM-Mg^{2+} in citrate, diethylbarbiturate, maleate or picoline at pH values between 4·5 and 8·0 and with either purified commercial YNA or YNA made in the laboratory.

Table 4. *The effects of different buffers on the rate of action of leaf ribonuclease on purified commercial YNA*

All the buffers were at pH 6·0; a purified pea-leaf enzyme was used at 0·05 unit/ml. of final reaction mixture and YNA at 20 mg. P/l. Temp. 25°.

Buffer		Percentage hydrolysis after (hr.) 1	2	4
Picoline	3 mM	16	20	33
	10 mM	19	25	35
Maleate	3 mM	20	31	44
	10 mM	25	40	57
Citrate	3 mM	24	38	63
	10 mM	33	54	76
Picoline 10 mM + citrate	1 mM	20	30	48
	3 mM	24	43	66
	10 mM	29	53	74

Table 5. *The effect of different buffers on the rate of action of pancreatic ribonuclease on purified commercial YNA*

All buffers were at pH 6·0, and the YNA at 20 mg. P/l. The enzyme was the 'main peak' fraction made by Martin & Porter (1951) at 0·1 unit/ml. of final reaction mixture. Temp. 25°.

Buffer		Percentage hydrolysis after (hr.) 1	2	4
Picoline	5 mM	5	10	17
	10 mM	10	14	22
	20 mM	10	17	28
Maleate	5 mM	7	10	17
	10 mM	10	16	29
	20 mM	24	31	41
Citrate	5 mM	22	28	40
	10 mM	35	42	60
Picoline maleate	10 mM + 10 mM	22	31	38
Picoline citrate	10 mM + 10 mM	25	36	57
Maleate citrate	10 mM + 10 mM	38	44	60

Table 6. *The effect of three salts on the action of leaf enzyme*

YNA, made from yeast in the laboratory, at 20 mg. P/l. in 3 mM citrate buffer, pH 6·0. Incubated at 25° with LRNase at 0·05 unit/ml.

Added salt		Percentage hydrolysis after (hr.) 1	2	4
None		21	36	68
NaCl	10 mM	27	47	86
	30 mM	30	54	90
	100 mM	33	63	91
$NaNO_3$	10 mM	28	47	84
$(NH_4)_2SO_4$	30 mM	29	54	90

This point has been studied in some detail because Mg^{2+} inhibits the spontaneous fission of leaf nucleoprotein and also its fission by LRNase and PRNase; these phenomena will be described more fully in a later paper. Furthermore, Lamanna & Mallette (1949) using YNA at 10 times the concentration we use, got 50 % inhibition by 0·5 mM-Mg^{2+}. Lamanna & Mallette suggest that their results are of physiological significance and that Mg may be part of the normal mechanism controlling PRNase action. In these experiments they used enzyme at 0·1 g./l. to get a rate of action similar to ours with enzyme at 0·1 mg./l. but more recently (Mallette & Lamanna, 1954) they found inhibition at pH 8, though not at pH 5, with much less concentrated enzyme. Ceriotti (1949) also found that Mg^{2+} was an activator. The reason for this disagreement is by no means clear.

To varying extents the metals often found to inhibit enzyme actions, e.g. Cu, Fe, Mn, Mo, Pb, Zn, inhibit both enzymes and all those tested have, like Mg, inhibited LRNase as much as, or more than, PRNase. Zinc is an outstanding example of this and an experiment with it is set out in Table 7. In maleate buffer the rate of action of LRNase is diminished to one-third by 16 μM-Zn^{2+}, whereas 2 mM is needed to cause this inhibition with

Table 7. *The effect of zinc on the action of RNase from leaf and pancreas*

YNA, made in the laboratory, at 20 mg. P/l. and buffers at 10 mM incubated with the enzyme and Zn^{2+} concentration stated at 25°.

Enzyme	Buffer	Zn^{2+} concn.	Percentage hydrolysis after (hr.) 1	2	4
LRNase, 0·1 unit/ml.	Maleate	0	29	52	91
		8 µM	19	28	76
		16 µM	9	17	35
		31 µM	0	7	25
		62 µM	0	0	16
	Citrate	0	34	57	98
		0·1 mM	33	55	98
		2 mM	17	37	90
PRNase, 0·06 mg./l.	Maleate	0	20	33	51
		0·5 mM	13	23	45
		2 mM	6	11	15
	Citrate	0	27	40	55
		2 mM	27	39	54

PRNase. The table also shows that the inhibition is very much less in citrate. The results with Mn are similar though less striking and an experiment is set out in Table 8. The inhibition of the two enzymes is in about the same ratio with Fe^{2+}; thus, in pH 6 maleate LRNase is 70% inhibited at 0·2 mM, whereas PRNase is only 25% inhibited at this Fe concentration but is 70% inhibited by 1 mM. Both enzymes are 50% inhibited in pH 6 maleate by 17 µM-Cu^{2+}.

