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REPRESSION OF ISOPEROXIDASE FORMATION IN EXCISED TOBACCO PITH BY EXOGENOUS, AUXIN-CONTROLLED RNA

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Abstract—Previous work has shown that tobacco pith tissue contains two constitutive isoperoxidases migrating toward the anode at pH 9·0. Within 24 hr of aseptic culture on basal medium, such tissue develops five new isoperoxidases, three cathodic and two anodic. The appearance of the new isoperoxidases involves *de novo* protein formation; it is inhibited by anaerobic conditions, by such inhibitors as Actinomycin D, and by the plant hormone indole-3-acetic acid (IAA). We now find that phenol RNA extracted from parent pith and injected or vacuum infiltrated into cultured pith explants prevents the appearance of the new isozymes; RNA from cultured pith has no such effect. Hydrolysis with 0·3 N KOH, ribonuclease or proteolytic enzymes partially destroys this activity, while treatment with both ribonuclease and proteolytic enzymes completely destroys it. Fractionation of the RNA indicates that part of the repressor activity is associated with an mRNA-like fraction.

INTRODUCTION

THE INTERACTION of the plant growth hormone, indole-3-acetic acid (IAA) and the enzyme system (IAA oxidase or peroxidase) which presumably controls endogenous levels of this substance has been described in detail.[1,2] The peroxidases of various species can be resolved into a series of isozymes by electrophoresis on starch-gel or polyacrylamide gel.[3-8] The number and relative quantities of such isozymes vary according to position on the plant, environmental factors during growth and age of the plant.[9]

In freshly excised tobacco pith, the peroxidase isozyme pattern at pH 9·0 is essentially anodic, showing two major bands, A_2 and A_3. After incubation in sterile culture medium for 24 hr, the pith develops five new isoperoxidases; three of these are cathodic, C_1, C_2 and C_3; and two anodic, A_0 and A_1.[7] The appearance of these new peroxidase isozymes is inhibited by Actinomycin D and thus probably involves *de novo* synthesis of RNA and protein.[1] The appearance of the new isozymes is also inhibited by IAA. Later on, after 48 hr, IAA acts to induce still another isozyme. Both IAA and Actinomycin D only have a slight depressive effect on the activity of the two constitutive isozymes in 24 hr culture, this presumably representing their turnover during the culture period.

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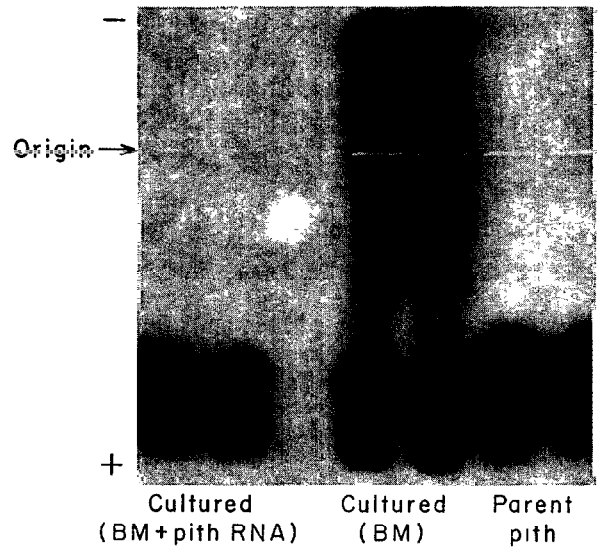

FIG. 1. ISOPEROXIDASE PATTERNS OBTAINED BY STARCH GEL ELECTROPHORESIS OF EXTRACTS OF TOBACCO PITH FROM INTACT PLANTS (RIGHT), PITH CULTURED IN BASAL NUTRIENT MEDIUM (BM) FOR 18 hr (CENTER), AND IN BASAL MEDIUM TO WHICH RNA FROM PARENT PITH HAS BEEN APPLIED (LEFT).

Arrow indicates origin, + the anodic, and − the cathodic ends of the zymogram. Each treatment was run in duplicate, with 40λ expressed liquid in each original slot.

