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ON THE NATURE OF INCREASE IN RIBONUCLEASE ACTIVITY IN MECHANICALLY DAMAGED TOBACCO LEAF TISSUES

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Abstract—Ribonuclease (RNase) activity was found to increase rapidly in mechanically damaged tobacco leaf tissues 2–4 hr after injury. The increased level of enzyme activity was maintained for several days. This rise in RNase activity appears to be due to protein synthesis as it was completely inhibited by various inhibitors of nucleic acid and protein synthesis. Anaerobic conditions or treatment with 2,4-DNP or KCN also lessened the increase in RNase level due to injury. Pretreatment of the tissues with actinomycin D before injury completely prevented the rise in RNase activity. The increase in RNase activity was greater in light than in darkness. The RNase was partially purified by gel-filtration. The molecular weight of the enzyme, as determined by molecular sieving, was 32,000. The enzyme is heat sensitive and has a pH optimum of 6·8. No evidence for the presence of different RNases in the tobacco leaf was found.

INTRODUCTION

THE 'wound respiration' of sliced tuber tissues appears to be an ideal system for the study of metabolic control in higher plant tissues. Click and Hackett[1] showed that the rapid rise of respiration in cut potato tuber tissues is dependent specifically on the synthesis of new proteins and nucleic acids. They concluded that the synthesis of nucleic acids and proteins must be released from some kind of repression soon after the slices are cut. This is supported by the following observations: The activity of a number of enzymes including cytochrome oxidase,[2] glucose-6-phosphate dehydrogenase,[3] invertase,[4, 5] ascorbate oxidase,[5] peroxidase,[6] phenylalanine ammonia lyase[7] is also increased in cut storage tissues. The rise of invertase activity in cut tuber tissues of Jerusalem artichoke[5] and of the peroxidases in sliced sweet potato roots[6] and white potato tubers[8] can be inhibited by various inhibitors of protein and nucleic acid synthesis. In cut carrot root tissues the increase of invertase activity parallels increased incorporation of labelled precursors of ribonucleic acid.[9]

Wounding and/or self-wounding (lesion formation due to hypersensitive reaction) is an important aspect of a wide variety of plant diseases. It therefore seems likely that the ubiquitous appearance of increased enzyme levels in diseased plant tissues[10, 11] is partly due to the wounding effect and concomitant derepression of enzyme synthesis. However, experimental

[1] R. E. CLICK and D. P. HACKETT, *Proc. Natl. Acad. Sci.* **50**, 243 (1963).
[2] D. P. HACKETT, D. W. HAAS, S. W. GRIFFITHS and D. J. NIEDERPRUEM, *Plant Physiol.* **35**, 8 (1960).
[3] F. ALBERGHINA and E. MARRÈ, *Rend. Accad. nazl. Lincei* **30**, 261 (1961).
[4] J. S. D. BACON, I. R. MACDONALD and A. H. KNIGHT, *Biochem. J.* **94**, 175 (1965).
[5] J. EDELMAN and M. A. HALL, *Biochem. J.* **95**, 403 (1965).
[6] Y. KANAZAWA, H. SHICHI and I. URITANI, *Agr. Biol. Chem.* (*Tokyo*) **29**, 840 (1965).
[7] T. MINAMIKAWA and I. URITANI, *J. Biochem.* (*Tokyo*) **57**, 678 (1965).
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[9] C. J. LEAVER and J. EDELMAN, *Biochem. J.* **97**, 27P (1965).
[10] F. SOLYMOSY and G. L. FARKAS, *Virology* **21**, 210 (1963).
[11] G. L. FARKAS and L. LOVREKOVICH, *Phytopathology* **55**, 519 (1965).

evidence to show that the increase in enzyme levels in diseased tissues is due to synthesis of enzyme protein is scanty.[11] Even less is known about the processes triggering these enzyme changes. In an approach to this problem the response of ribonuclease (RNase) level to mechanical wounding and/or infection is being studied in our laboratory. Indications that RNase activity is increased on mechanical injury and/or infection has already been obtained.[11–14] In the present paper a more detailed analysis of the development of RNase activity in mechanically damaged tobacco leaf tissues is presented and some properties of the enzyme are described.