Table 8. *The effect of manganese on the action of RNase from leaf and pancreas*

YNA, made in the laboratory, at 20 mg. P/l. in 10 mM maleate buffer incubated with the concentration of enzyme and Mn^{2+} stated at 25°.

Enzyme	Mn^{2+} concn. (mM)	Percentage hydrolysis after (hr.) 1	2	4
LRNase, 0·1 unit/ml.	0	40	61	90
	1	15	26	80
	5	9	13	43
PRNase, 0·06 mg./l.	0	27	32	56
	5	24	26	38
	20	13	19	31

With all these metals the inhibition is greater in maleate or picoline at pH 7 than it is at pH 6 and in citrate the inhibition is small. For example, the inhibition of LRNase by 8 µM-Cu^{2+} is nearly complete in pH 7 maleate, whereas there is only 50% inhibition by 5 mM-Cu^{2+} in pH 6 citrate and this Cu concentration is sufficient to make the solution obviously blue. The pH dependence of metal inhibition, as has already been pointed out, makes the determination of the optimum pH somewhat uncertain. The effect is particularly striking with PRNase which has the more alkaline optimum, and an experiment illustrating this is set out in Table 9. The conditions are here extreme because the ratio of Fe to P in solutions containing 0·5 mM-Fe is 28:20, i.e. greater than in even the worst commercial nucleic acid preparation, but they illustrate the way in which, in this pH range and in the absence of added Fe, the rate of action increases with increasing pH while in the presence of Fe it falls.

The counteraction of the inhibitory effect of metals by citrate, ethylenediaminetetraacetate, etc. has already been mentioned. Various other materials able to bind metals act similarly, and of them proteins and commercial peptone are important because they are likely to contaminate nucleic acid

Table 9. *Shift of the apparent* pH *optimum of PRNase action in the presence of* Fe

YNA, made in the laboratory, at 20 mg. P/l. in 10 mM maleate buffers at the three pH values stated. Incubated with PRNase at 25°.

Concentration of enzyme (mg./l.)	Concentration of Fe^{2+} (mM)	pH	Percentage hydrolysis after (hr.) 1	2	4
0·06	0	5·5	12	21	39
		6·0	18	33	46
		6·5	26	45	48
0·20	0·5	5·5	24	33	38
		6·0	23	31	38
		6·5	21	29	31

preparations. This then is another factor that has to be considered in trying to account for differences in the behaviour of different samples of nucleic acid and enzyme preparations carrying with them different amounts of contaminating protein. This factor is only likely to become significant when unfractionated tissue extracts are being used so that the ratio of protein to enzyme activity is high. Under the conditions used in most of these experiments our best LRNase preparations introduce a little more protein into the system than PRNase preparations do to get the same rate of action (Holden & Pirie, 1955*a*); nevertheless, it is the LRNase that is, throughout, the more easily inhibited and this is true even with crude LRNase preparations which introduce much more protein.

The effect of some possible inhibitors. Some stress has been laid by Ledoux (1954) on the importance of the state of oxidation of the enzyme in controlling the action of PRNase. Under the conditions of our experiments the addition of cysteine, glutathione, ascorbic acid, KCN or H_2O_2 had no effect on either enzyme. These substances were neutralized and tested at several concentrations between 10 and 1 mM. Ledoux found that iodosobenzoate inhibited PRNase slightly if the enzyme is incubated with the reagent before adding the substrate. We are not satisfied that this effect, under our conditions, is real but iodosobenzoate inhibits LRNase strongly at 10 mM; at 2·5 mM there is 50 % inhibition and it is perceptible at 1 mM.

Comparison of different nucleic acids. Three types of comparison have been made; those between YNA preparations made in different ways, those between YNA preparations and products derived from them by partial hydrolysis, and those between nucleic acid preparations made from yeast, normal tobacco leaf and tobacco mosaic virus (TMV). Comparisons of the first type, and the effect on them of varying degrees of contamination by inhibitory metals, have already been discussed. When the rates of hydrolysis are measured in citrate buffer so that this inhibition is minimized, no significant differences have been found in the rate of action on different YNA fractions with either enzyme. The part of YNA that remains acid-precipitable and indiffusible after the action of PRNase, commonly called 'core', is a substrate for LRNase (Holden & Pirie, 1955*a*). The experiment set out in Table 10 confirms this and shows that its rate of hydrolysis is similar to that of YNA. We consistently find a small and probably significant difference in favour of 'core' but attribute this to differences in the amount of inhibitory metal present and to the small particle size of 'core' facilitating hydrolysis. Nucleic acids made from NP and TMV are also compared in Table 10 and it is clear that, unlike 'core', they are nearly as readily attacked as YNA by PRNase, though the action on several different NP nucleic acid preparations has consistently been slower to an extent that is probably significant. With LRNase, on the other hand, the greater rate of action on YNA and 'core' is definite.