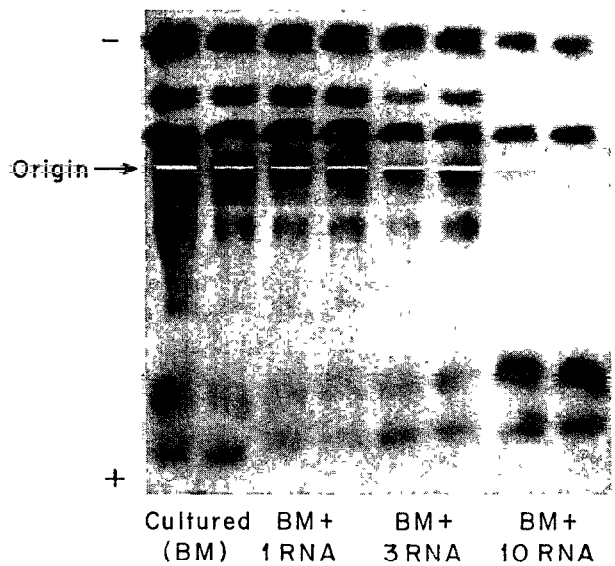

FIG. 2. EFFECT OF CONCENTRATION OF RNA ON REPRESSOR ACTIVITY.

Details as in Fig. 1. Relative concentrations (based on O.D.) were 1, 3 and 10, the latter corresponding to 4·8 mg RNA/ml. Control pith was treated with 10 mM tris buffer pH 7·4.

The appearance in culture of isoperoxidases absent from the parent tissue lends itself to several possible interpretations. One would involve the concept that peroxidase formation is prevented directly by high endogenous levels of IAA in the parent tissue; upon removal of tissue to culture, IAA levels are reduced and peroxidase appears. An alternate possibility is that a macromolecular repressor controlled by IAA prevents expression of genetic loci specific for the new peroxidases. We have examined these two possibilities and have found that a phenol RNA extract of pith tissue inhibits and may completely prevent development of the new peroxidase isozymes during aseptic tissue culture. Since the level of the effective fraction appears to be under the control of IAA, the RNA may represent the molecular means through which the hormone exerts its effect on the enzyme.

RESULTS

Effects of RNA Application

Extracted RNA was applied to excised cultured pith in two basic experiments: the first involved 24 mg RNA in 5 ml buffer extracted from 120 g fresh pith; the second involved a 1:3:10 dilution series of a second RNA extract prior to application. Typical results are presented in Figs. 1 and 2. From Fig. 1 it is evident that RNA from fresh pith tissue inhibits formation of the induced anodic and cathodic isozymes which normally appear during culture. Figure 2 shows a diminishing effect with increasing dilution of a less effective extract.

Phenol-extracted RNA obtained from pith explants which have been incubated for 24 or 48 hr (and have thus formed the new isoperoxidases) is virtually without effect on isoperoxidase formation. This indicates that the effective RNA found in fresh tissue disappears during *in vitro* culture. However, RNA extracted from cultured pith exposed to 10^{-4} M IAA in the medium (i.e. pith in which the induction of the new isozymes is inhibited) has a clear inhibitory action on the development of the new isozymes. Thus, addition of IAA to the medium either increases the synthesis or the persistence of the active macromolecule.

It might be argued that the repressor activity of the macromolecular extracts is due to IAA or to other low molecular weight compounds present as contaminants. This possibility was virtually ruled out by the demonstration that hydrolysis with 0·3 N KOH significantly lowered the repressor activity of the extract (Fig. 3). Such treatments should not destroy

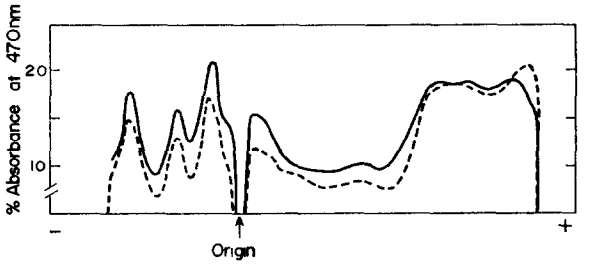

FIG. 3. EFFECT OF TREATMENT WITH KOH UPON RNA ACTIVITY.

Scan diagram of starch gel electrophoretic patterns of peroxidase isozymes: (------) represents isoperoxidase pattern produced by cultured pith treated with extracted RNA. (———) represents the same treatments except that the RNA was treated for 18 hr with 0·3 M KOH at 30° before application. Symbols as in Fig. 1.