RESULTS

Comparison of the Effect of Various Kinds of Mechanical Damage

Freshly harvested tobacco leaves usually had relatively low RNase activity. Incubation of leaf disks in Petri dishes on wet filter paper for 24 hr resulted in a marked increase in RNase level. Part of the increase was clearly due to the rise of RNase activity at or close to the cut surface of leaf disks. This was indicated by a comparison of RNase activities in homogenates

TABLE 1. THE EFFECT OF VARIOUS KINDS OF MECHANICAL DAMAGE ON THE RNASE LEVEL OF TOBACCO LEAF TISSUES

Treatment	RNase activity in enzyme units/1g fr. wt.	Percentage increase control = 100%
Untreated (control)	170	0
Incubated normally*	310	82
Inner parts of disks incubated normally*	260	53
Rubbed with carborundum*	472	177
Infiltrated with H_2O*	435	150
Infiltrated with H_2O and rubbed with carborundum*	510	200

* The disks were incubated in Petri dishes on wet filter paper for 24 hr.

from whole disks and from "inner disks" obtained by removing the edge of the large disks by a smaller cork-borer. The removal of the edge of the disks diminished the increase in RNase activity considerably but not altogether (Table 1). The rest of the increase might be attributable to the effect of tissue isolation (induced ageing) which is in line with the previously observed increase in RNase activity in detached *whole* barley leaves.[15]

Gentle rubbing of leaf disks with carborundum that caused damage invisible to the naked eye led to a marked increase in RNase activity over the non-rubbed "controls". The rapid infiltration of leaf disks with distilled water, which apparently exerts a damaging stress on the cytoplasmic structure, also resulted in an increase of RNase activity (Table 1).

Kinetics of the Increase of RNase Activity

The kinetics of increase in RNase activity was followed both in leaf disks rubbed with carborundum and in those infiltrated with water. With both treatments the rapid increase in enzyme activity took place during the first 4 hr after damaging the tissues. There ap-

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[15] C. KISBÁN, M. HORVÁTH, L. DÉZSI, J. UDVARDY and G. L. FARKAS, *Acta Botan. Acad. Sci. Hung.* **10**, 275 (1964).

peared to be a short lag period of about 1 hr followed by the rapid rise of RNase activity levelling off after 4 hr (Fig. 1). The lag period was somewhat longer in the infiltration experiments.

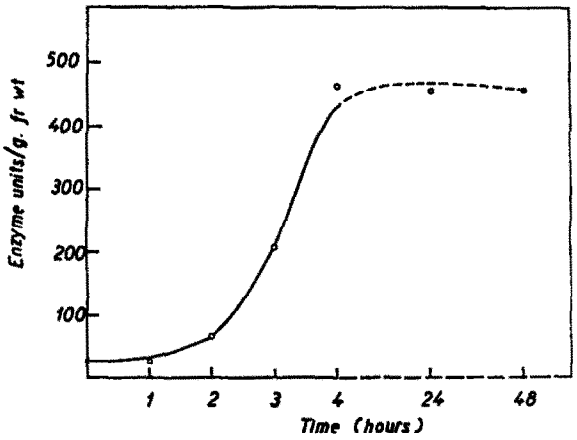

Fig. 1. TIME COURSE OF THE DEVELOPMENT OF RIBONUCLEASE ACTIVITY IN TOBACCO LEAF TISSUE DAMAGED BY RUBBING WITH CARBORUNDUM.

Effect of Inhibitors

To throw some light on the nature of changes in enzyme activity various inhibitors of nucleic acid and protein synthesis were tested for their effectiveness in inhibiting the increase in RNase activity. The rise of RNase in leaf disks infiltrated with water (control) was compared with that in disks infiltrated with various inhibitors dissolved in water. Table 2 shows that the increase in RNase activity that occurred in 24 hr after infiltration was cut down by

TABLE 2. EFFECT OF INHIBITORS OF NUCLEIC ACID AND PROTEIN SYNTHESIS AND OF SOME OTHER SUBSTANCES ON THE DEVELOPMENT OF RNASE ACTIVITY IN TOBACCO LEAF TISSUES INFILTRATED WITH DISTILLED WATER

