

## Plant Nucleases

### III. POLYACRYLAMIDE GEL ELECTROPHORESIS OF CORN RIBONUCLEASE ISOENZYMES<sup>1</sup>

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#### ABSTRACT

Isoenzymes of RNase were detected in plant extracts after polyacrylamide gel electrophoresis with a new buffer system. The gels were incubated in an RNA solution, then dipped for 30 seconds into 0.2% toluidine blue. The method is rapid and is sensitive to very small amounts of RNase. The effects of buffers and ethylenediaminetetraacetate on the different enzymes are illustrated by photographs and scans of the gels.

RNase I, from endosperms and roots, was the fastest-moving corn RNase. Two isoenzymes of corn RNase II, from microsomes, were detected in the hybrid WF9 × M14, while each parental inbred had one of the isoenzymes. Three isoenzymes of corn Nuclease I, from crude mitochondria, had about the same mobility as the RNase II isoenzymes, but were inhibited by ethylenediaminetetraacetate. RNases were also detected in tobacco and wild carrot tissue cultures.

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Plants contain a number of enzymes which degrade RNA. The enzymes found in corn fall into three general classes: (a) RNase I (ribonucleate nucleotido-2'-transferase (cyclizing) E.C. 2.7.7.17), which has a pH optimum near 5, produces 2',3'-cyclic nucleotides, and is a soluble enzyme; (b) RNase II (also E.C. 2.7.7.17), which has a pH optimum at 6 or above, produces 2',3'-cyclic nucleotides, and is associated with crude microsomes; and (c) Nuclease I (E.C. 3.1.4.x), which has a pH optimum near 6, produces 5'-nucleotides from both RNA and DNA, and is associated with large cell particles (20, 21). Pure RNase I has been obtained from corn endosperm (22), while partially purified RNase II and Nuclease I have been obtained from corn roots (20).

Changes in RNase levels in plants have been associated with injury (2, 7), infection (7, 12, 13), growth or growth inhibition (1, 14), RNA synthesis (9), senescence (11, 15), and endosperm maturation (8, 5). However, no widely accepted *in vivo* function for plant RNases has yet been found. Barnard (3) discussed some of the possible functions of RNases.

Unfortunately, most studies on plant RNases have depended upon assays which measure total RNase activity, rather than the activities of the individual RNases, which might have unique

functions *in vivo*. Sodek *et al.* (16) found that EDTA specifically inhibited corn Nuclease I and a similar wheat RNase. This allowed Sodek and Wright (15) to determine that a wheat RNase similar to corn RNase I increased in illuminated, detached wheat leaves, and that this increase was prevented by kinetin. Acton *et al.* (1) found that a light-mediated increase in RNase of lupine hypocotyls was associated with polysomes and ribosomes. This RNase appeared to be either RNase I or II.

I have reported that mixtures of corn nucleases may be partially separated by differential centrifugation and by gel filtration, and that further information on the levels of different nucleases may be gained by determining the activity on RNA at pH 5 and pH 6.2 as well as the activity on DNA (20, 21).

Wolf (24) detected a number of RNases from bean, wheat, and sugar beet after electrophoresis, using a methylene blue stain technique. An assay for phosphodiesterase in the gels (10) showed that some of these plant RNases were also capable of hydrolyzing the  $\alpha$ -naphthyl ester of uridine-5'-phosphate (24). Randles (12) detected changes in RNase isoenzymes in virus-infected Chinese cabbage after polyacrylamide gel electrophoresis, but had to first inhibit the RNase with copper and later restore activity with EDTA. A more rapid method was developed which separated and detected the different partially purified corn RNases after polyacrylamide gel electrophoresis, but it was not suitable for crude preparations (18). This method has now been improved so that crude enzyme preparations from corn and other plants may be separated and detected by gel electrophoresis. The use of specific inhibitors gives further information on the types of enzymes involved. These methods are able to detect the differences in the RNA-degrading enzymes associated with crude mitochondrial and microsomal preparations from corn.

#### MATERIALS AND METHODS

**The Preparation of Enzymes.** The corn (*Zea mays* L., "WF9 × M14") RNase I, RNase II, and Nuclease I enzymes have been described previously (20, 22). Crude extracts of corn endosperm were made by grinding a single mature endosperm with a mortar and pestle, then soaking the tissue in 1 ml of sample buffer (see below) for 2 hr. The extracts were centrifuged, and from 20 to 100  $\mu$ l were applied to the top of the gels. Half-seeds were used when high RNase levels were expected.