IAA. Crystalline ribonuclease and the proteolytic enzymes trypsin and pronase also destroyed a part of the repressor activity (Table 1), indicating the presence of some residual protein in the 'phenol RNA'. Such protein could either function as a repressor itself, or

TABLE 1. THE EFFECT OF VARIOUS HYDROLYTIC AGENTS ON THE REPRESSOR ACTIVITY OF EXTRACTED RNA

Treatment	Percentage repression of new isoperoxidases
None	100
0·3 N KOH	51
RNAse	49
Trypsin	18
Trypsin and RNAse	−72*
Pronase	33
Pronase and RNAse	−50*

* Stimulation.

could merely protect the RNA from degradation by the ribonuclease. Digestion of the repressor fraction with both proteolytic enzyme and RNase caused it to promote, rather than inhibit, isoperoxidase formation. This may be due to the cytokinin content of the digested RNA, since cytokinins have previously been shown to promote isoperoxidase formation.[1]

Characterization of the Active RNA

Zubay procedure. The activities of the *s*RNA of the supernatant and ribosomal RNA pellet are compared in Fig. 4. The pellet contained approximately 80 per cent of the 24 mg of RNA extracted. The major repressor activity is clearly in the supernatant fraction, which

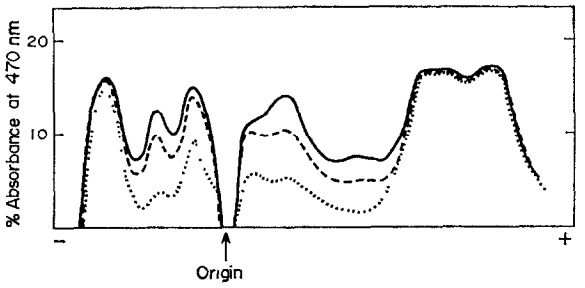

FIG. 4. COMPARISON OF THE REPRESSOR ACTIVITIES OF THE VARIOUS RNA FRACTIONS OBTAINED BY SEPARATION IN 1 M NaCl.

Details as in Fig. 1. (———) represents the peroxidase isozyme pattern of pith cultured for 18 hr in basal medium, (------) basal medium + RNA from ribosomal pellet, and (.....) basal medium + the *s*RNA supernatant.

may contain several RNA components; the redissolved ribosomal pellet fraction had lesser activity, especially on a per unit RNA basis.

Sephadex fractionation. Since most of the active component was detected in the Zubay supernatant, the latter was applied to a Sephadex G-100 column which has been found

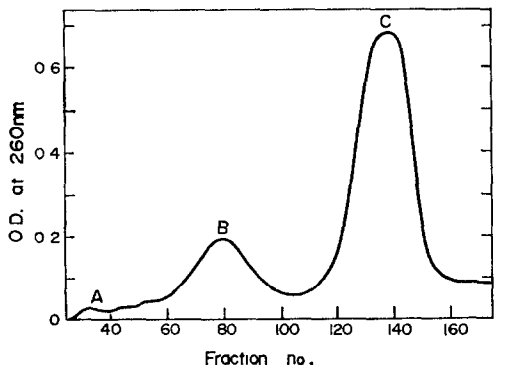

FIG. 5. ELUTION PROFILE OF THE *s*RNA FROM A SEPHADEX G-100 COLUMN.

Elution medium was 1 M NaCl m 10 mM tris pH 7·4. Fractions A, B and C are described in the text; 5 ml fractions were collected.

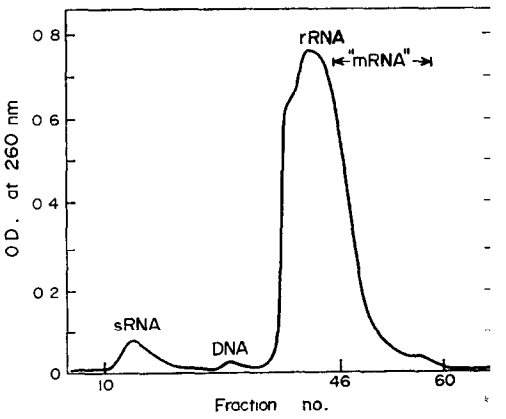

FIG. 6. MAK COLUMN FRACTIONATION OF TOTAL RNA EXTRACT FROM 120 g PITH OF INTACT TOBACCO PLANTS.