Treatment	Concentration (M)	RNase activity in enzyme units/1g fr. wt.		
			Tissue treated with	
		Untreated	H_2O	Inhibitor
Actinomycin D	5×10^{-5}	360	644	272
8-Aza-adenine	$1{\cdot}5 \times 10^{-2}$	480	932	240
p-Fluorophenylalanine	2×10^{-2}	400	800	426
Puromycin	10^{-4}	256	420	108
Chloramphenicol	7×10^{-3}	290	525	44
2,4-DNP	10^{-4}	175	365	264
Mitomycin	10^{-4}	360	644	565
KCN	10^{-3}	175	340	125
Kinetin	10^{-5}	180	345	270
Anaerobic conditions	—	180	356	250

All the compounds tested were infiltrated into leaf disks 24 hr before testing the tissues for RNase activity.

p-fluorophenylalanine, puromycin, chloramphenicol, actinomycin D and 8-azaadenine. The inhibitors not only stopped the rise of RNase activity but in the inhibitor-treated samples the RNase activity dropped well below the level of the controls. Less specific inhibitors of protein synthesis such as 2,4-dinitrophenol (DNP), KCN, and anaerobic conditions also inhibited the rise in RNase activity though to a lesser extent. Mitomycin, an inhibitor of DNA re-duplication, was only slightly effective. Kinetin, a compound known to stimulate RNA and protein synthesis in isolated leaf tissues, also exhibited a smaller but reproducible inhibitory effect (Table 2).

As most of the increase in RNase activity occurred during the first 4 hr after damaging the tissues (Fig. 1) the effectiveness of some of the inhibitors in short-term experiments was also tested (Table 3). Four inhibitors that have different modes of action completely prevented the rise in RNase activity induced by cellular damage.

TABLE 3. EFFECT OF INHIBITORS OF NUCLEIC ACID AND PROTEIN SYNTHESIS ON THE DEVELOPMENT RNASE ACTIVITY IN TOBACCO LEAF TISSUES INFILTRATED WITH WATER

Compound	Concentration (M)	RNase activity in enzyme units/1 g fr. wt.		
		Untreated	Tissue treated with	
			H_2O	Inhibitor
8-Aza-adenine	$1{\cdot}5 \times 10^{-2}$	192	252	52
Actinomycin D	5×10^{-5}	112	216	128
p-Fluorophenylalanine	2×10^{-2}	192	252	88
Chloramphenicol	7×10^{-3}	112	216	80

Treatment with water or inhibitor lasted for 3 hr.

In one experiment the effect of pretreatment with actinomycin D on the development of RNase activity in leaf disks rubbed with carborundum was tested. Disks were infiltrated either with water of 5×10^{-5} M actinomycin D and then incubated in Petri dishes for 18 hr. After this preincubation period some of the disks were rubbed with carborundum. Three hours after the rubbing the tissues were assayed for RNase activity. Pretreatment of the tissues with actinomycin D completely inhibited the development of RNase activity in the mechanically damaged tissues (Table 4).

TABLE 4. EFFECT OF PRETREATMENT WITH ACTINOMYCIN D ON THE RNASE LEVEL IN CONTROL AND MECHANICALLY INJURED TOBACCO LEAF TISSUES

Treatment of disks*	RNase activity in enzyme units/1 g fr. wt.
Infiltration with H_2O	840
Infiltration with H_2O and rubbing with carborundum	1260
Infiltration with actinomycin D†	60
Infiltration with actinomycin D† and rubbing with carborundum	64

* Disks were pretreated with actinomycin D (or water) for 18 hr before rubbing them with carborundum. RNase activity was assayed 4 hr after rubbing the tissues.

† 50 μM.

(mol. wt. 12,400). The molecular weight of tobacco leaf RNase was calculated from the standard curve shown in Fig. 3 where elution volumes (Ve) of reference proteins are plotted against the log molecular weights of the same substances. The standard curve agrees well with that obtained by Andrews.[16]