Crude enzyme preparations from tobacco (*Nicotiana tabacum* L., var. Xanthi) and wild carrot (*Daucus carota* L.) tissue cultures were provided by Dr. J. Widholm. The tobacco cells were homogenized, centrifuged, and then dialyzed against sample buffer without sucrose. The wild carrot culture medium, dialyzed against sample buffer, was the source of enzymes. It was not determined whether the enzymes had been secreted by the cells or had come from dying or damaged cells.

RNase and DNase test tube assay methods were the same as

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previously described (19, 20). One unit represents the liberation of approximately 0.015  $\mu$ mole of acid-soluble nucleotide per min at 37 C.

**Polyacrylamide Gel Electrophoresis.** The electrophoresis system of Davis (6) was modified by following some of the ideas of Williams and Reinfeld (17). The main gel buffer was changed to 71 mM tris-HCl, pH 7.5, while the spacer gel buffer was changed to 15 mM citric acid and 42 mM HEPES, adjusted to pH 5.5 with NaOH (concentrations are those in the gel). The acrylamide concentration was 7.5% in the main gel and 2.5% in the spacer gel. Samples were layered on the gel in a sample buffer which contained 10% sucrose in the spacer gel buffer. The electrode buffer contained 30 mM barbital and 4.2 mM HEPES adjusted to pH 7.5 with NaOH. Electrophoresis was carried out for 10 min at 2 ma/tube, then for 35 to 40 min at 4 ma/tube until the bromphenol blue dye front had reached the bottom of a 50 mm gel, the anode end.

The method for detection of RNase in polyacrylamide gels has been presented (18) but will be described here in more detail. After electrophoresis, the gels were removed from the tubes at timed intervals and dropped into small test tubes containing 4 ml of a preincubation buffer (see "Results and Discussion" for selection of appropriate buffers). After incubation at 37 C for a fixed period (usually between 10 and 20 min), the solution was changed for one containing 4 mg/ml dialyzed yeast RNA (not high molecular weight). After 15 min at 37 C, the RNA solution was replaced by a postincubation buffer (which is usually the same as the preincubation buffer) for 2 to 3 min, depending upon the time schedule which has been established. Finally, the gels were slid into a small basket made from plastic screen and dipped into a solution of 0.2% toluidine blue and 0.5% acetic acid, adjusted to pH 3. The gels were removed from the dye after 30 sec, rinsed in tap water, and put back into small test tubes containing 0.5% acetic acid, pH 3. The acetic acid was changed two or three times in the next hour, while the dye-free bands of enzyme activity became visible within 10 min. The acetic acid may be replaced by distilled water after 1 hr. With low amounts of enzyme, the stain may remain unchanged for some days. High amounts of enzyme, especially RNase I, will broaden the unstained bands and eventually cause the loss of the blue color from large areas of the gels. The stain may be preserved for some months in 5% perchloric acid, which also changes the color to pinkish violet.

Gels stained with toluidine blue were scanned with the linear transporter of a Gilford Model 240 Spectrophotometer.<sup>2</sup> Blue gels were scanned at 580 nm, the pinkish violet gels at 520 nm. The blue gels have a background absorbance of 1.5 to 2.0, which is reduced in the regions of RNase activity. As little as 0.1 unit of RNase I produced a change detectable by scanning, while it could not be located visually. The background did not produce a horizontal trace, possibly because the cylindrical gels did not maintain the same relative positions passing the slit of the spectrophotometer. Some gels had peaks at the top of the main gel. This seems to be the result of either the discontinuity between the spacer gel and the main gel, or with some crude preparations, the inability of part of the RNase to enter the main gel.