Elution was with a 10 mM tris pH 7·4-buffered 0·4–1·25 M NaCl gradient. The area marked '*m*RNA' indicates pooled fraction numbers checked for repressor activity and *m*RNA-like properties. 5 ml fractions were collected.

effective in detecting *s*RNA with amino-acid acceptor activity.[10-12] A typical elution profile is presented in Fig. 5. Peak C presumably corresponds to breakdown products or monomer *t*RNA, peak B to *s*RNA with amino acid acceptor ability or dimer *t*RNA, and peak A to DNA and the remainder of the *r*RNA not precipitated in the Zubay procedure or possibly to 5 S RNA. Neither the pooled B (tubes 62–91) nor C (tubes 110–150) fractions possessed appreciable repressor activity and there was insufficient material in fraction A to test.

MAK column fractionation. Since MAK column chromatography has been used for the isolation of an *m*RNA-like component from various plants,[13-15] this procedure was adopted in the present investigation. According to most workers, the *m*RNA fraction appears as a shoulder adjacent to the second ribosomal elution peak. This peak does not appear in the O.D. profile but can be traced by other means, including isotopic pulse-chase labelling. In our experiments, the total RNA extract was applied to the MAK column. Figure 6 shows the elution profile obtained and also the region possibly containing *m*RNA (tubes 46–60). All fractions to the left of this (10–46) were pooled and the biological activity compared to that of the putative messenger region. Results shown in Fig. 7 indicate that while the pooled tube 10–46 fraction had some inhibitory activity as compared to controls in basal medium, the 46–60 region produced the most marked inhibitory effect.

Incorporation of uracil ^{14}C. The turnover of *m*RNA is believed to be much more rapid than that of any other type of RNA.[16] Moreover in *E. coli*, Gros *et al.*[17] have shown that in

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pulse-chase experiments with ^{32}P or uracil ^{14}C the pulse was initially incorporated in the *m*RNA and only later into other regions. Similar behaviour in the present system would lend more support to the assumed location of a *m*RNA region in plant systems.[13–15] In order to obtain a finer resolution in this experiment and aid uracil-2-^{14}C detection in relation to peaks, the amount of the 10 mM tris pH 7·4 buffered 0·4–1·25 M NaCl elution medium was doubled as compared to the previous experiment. This permitted us to obtain a greater number of fractions over the profile.

Results of a 1-min and 3-hr incorporation period are shown in Fig. 8. It can be seen that while counts were detected in several regions after short term labelling, the major peak was

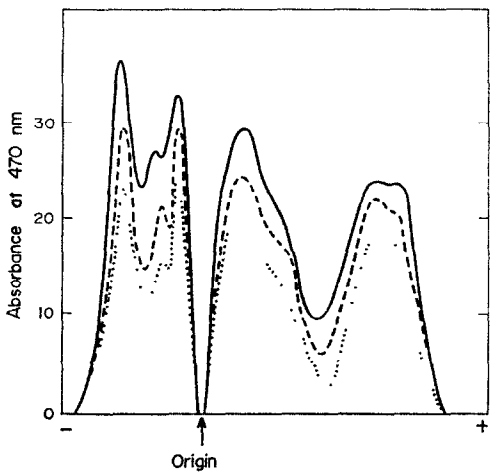

FIG. 7. EFFECTS OF MAK COLUMN ELUATES ON ISOPEROXIDASE PATTERNS.

Details as in Fig. 4. (———) — pith cultured for 18 hr in basal medium; (------) in basal medium + fractions 10–46 of Fig. 7 pooled, dialysed and ultrafiltered; (.....) basal medium + fractions 46–60 ('*m*RNA') pooled, dialysed and ultrafiltered.