Table 10. *Comparison of four different nucleic acids with LRNase and PRNase*

The substrates were made by the methods described by Holden & Pirie (1955*b*) and were incubated at 25° in 10 mM pH 6 citrate buffer.

Enzyme	Substrate	Percentage hydrolysis after (hr.) 1	2	4
LRNase, 0·10 U/ml.	YNA	26	46	75
	YNA 'core'	27	46	78
	NP nucleic acid	16	33	57
	TMV nucleic acid	21	40	62
PRNase, 0·06 mg./l.	YNA	32	45	49
	YNA 'core'	<5	<5	<5
	NP nucleic acid	22	43	46
	TMV nucleic acid	32	42	49

In other respects the kinetics of the action on all these substrates is similar. Not all the phenomena described earlier in this paper with YNA have been demonstrated on them because of shortage of material, but all those looked for, e.g. the activating effect of many salts and the inhibitory effect of Cu, Fe and Zn particularly on LRNase, have been confirmed.

It is clear from Table 10 that the course of the action of PRNase on YNA is similar to that on the other two nucleic acids and that they too leave a 'core'. This has been found with nucleic acid made from TMV by all three methods of fission and the 'core' has been recognized both by its acid-precipitability and by its failure to diffuse through cellophan. Using the first criterion the amount of 'core' is the same with each nucleic acid source; using the second we find that TMV nucleic acid gives only a 30 % yield of 'core' whereas YNA gives 40 %. The dialysis technique is not sufficiently quantitative for this to be considered a significant

difference. It is clear, however, that the action of PRNase on TMV nucleic acid leaves a 'core' and we do not therefore agree with the contrary conclusion come to by Schramm (1954).

DISCUSSION

Nothing is known about the action of RNase in any tissue *in vivo*. There is, therefore, no reason to expect any similarity between the properties of the predominant RNase, or RNases, of leaf and the two predominant RNases in pancreas. The similarities that have been found are therefore more striking than the differences; each is more thermostable than most other enzymes in the same tissue; the same inhibitors act on each, though LRNase is the more easily inhibited. The enzymes were already known to have different ranges of specificity so that a detailed comparison between them may seem rather artificial. It was undertaken because of the observation (Holden & Pirie, 1952) that PRNase attacked leaf nucleoprotein more rapidly than LRNase when quantities of the enzymes were taken which had the same rate of action on YNA. This difference was interesting because it was the enzyme with the more restricted specificity working on what could not conceivably be its normal substrate, that gave the faster rate.

The difference between the two enzymes is readily explained by the results in this paper. Diminution of the substrate concentration affects the rate of action of LRNase more than that of PRNase and, in the early work, leaf nucleoprotein was always used more dilute than YNA so that the apparent efficacy of PRNase was enhanced. Furthermore, the inhibitory metals affect LRNase more than PRNase so that again the efficacy of PRNase would be enhanced if NP carried inhibitory metals with it. The investigation of the metals in leaf nucleoprotein is still incomplete but it is clear that besides Ca and Mg it has a Zn content that may be significant. Both enzymes are, in general, protected from these metal inhibitions by citrate so that the presence of inhibitory metals in leaf nucleoprotein would also account for the activation by citrate of its enzymic fission (Holden & Pirie, 1952). This had seemed anomalous because with YNA as substrate neither enzyme was activated much by citrate; it seemed possible, therefore (Pirie, 1951), that there was another enzyme which was responsible for separating protein from nucleic acid, that this was activated by citrate and that the separation was a necessary prelude to RNase action. There is now no reason to consider this suggestion further.

The fact that the action of PRNase is affected by the ionic composition of the medium has been recognized by others but there is considerable disagreement over the details of the effect. The results in this paper suggest several possible origins for the disagreement. Preparations of YNA vary; even when the same preparation is used throughout, different concentrations of Na are present according to the concentration of YNA used; even at the same concentration differences are introduced by the use of different buffers. In spite of this there are some pieces of good agreement. Thus Zittle (1946*a*), though working in bicarbonate buffer and with 40 g./l. YNA, got 50% inhibition of PRNase by 0·45 mM-Zn^{2+}. This is what we find for that enzyme in maleate buffer at 0·005 the YNA concentration. In contrast to this there is disagreement, as we have already pointed out, over the effect of Mg. In another paper in this series (Holden & Pirie, 1955*b*) we question the assumption that free nucleic acid is a normal component of tissues so that these studies may have no immediate relevance. It would be convenient, however, if the differences could be resolved before an attempt is made to assign a function to RNase *in vivo*.