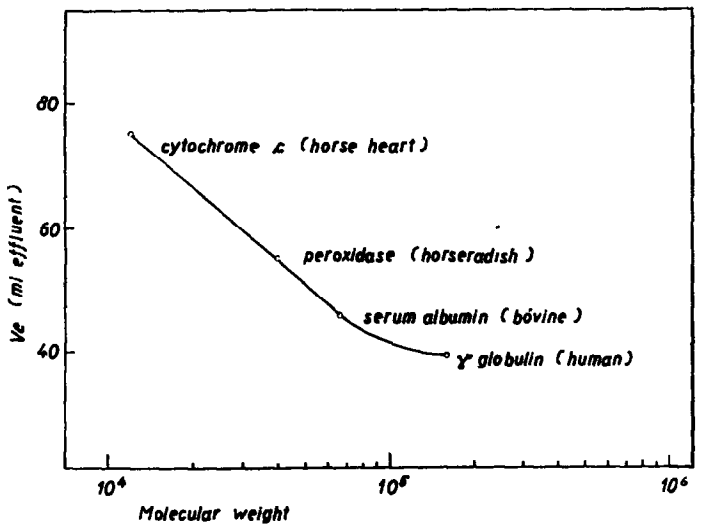

FIG. 3. CALIBRATION CURVE FOR THE DETERMINATION OF MOLECULAR WEIGHT ON A SEPHADEX G-100 COLUMN.

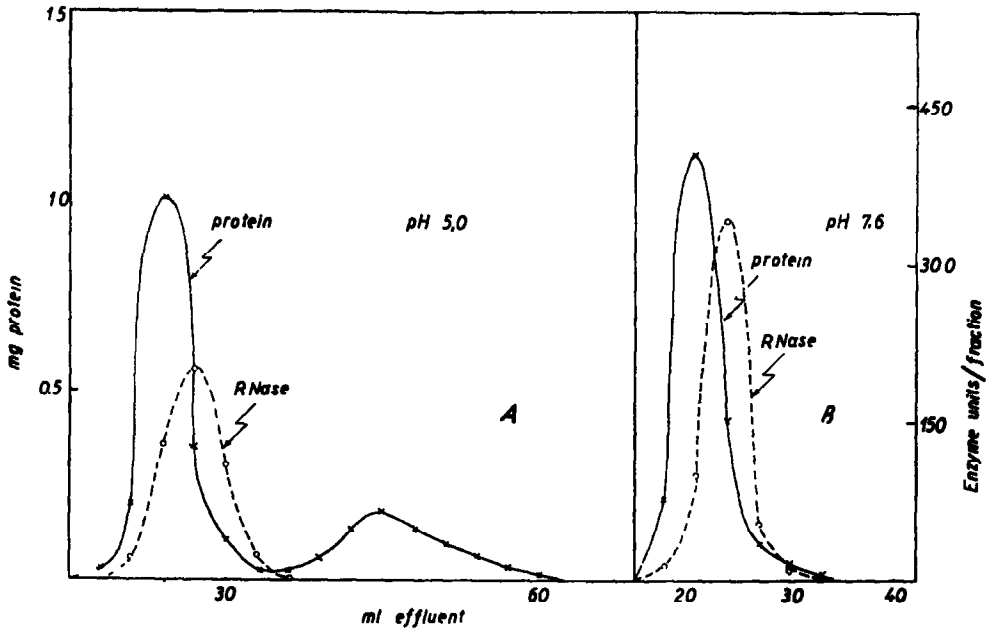

FIG. 4. ELUTION PATTERN OF TOTAL PROTEINS AND RIBONUCLEASE ACTIVITY OF TOBACCO LEAF EXTRACTS AT pH 5·0 (A) AND pH 7·6 (B) RESPECTIVELY ON A SEPHADEX G-50 COLUMN.

[16] P. ANDREWS, *Biochem. J.* **91**, 222 (1964).

Effect of Illumination

All the experiments reported above were carried out in dim light under ordinary laboratory conditions. Further investigations on the possible role of illumination indicated that light strongly promotes the development of RNase activity (Table 5).

TABLE 5. THE EFFECT OF ILLUMINATION ON THE DEVELOPMENT OF RNASE ACTIVITY IN MECHANICALLY DAMAGED TOBACCO LEAF TISSUES

Treatment	RNase activity in enzyme units/1 g fr. wt.
Control, dark	120
Control, illuminated	132
Rubbed with carborundum, dark	188
Rubbed with carborundum, illuminated	248

Disks were kept in Petri dishes in complete darkness or illuminated by 10,000 lx (fluorescent light) for 6 hr after injury.