## RESULTS AND DISCUSSION

The new electrophoresis buffer system produced separations of RNases I and II similar to those using the Davis buffer system (18), while the separation of Nuclease I was improved (Fig. 1). The RNase I preparation with two bands was subsequently used

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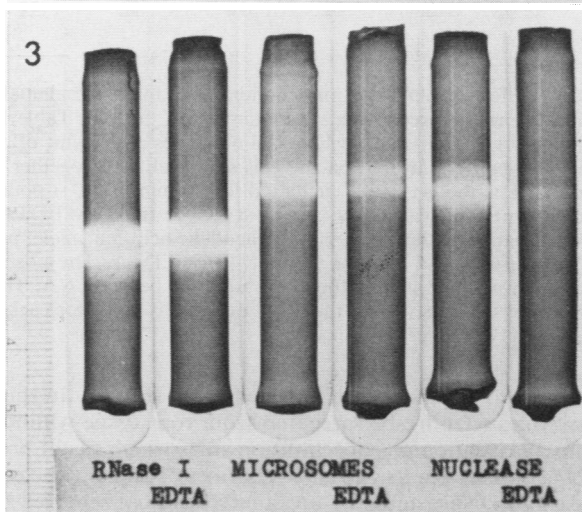
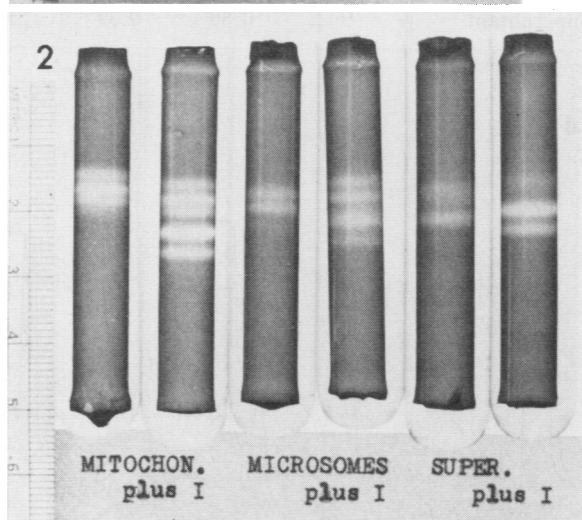
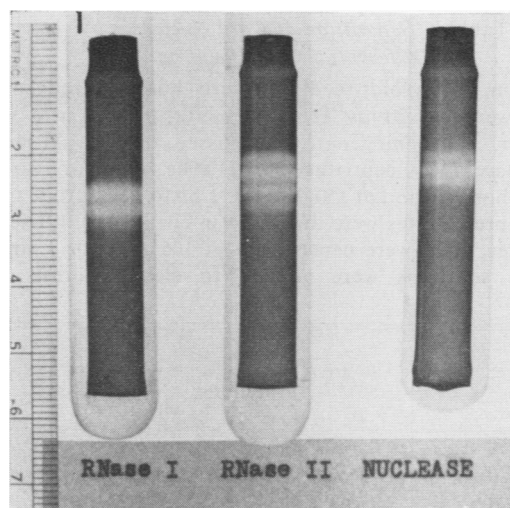


FIG. 1. Polyacrylamide gel electrophoresis of purified corn nuclease enzymes. RNase I: 8 units; RNase II: 10 units; Nuclease I: 9 units.

FIG. 2. Relative mobility of purified corn RNase I and crude mitochondrial nuclease, crude microsomal nucleases, and soluble nucleases. See Table I for details of enzyme preparations. About 2.4 units of RNase I were added where indicated.

FIG. 3. Effect of EDTA on corn nuclease enzymes. RNase I: 12 units; microsomal enzymes 4 units; Nuclease I: 3 units. After electrophoresis the gels were incubated in 0.1 M potassium cacodylate, pH 5.8, and 4 mM EDTA for 25 min, then in an RNA solution with 50 mM potassium cacodylate, pH 6, and 4 mM EDTA for 12 min.

Table I. Separation of Corn Nucleases by Differential Centrifugation

Roots from 3-day-old seedlings were homogenized in two times their weight of 20 mM Tris-HCl buffer, pH 7.5, in a VirTis homogenizer. The homogenate was passed through Miracloth, then was successively centrifuged at 21,000g for 20 min to yield crude mitochondria and at 150,000g for 1 hr to yield crude microsomes. The precipitates were dispersed in citrate-HEPES-sucrose sample buffer, then were centrifuged at 105,000g for 1 hr. The supernatant solutions were applied to electrophoresis tubes (Fig. 2).