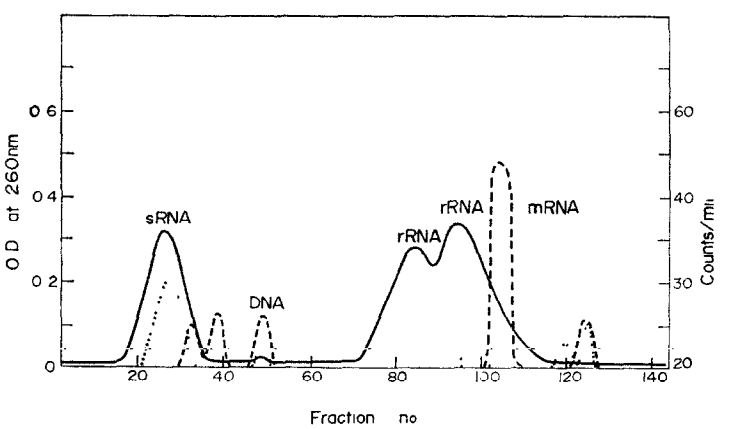

FIG. 8. PROFILE OF URACIL-2 ^{14}C INCORPORATION INTO TOBACCO PITH AS REVEALED BY MAK COLUMN FRACTIONATION (———) DURING SHORT TERM, 1 min (------) AND EXTENDED, 3 hr (.....) PERIOD.

0·01 mc uracil-2 ^{14}C was applied by injection into 5 cm mid-stem sections of each of 10 plants, and extracts subsequently made of cm stem sections. 5 ml fractions collected.

in the '*m*RNA' region, which in the previous experiment contained most of the biological activity. After 3 hr the counts appear in several regions of the elution profile including the *s*RNA and the *r*RNA.

DISCUSSION

The function of various types of RNA in the regulation of activities in bacterial and viral systems is well known. In eucaryotic organisms such as mammals and angiosperms, exogenous RNA has been reported to induce several effects in cell-free systems, in cell cultures and even in intact organisms. While some of these reports have been challenged, others seem sufficiently substantial to merit acceptance. The reported effects include the induction of specific proteins and enzymes, including hemoglobin,[18-20] serum albumin, tryptophane pyrrolase, glucose-6-phosphatase,[21] uterine alkaline phosphatase[22] and α-amylase.[23] The second group of RNA-induced effects, which may well be a concomitant of the first, includes growth effects in angiosperm cell cultures similar to those caused by the phytohormone, gibberellic acid.[24-27] In mammalian tissues, RNA has been reported to function in the transfer of either estrogenic or androgenic effects.[22,28-32] A third group of exogenous RNA-elicited changes is the induction of immunological responses such as antibody production[33-35a] and homograft immunity.[36]

Imsande and Ephrussi[37] were unable to repeat the experiment of Niu *et al.*[21] concerning the RNA induced transfer tryptophane–pyrrolase and glucose-6-phosphatase and have questioned the sensitivity of the latter assay method used by Niu *et al.* The estrogen effects have been questioned by Hechter and Halkerston[38] who attribute both the effect of the alleged RNA and its negation by RNAase to other factors. Moreover, the RNA-induction of some of the immunological responses were possibly[39,40] due to the presence of antigen fragments in the RNA extracts. However, RNAase nullification of the immuno-responses indicates that the effect was elicited by the RNA itself and not antigen fragments. Subsequent

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investigation on the steroid response (see review by Segal[32]) and the recent partial characterization of the biologically active RNA component in testosterone treated tissues[31] indicate that the RNA effect is real. Complete characterization of a biologically active RNA component as *m*RNA in higher organisms has as yet been achieved only in the hemoglobin system (see Labrie[19] and the review by Chantrenne *et al.*[20]). In angiosperms very little has been accomplished in this field and only Masuda[41] has thus far achieved promising but as yet only partial results.

Results of the present investigation (Figs. 1 and 2), show that RNA extracted from parent pith tissue prevented the formation of the usually formed new anodic and cathodic isoperoxidases. This effect in culture is also induced by IAA[1] and the hormonal effect may in like manner be transferred by exogenously applied RNA extracted from IAA-treated cultures. The phenomenon shows greater effect as the concentration of applied RNA is increased (Fig. 2), is diminished by KOH hydrolysis (Fig. 3), and is specific, since exogenous RNA from cultured pith elicits no such response. Diminution of the effect by KOH hydrolysis also makes it unlikely that IAA in the extract is responsible for the effect, since IAA is very stable to alkaline reagents.