Properties of the Enzyme

The enzyme was found to be localized almost entirely in the soluble fraction of tobacco leaf homogenates (cf. Experimental). The RNase of White Burley tobacco leaves was heat labile. Determination of the molecular weight of the partly purified enzyme by the gel filtration method gave a value of 32,000. In Fig. 2 the elution pattern of marker proteins and tobacco leaf RNase on Sephadex G-100 is shown. The RNase activity came off after the horse-raddish peroxidase peak (mol. wt. 40,000) and before the cytochrome *c* peak

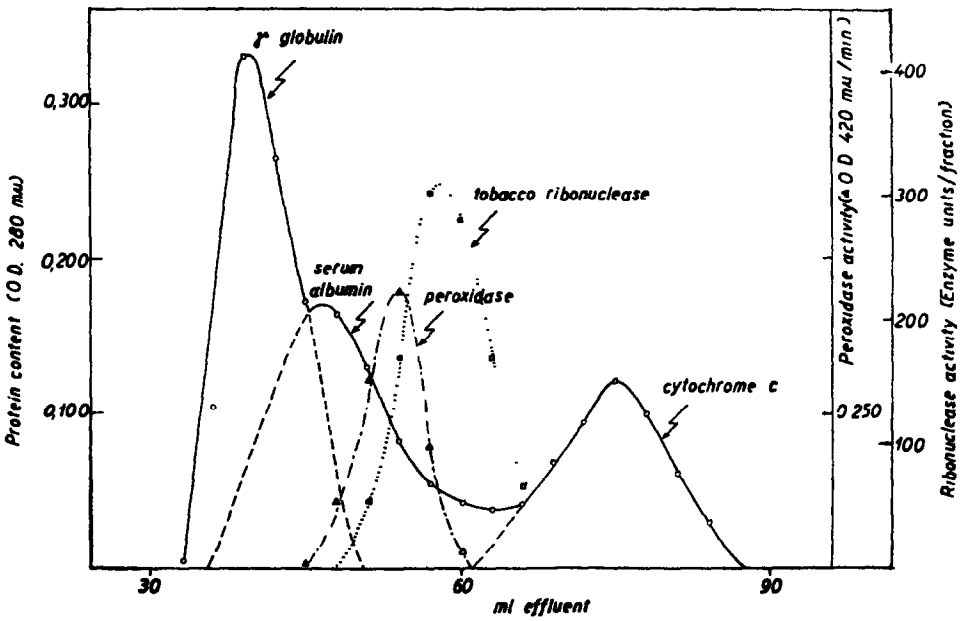

FIG. 2. ELUTION PATTERN OF VARIOUS MARKER PROTEINS AND TOBACCO RIBONUCLEASE ON A SEPHADEX G-100 COLUMN.

Multiple forms of RNase have been reported in some plants,[17, 18] and attempts were therefore made to resolve the RNase activity of tobacco leaf extracts into more than one component. By varying the conditions of gel filtration, total proteins of the tobacco leaf extracts were resolved into two distinct peaks (chromatography at pH 5·0 in 0·02 M acetate buffer) without resolution of the RNase activity (Fig. 4A). Thus no indication was obtained for the presence of several tobacco RNases differing markedly in molecular weight.

At pH values above neutrality (Tris buffer pH 7·6) all the proteins appeared as a single fraction (Fig. 4B). The concomitant determination of RNase activity indicated that most leaf proteins have a larger molecular weight than tobacco leaf RNase. Fractionation of total proteins on Sephadex G-100 is therefore a useful method for the purification of tobacco RNase (cf. Materials and Methods). The pH optimum of the tobacco leaf RNase was between pH 6·5 and 7·0.

DISCUSSION

One of the most striking features of the results presented is the early increase in RNase activity in mechanically damaged tobacco leaf tissues. This takes place about 2 hr after injury whereas 24 hr elapsed before the development of "wound respiration" and increase in RNA and proteins synthesis in the experiments of Click and Hackett.[1] The rise in invertase activity of sliced *Beta vulgaris* roots studied by Bacon *et al.*[4] is also slow to start and the rapid increase began only after washing the slices for 9–10 hr. More or less the same is true for the invertase system of Edelman and Hall[5] in *Helianthus tuberosus* storage tissues and for the formation of peroxidase in sliced sweet potato roots.[6] The earliest increase of enzyme activity in wounded tissues that had been reported previously was by Minamikava and Uritani.[7] In their experiments the rapid increase in phenylalanine deaminase and tyrase activities in cut sweet potato root tissues was observed 5–6 hr after slicing.