	RNase, pH 6.2	RNase, pH 5.0, and RNase, pH 6.2	DNase and RNase, pH 6.2	Gels
	units/ml	ratio	ratio	$\mu$ l
Mitochondria	158	0.49	0.71	20
Microsomes	90	0.53	0.07	30
Supernatant	56	0.80	0.39	50

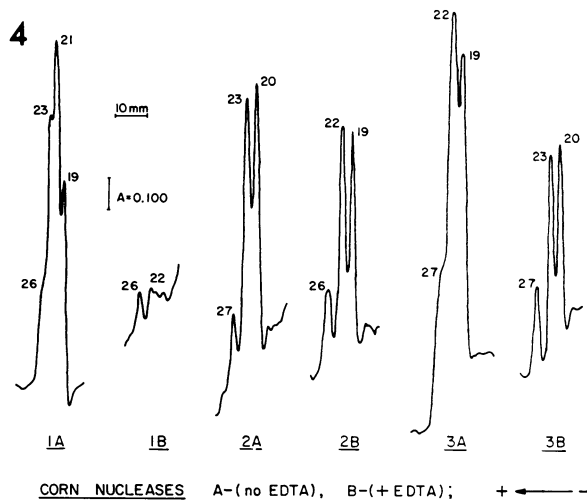


FIG. 4. Effect of EDTA on mitochondrial and microsomal nucleases. The crude enzyme preparations were similar to those in Table I. Approximately 1.25 unit of mitochondrial nucleases and 1 unit of microsomal RNases were layered on the gels separately and together. After electrophoresis the gels were incubated for 20 min in 0.1 M potassium cacodylate, pH 5.8, with either (A) 0.1 M KCl or (B) 4 mM EDTA. The RNA incubation buffers were 50 mM cacodylic acid-imidazole, pH 6.2, with 50 mM KCl and with or without 4 mM EDTA. The post-RNA incubation was for 3 min in the same buffer as before RNA. The distance in mm from the top of the small pore gel is given for each peak. The anode is at the left.

as a standard in mixtures of other enzymes (Fig. 2). In this case the enzymes were freshly prepared from root tissue without any lengthy purification or concentration steps which might produce artifacts or selective loss of some enzymes (Table I). The relative RNase and DNase activities are similar to those reported before (20), with both mitochondria and microsomes having high RNase activity at pH 6.2, while only the mitochondria have appreciable DNase activity. The higher RNase activity at pH 5 in the supernatant solution is due to RNase I, which is soluble (19), though this fraction also contains some Nuclease I and possibly RNase II. The crude mitochondrial RNases (Fig. 2) separated into three bands very similar to those in the purified Nuclease I (Fig. 1). A very similar mitochondrial preparation was the source of the purified Nuclease I. Both RNase I enzymes moved faster than the mitochondrial enzymes, with the possible exception of a

faint fourth band. The microsomal enzymes separated into two major bands plus a very faint faster moving band which corresponded with the slower moving RNase I band. This is in contrast with the purified RNase II, isolated from microsomes (20), with four strong bands (Fig. 1). The fastest of the four bands might be RNase I, which probably has a greater stability through the purification process. It is apparent from the relative mobilities compared to RNase I that the three bands from mitochondria and the two bands from microsomes would overlap on the same gel. The supernatant enzymes included a fast moving band which had the same relative mobility as the slow RNase I, while the slower supernatant enzymes could be a mixture of mitochondrial and microsomal enzymes.

The selective inhibition of Nuclease I by EDTA (16) serves to distinguish the noninhibited RNase I and microsomal RNase from the inhibited Nuclease I preparation (Fig. 3). The residual enzymatic activity may be the result of trace amounts of the RNases or because insufficient time was allowed for complete EDTA inhibition (16). EDTA can help to distinguish the enzymes present in mixtures, as shown in Figure 4. Gel 1A, from mitochondria, had four bands of activity, although the shoulder, 26 mm from the top of the main gel, was distinct only visually. If the gels were incubated in EDTA (1B), very little activity was left except at 26 mm. Comparison with Figure 2 suggests that the 26 mm band is RNase I. These mitochondria were not washed, so that some soluble enzymes would be present. EDTA had little effect on the microsomal RNases (gels 2A and 2B). The peak at 27 mm may also be RNase I. The enzymes from the two sets of cell particles were mixed and separated by electrophoresis, with the same amount of each as in the first two sets of gels (gels 3A and 3B). Although the original enzyme preparations had a total of seven bands, only three were seen for the mixture (gel 3A). After EDTA incubation, three distinct bands were detected, very much like those in gel 2B. The peak at 27 mm, thought to be RNase I (Fig. 2) was larger than in either enzyme preparation alone, suggesting that the same enzyme was present in both preparations. Differential centrifugation has clearly separated the five particle-bound RNases into two classes. The three major enzymes in crude mitochondria (gel 1A) are the precursors of purified Nuclease I, and all three are inhibited by EDTA. The microsomes contain two major RNases (gel 2A), neither inhibited by EDTA. Corn roots appear to contain six different enzymes with RNase activity.