A question which has to be posed *a priori* is the manner of the entrance of large molecules like RNA and proteins into the cell. This may be explained by the nature of tobacco pith tissue employed. Pith sections used were cylinders upon which all external surfaces had freshly cut cells. These were immediately immersed in solution, allowing no wound healing process to seal off the cut cells. RNA in contact with the latter could penetrate easily, especially when aided by vacuum infiltration, which was applied without detrimental effects to the cultures. Upon entering the peripheral cells the RNA could migrate intercellularly via plasmodesmata. Alternatively, the large molecules could enter via a pinocytotic mechanism, recently demonstrated in plant cells.[42]

The series of experiments undertaken in an attempt to characterize the active RNA component indicate that it is primarily in the Zubay supernatant (Fig. 4), is probably not in *t*RNA (Fig. 5), and is probably included in that portion of a MAK column elution profile (Figs. 6 and 7) which has, in plant extracts of several species, been reported to contain *m*RNA.[13-15] This fraction also has a higher rapid uracil-2-^{14}C incorporation (Fig. 8), as *m*RNA should.

Doubtless the *m*RNA here characterized is only part of the total cellular messenger fraction and, as seen in Figs. 2 and 4, other regions have some activity as well. This is in accord with the accepted concept of *m*RNA heterogeneity and may, in addition to the longevity factor mentioned previously, be a contributing factor to the considerably diminished effects of characterized RNA as compared to that elicited by 'total RNA' application. This is all the more so since peroxidase is not one uniform protein unit but has protein, heme and carbohydrate components.

EXPERIMENTAL

(*a*) *Peroxidase determinations*

Tobacco plants *cv* Wisconsin 38 were used as previously described.[1] Each replicate culture flask contained 25 cylinders of pith tissue in 50 ml of culture medium. After an 18-hr culture period, cylinders were removed from culture medium, gently washed 3 times with distilled H_2O, blotted with filter paper and ground with solid CO_2. After thawing, the expressed sap was centrifuged for 15 min at 14,000 *g* and two to three replicates of 40λ each used for vertical starch gel electrophoresis. The gel plate for starch gel electrophoresis was made

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up in a modified Buchler apparatus having the origin in the center of the plate. It contained 18·4 ml 0·2 M borate buffer pH 9·0 and 381·6 ml distilled H_2O in which 40 g of hydrolysed starch has been solubilized by heating. The hot sol was degassed by vacuum before pouring and allowed to set for at least 1 hr at 4° before use. After insertion of the 40 samples into the origin slots with a microsyringe, the origin opening and slots were sealed off with warm vaseline and the plates subsequently placed vertically in the bridge containing NaOH–borate buffer pH 8·3 as the mobile phase and run at 300 V (10 V/cm) for 3½ hr at 4°. After completion of the separation, the gels were developed with guaiacol–H_2O_2 reagent (50 ml 0·1 M KH_2PO_4, 3·6 ml 0·1 M NaOH, and one drop each of 30% H_2O_2 and 5 mM guaiacol) for 10 min and finally fixed with Smithies' solution,[43] containing 2 parts glycerine, 2 parts HOAc, 5 parts H_2O and 5 parts MeOH. Gels were scanned for optical density at 470 nm by excising longitudinal gel strips representing each 40 slot placing each in a Gilford model 2410 spectrophotometer equipped with a linear transport scanner attachment.

(*b*) *RNA extraction*

RNA was extracted from *ca.* 120 g tobacco pith tissue pooled from 4 different plants by the procedure of Loening and Ingle,[44] which utilizes a phenol reagent containing 0·1% by wt. hydroxyquinoline and 10% *m*-cresol, together with an equal volume of 10 mM tris pH 7·4 buffer containing 1% sodium lauryl sulphate and 12 mg/ml bentonite. Two additional cycles of solution in buffer and cold EtOH precipitation[45] were included. The absorption pattern of the final RNA precipitate redissolved in 10 mM tris buffer pH 7·4 was checked with a Model 350 Perkin–Elmer Spectrophotometer; it showed a sharp peak at 257 nm and a 260:280 nm O.D. ratio of 2·8:1. For application to tissues the 24 mg RNA precipitate thus obtained was dissolved in 5 ml of the above buffer and successive 0·05 ml aliquots applied by microsyringe injection into pith cylinders, followed by vacuum infiltration for 3 min in the excess solution which did not enter tissue upon injection. Controls were similarly treated with buffer. Where the RNA solution was divided into several treatments, equal aliquots or aliquots of differing concentration were assessed by relative O.D. at 257 nm. All manipulations were performed aseptically in a sterile culture room. When hydrolysed with KOH, the RNA extracted was divided into two aliquots of 2·5 ml each, one of which was incubated for 18 hr at 30° with 0·3 M KOH and pH restored to 7·4 with 0·1 HCl before application. The other treatments in this experiment received identical amounts of KOH and acid in the same buffer system. Enzymatic hydrolytic techniques are described below.