The lag period preceding the increase in protein synthesis was interpreted by Click and Hackett[1] as being necessary for the development of new RNA. This may be true for the tobacco RNase as the increase of its activity in the wounded tobacco leaf is affected by inhibitors of both protein and nucleic acid synthesis.

The second important feature of the results is the extreme sensitivity of the tobacco RNase to almost all the inhibitors tested and also the speed with which they affected the RNase level. Our results are indicative not only of a very rapid RNase synthesis in damaged tobacco tissues but also of a rapid turnover of the RNase molecule. This is suggested by the observation that not only the rise of RNase activity is inhibited but also the normal level of RNase decreases considerably in the inhibitor-treated samples (Table 2). In extreme cases RNase activity almost disappeared in tissues treated with actinomycin D for 24 hr (Table 4). Experiments with inhibitors are, of course, only suggestive of a *de novo* synthesis of RNase and do not constitute final proof.

It is interesting to speculate on why the maximum potential for RNase formation of tobacco leaf tissues is not fully realized under normal conditions. An obvious interpretation might be based on the current repressor hypothesis. If the results can indeed be interpreted in terms of the Jacob–Monod theory one has to postulate that the factor(s) leading to derepression of enzyme synthesis can become available under a wide variety of conditions associated with cellular damage. In fact, slicing, rubbing, wounding or self-wounding of plant tissues due to

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lesion formation in diseased plants do all lead to increased RNase levels.[19] Even very slight damage ("stress") is sufficient to induce the increase of some enzyme activities as shown for RNase in the present work and for glucose-6-phosphate dehydrogenase in an earlier paper.[20] The nature of substances which might act as derepressors and arise on damage is unknown. Their effect appears to be very fast and irreversible. The rapid and prolonged washing of tobacco leaf tissues rubbed with carborundum did not eliminate or even diminish the increase in RNase activity. In this sense the system is certainly different from the classical wound hormone of Haberlandt.

Santilli[21] has shown that the effect is translocated. We also observed that the effect of local injury is translocated in the bean leaf and that RNase activity is increased to some extent also in the non-treated parts of the damaged leaf.[8]

We have only some preliminary information on the properties of the RNase concerned. The enzyme appears to be confined primarily to the soluble fraction as most plant RNases are.[22–25] Its pH optimum (at pH 6·8) is higher than that reported for most plant RNases[26] and does not correspond to the second slightly alkaline pH optimum found with some crude systems.[18, 24] The remarkable heat stability reported for a number of plant RNases[26, 27] was not observed.

An interesting feature of the tobacco RNase appears to be its relatively large molecular weight (approx. 32,000).

EXPERIMENTAL

Plant Material

Nicotiana tabacum var. White Burley plants were grown in a greenhouse. Leaves 20 to 25 cm long were harvested. Disks 1·2 cm in diameter were cut out from a single leaf and used, after randomization, for the various treatments.

Methods

The disks were infiltrated with water or inhibitors in Thunberg tubes *in vacuo*. The excess of water was evaporated before incubating the disks in Petri dishes on wet filter paper, except when disks with water-filled intercellular spaces were incubated to ensure partially anaerobic conditions.

For the extraction of the enzyme the tissues were homogenized in distilled water in a mortar with quartz sand. The brei was filtered through four layers of cheese cloth and the extract centrifuged in a Spinco Model L 50 preparative ultracentrifuge at 20,000 × *g* for 10 min. Preliminary studies on the localization of the enzyme by fractional centrifugation in isotonic media indicated that up to 98% of the activity could be recovered in the soluble fraction. Therefore, the 20,000 × *g* supernatant obtained in hypotonic medium was used for most experiments.

The method adopted for the determination of RNase activity was based on those of

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[23] B. KESSLER and N. ENGELBERG, *Biochim. Biphys. Acta* **55**, 70 (1962).
[24] H. SCHWEIGER, *Planta* **68**, 247 (1966).
[25] B. G. SHINDE, B. K. CHANDRASEKHAR and V. SANTILLI, *Phytopathology* **54**, 908 (1964).
[26] CH. B. ANFINSEN and F. H. WHITE, In *The Enzymes* (Edited by P. D. BOYER, H. LARDY and M. MYRBÄCK), Vol. 5, p. 95. Academic Press, New York (1961).
[27] W. FRISCH-NIGGEMEYER and K. K. REDDI, *Biochim. Biophys. Acta* **26**, 40 (1957).