SUMMARY

1. The effect of variation in the conditions on the completeness of the precipitation of yeast nucleic acid by uranyl nitrate and trichloroacetic acid, has been investigated.

2. Ribonuclease from pea leaves hydrolyses nucleic acid more extensively than the pancreatic enzyme. There are also differences in thermostability and pH optimum.

3. The leaf enzyme is the more easily affected by several inhibitors.

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Metabolism of Polycyclic Compounds

9. METABOLISM OF 2-NAPHTHYLAMINE IN RAT TISSUE SLICES*

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The increased excretion of ethereal sulphate and glucosiduronic acid found in the urine of dogs treated with 2-naphthylamine was considered by Engel (1920, 1924) to be due to conjugation products of 2-amino-1-naphthol, since this compound was identified in the acid-hydrolysed urine by a colour test described by Liebermann & Jacobson (1882). Quantitative studies showed that more ethereal sulphate and glucosiduronic acid was produced than was expected from the 2-naphthylamine administered. Wiley (1938*a*) isolated 2-amino-1-naphthyl sulphuric acid from the urine of dogs which had been dosed with 2-naphthylamine and confirmed the structure of this product by synthesis. Dobriner, Hofmann & Rhoads (1941) isolated 2-naphthylamine, 2-acetamidonaphthalene and 2-acetamido-6-naphthol from the urine of rats, rabbits and monkeys and obtained evidence for the presence of conjugated derivatives and of 2-amino-6-naphthol.

Manson & Young (1950) administered 2-naphthylamine to rats and isolated 2-amino-1-naphthyl sulphuric acid from the urine. They also confirmed the presence of 2-naphthylamine and 2-acetamido-6-naphthol, but concluded that 2-acetamidonaphthalene was either absent or present only in very small amounts. When the rats were dosed with 2-acetamidonaphthalene by stomach tube, 2-acetamido-6-naphthol and small amounts of the unchanged compound and of 2-naphthylamine were present in the urine. When 2-acetamidonaphthalene was administered by subcutaneous injection 2-acetamido-6-naphthol only was found in the urine.

* No. 8 of this series: Boyland & Solomon (1955).

Wiley (1938*b*) found normal Q_{O_2} values for rat liver and kidney slices in Ringer solution saturated with 2-naphthylamine, and for liver and kidney slices from animals which had been injected daily with 2-naphthylamine for 40 days. Rat liver homogenates are able to synthesize *m*-aminophenyl sulphuric acid from *m*-aminophenol (Bernstein & McGilvery, 1952). The hydroxylation of aromatic compounds by isolated liver tissue has been demonstrated by the conversion of benzene into phenol (Tschernikow, Gadaskin & Gurewitsch, 1930), and the conversion of naphthalene into dihydrodihydroxy derivatives (Boyland & Wiltshire, 1953).

MATERIALS

2-Naphthylamine was purified by the method described by Case (1951). 2-Acetamidonaphthalene was prepared by the acetylation of 2-naphthylamine in pyridine with acetic anhydride. 2-Amino-1-naphthol hydrochloride was prepared by the reduction of 2-nitroso-1-naphthol with $SnCl_2$ in HCl (Grandmougin & Michel, 1892). 2-Acetamido-1-naphthol was prepared by the alkaline hydrolysis of 2-acetamido-1-acetoxynaphthalene (Grandmougin, 1906). 2-Amino-1-naphthyl sulphuric acid was prepared by oxidation of 2-naphthylamine with $K_2S_2O_8$ as described by Boyland, Manson & Sims (1953). 2-Acetamido-1-naphthyl sulphuric acid: acetylation with acetic anhydride in pyridine caused hydrolysis of the sulphate group of 2-amino-1-naphthyl sulphuric acid but acetylation with thioacetic acid was effective. 2-Amino-1-naphthyl sulphuric acid (1 g.) was dissolved in pyridine (10 ml.) and thioacetic acid (0·3 g.) was added. After standing overnight a slight excess of 40% KOH was added, the solution evaporated to dryness in a desiccator over H_2SO_4 and the residue crystallized twice from ethanol to give pink prisms of the *potassium salt of 2-acetamido-1-naphthyl sulphuric acid.* (Found: N, 4·4. $C_{12}H_{10}O_5NSK$ requires N, 4·4%.)