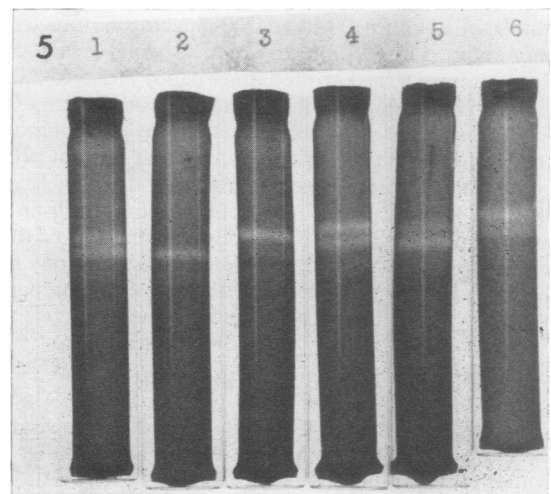


FIG. 5. RNase II isoenzymes. Microsomes were prepared from seedlings of the hybrid WF9  $\times$  M14 and each inbred, using the procedure given for Table I. Approximately one unit of each microsome preparation was applied to the gels: 1: Hybrid; 2: WF9; 3: M14; 4: mixture of WF9 and M14; 5: mixture of the hybrid and WF9; 6: mixture of the hybrid and M14.

A preliminary experiment on the inheritance of RNase II is illustrated in Figure 5. Microsomal preparations were made from WF9, M14, and F1 hybrid seedlings. The hybrid (gel 1) produced two major RNase II bands after gel electrophoresis, while WF9 (gel 2) produced one band and M14 (gel 3) also produced one band. Runs made with mixtures of the two inbred preparations (gel 4) and of each inbred with the hybrid (gels 5 and 6) showed that the two inbred bands were different. The WF9 enzyme corresponds with the faster hybrid band while the M14 enzyme corresponds with the slower band. Attempts to follow genetic changes in the mitochondrial preparations were inconclusive.

Figure 5 also reveals that the background stain is reduced from the top of the small pore gel to a point beyond the faster RNase II band. Similar changes in background staining are apparent in other figures, both photographs and scans. The cause is unknown, but it does not affect the identification of bands of intense RNase activity.

RNase I is found in large amounts in developing and mature corn endosperms (8, 22) and is readily purified from this tissue (22). The RNase concentration in the endosperm is genetically controlled. It is very high in endosperms homozygous for the opaque-2 mutation (4, 5, 23), but it also differs considerably in different inbreds and hybrids (C. M. Wilson, in preparation). Extracts were made from a number of "normal" inbreds and hybrids plus several homozygous for the opaque-2 and floury-2 genes, and from the strains Illinois High Protein and Illinois Low Protein. Extracts from single endosperms or mixed extracts from endosperms of different seeds all produced a single band after electrophoresis. One example, from an M14 inbred endosperm, is shown in Figure 6. It was concluded that only a single RNase I species exists in corn endosperm. This conclusion may appear to be in conflict with the appearance of two bands in one preparation of purified RNase I (Figs. 1, 2, 3). However, this may be an artifact of the purification process (22). Unfortunately, the original batch of seed was not available for testing. RNase was extracted from viable seeds which had been stored at room temperature for 5 years. Upon electrophoresis two or three additional minor bands were seen, usually ahead of the main band. Only single bands have been recovered from fresh or frozen seeds. An endosperm extract left at 3 C for 2 weeks did not develop multiple RNase bands. The RNase I preparation with the artifact band is a convenient standard, since the fast band is always separated from the bands found in fresh extracts from well-preserved samples of either seed or seedling.