RNA characterization

Soluble RNA. Total RNA was divided into ribosomal and soluble fractions by the Zubay[46] technique employing 1 M NaCl. To prevent any deleterious effects of excess of NaCl on the tissue, the final buffered RNA solutions were exhaustively dialysed against buffer at 2–4° before application to tissue.

Sephadex fractionation. Since *s*RNA with amino-acid acceptor ability can be separated by Sephadex G-100 column chromatography,[10–12] the Zubay supernatant obtained as above was applied to such a column and the biological activity of the assumed amino-acid accepting *s*RNA peak tested. The pith of 4 tobacco plants finally yielded 6 ml of Zubay supernatant in 1 M NaCl in 10 mM tris pH 7·6. This was applied to a 50 mm × 40 cm Sephadex g-100 column and 5 ml fractions collected during elution with 1 M NaCl in the above buffer. Transmission in UV was automatically recorded. Pooled peaks were dialysed against buffer to get rid of excess NaCl; in order to reduce volume and exclude any possible low M.W. molecules, the solutions were passed through an Amicon Diaflo Ultrafiltration unit with 10,000 M.W. exclusion filter. The volume was reduced to 5 ml and thereupon applied to tissues as outlined previously.

MAK column separation. Total RNA extracted as above was placed on a MAK column prepared as described by Mandell and Hershey[47] and eluted with 0·4–1·25 M NaCl buffered with 10 mM tris pH 7·4, and 5 ml fractions collected, their UV absorption properties being recorded as above. Pooled fractions were dialysed and subjected to ultrafiltration in the same manner as in the previous step.

An attempt was made to obtain the so-called 'tenaciously bound' RNA which may also contain some of the *m*RNA [48,49] by eluting the MAK column with 2% sodium dodecyl sulphate after the 0·4–1·25 M NaCl gradient run, although some RNA was detected it was found to be contaminated with components of the MAK itself and biological activity could therefore not be credited to the RNA.

Incorporation of uracil ^{14}C. 0·1 mc uracil 2-^{14}C of specific activity of 47 mc/ml (Tracerlab lot G1-243) was dissolved in 50 ml 10 mM tris pH 7·4 and 5 ml aliquots injected into the pith along a 5 cm mid-stem section in each of 10 intact plants placed in a controlled environmental chamber at 20°. Half of the plants were harvested 1 min after application and the others 3 hr later. Upon harvesting, 15 cm mid-stem sections

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were removed from the rest of the plant, plunged into liquid N_2 and total RNA extracted as described above. This was then passed through a MAK column, 5 ml fractions collected and an UV O.D. profile of the fractions obtained as described. ^{14}C incorporation in the fractions was determined on an Ansitron Liquid Scintillation Spectrometer, in Bray's[50] scintillator solution comprised of 60 g naphthalene, 4 g 2,5-diphenyl-oxazole, 0·2 g *p*-bis[2-(5 phenyloxazolyl)] benzene, 100 ml methanol, 20 ml ethylene glycol made up to 1 l. with 1–4 dioxane.

Enzymatic Hydrolyses

RNAse (Worthington Biochemical Co.) was heated 5 min at 100° prior to use to eliminate DNAse and protease activity. 10 μg/mg RNA were incubated in pH 5·0 acetate buffer at 37° for 1 hr. Trypsin (Worthington Biochemical Co.) was used at 30 μg/mg RNA in pH 8·1 Tris buffer. Incubation was at 37° for 1 hr. Pronase (Calbiochem.) was self digested at 37° for 1 hr to inactive DNAse and RNAse prior to use. 60 μg/mg RNA was used in pH 8 5 Tris buffer; incubation was at 37° for 1 hr.

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