MacDonald[28] and Tuve and Anfinsen.[29] The reaction mixture contained 0·5 ml leaf extract (or suitably diluted purified fraction), 0·5 ml of a 7·5 mg/ml yeast RNA solution (British Drug Houses Ltd.) and 0·5 ml of 0·1 M acetate buffer at pH 5·0, and was incubated at 37° for 30 min. The reaction was stopped by adding McFadyen's reagent (0·25% uranylacetate in 2·5% trichloroacetic acid). The mixture was kept overnight at 4°, centrifuged and diluted 1:10. The absorptivity of the supernatant was measured at 260 nm in a MOM 201 u.v. spectrophotometer. The results are expressed in enzyme units, one enzyme unit corresponding to an increase in absorptivity of 0·010 over the zero time control.

The protein content of fractions was determined turbidimetrically. To 1 ml of each fraction 1 ml of 10% trichloroacetic acid and 1 ml of distilled water were added. The absoptivity (turbidity) of the solution was determined at 550 nm. The standard curve was prepared using bovine serum albumin (Sigma, Fraction V).

The crude extract was partly purified on a 2·2 × 14 cm Sephadex G-50 column. The Sephadex beads were swollen in distilled water for 24 hr followed by equilibration with either 0·02 M acetate buffer at pH 5·0 or 0·02 M Tris (hydroxymethylaminomethane) buffer at pH 7·6 as indicated in the text. For the chromatographic fractionation of proteins the leaf tissues were extracted in 0·02 M acetate buffer (2 g fresh weight/1 ml buffer) containing 0·1% cysteine, 0·1% ascorbic acid and 17% sucrose (cf. Farkas and Stahmann[30]) in a mortar and centrifuged twice as described above. 3 ml extract was layered on the column. Proteins were eluted by 0·02 M acetate buffer and 3 ml fractions were collected. The flow rate was 7·8 ml/cm^2/hr. For determining the molecular weight three RNase-rich fractions containing little protein (cf. Fig. 4) were pooled and concentrated threefold by the addition of dry Sephadex G-25 beads to the solution. Sucrose (17%) was added to 3 ml of the concentrated solution, the mixture was applied to a calibrated 2·2 × 24 cm Sephadex G-100 column and chromatographed.

Calibration of the column and the estimation of the molecular weight of the partly purified tobacco leaf RNase was carried out as described by Andrews.[16] To calibrate the columns equilibrated with 0·02 M acetate buffer the elution volumes of proteins of known molecular weight (dissolved in 3 ml 0·02 M acetate buffer at pH 5·0 containing 17% sucrose) were chromatographed: 3 mg cytochrome *c* (Sigma, Type II), 0·05 mg horse-radish peroxidase (Worthington, crystalline), 1 mg bovine serum albumin (Sigma, Fraction V), and 1 mg γ globulin (Sigma, Fraction II). The molecular weights of the proteins used are as follows: cytochrome *c* 12,400, horse-radish peroxidase 40,000, bovine serum albumin 67,000, γ globulin 160,000. The distribution of γ globulin, cytochrome *c* and bovine serum albumin in the fractions was determined by measuring the absorbance at 280 nm. The distribution of peroxidase and RNase was assayed by enzyme activity measurements. The system for peroxidase determination contained 0·5 ml of a 0·22% guaiacol (Merck) solution, 0·5 ml of 0·3% H_2O_2 (Merck), suitable amount of enzyme, 0·1 M Tris buffer at pH 7·5 in a total volume of 3·0 ml. The increase in absorptivity at 420 nm was read against a blank containing the complete system minus peroxide. The enzyme activity in Fig. 2 is expressed as Δ A at 420 nm/min under the conditions described.

In some experiments pancreatic RNase (Armour) was also included as a standard for the calibration of columns.

Acknowledgement—The excellent technical assistance of Miss Mary Göbel is acknowledged with thanks.

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