The RNases found in the medium in which wild carrot cells were cultured formed two bands upon electrophoresis (Fig. 6). The band at 33 mm moved further than any other RNase reported here.

Cultured tobacco cells yielded four bands of RNase which illustrate the sensitivity of different enzymes to EDTA and buffers (Fig. 7). The bands at 27 and 29 mm were just barely separated to the eye. These two RNases were relatively unaffected by changes in the buffers used during incubation of the gels or by including EDTA in the solutions. The two enzymes at 12 and 16 mm, however, showed high activity only if preincubated and postincubated in potassium cacodylate. EDTA inhibited these two nucleases completely, while citrate at two pH values greatly reduced their activity.

The attention which must be paid to details of timing and buffers depends upon the results wanted. If large amounts of enzyme (5–30 units) can be applied to each gel and qualitative results are sufficient, the different bands may be detected over quite a range of conditions. Semiquantitative results may be obtained if careful attention is paid to details, especially if the gels can be scanned after staining. A time schedule can be established so that the gels move through the incubation and staining procedure at the rate of one a minute. The preincubation step is

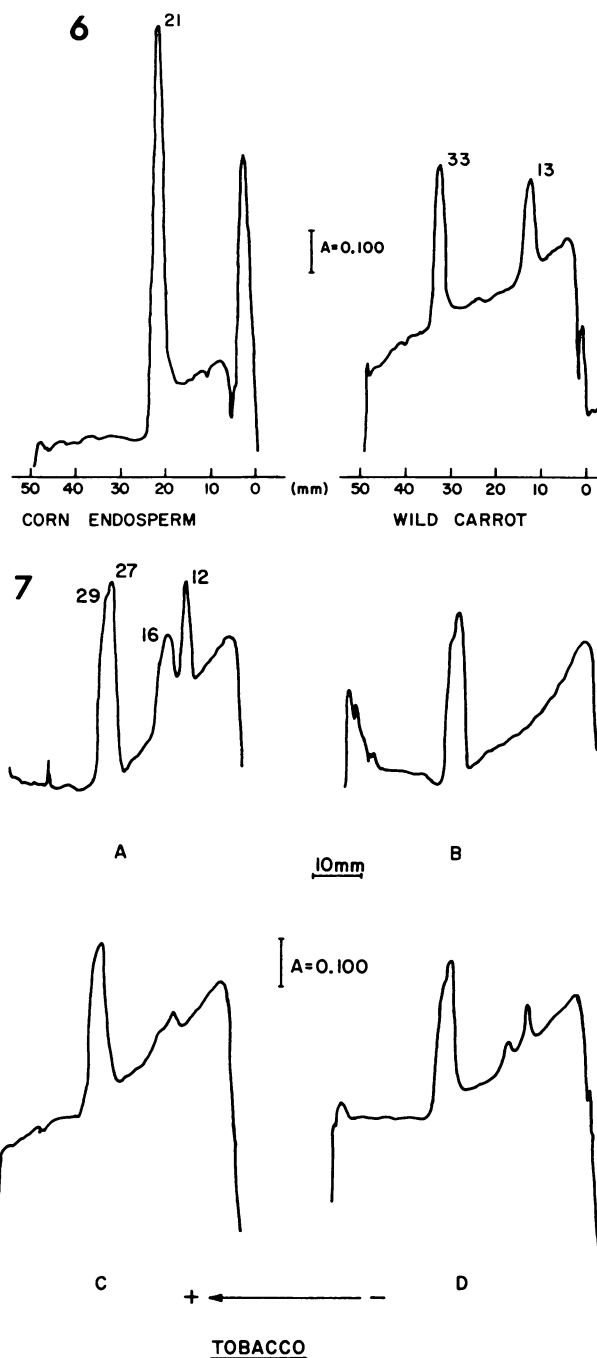


FIG. 6. Gel electrophoresis of nucleases in corn endosperm and in wild carrot culture medium. One seed from an M14 inbred ear with an average RNase content of 42 units per kernel was ground to a powder with a mortar and pestle. The whole endosperm was extracted for 2 hr with 1 ml of sample buffer containing 10% sucrose. After centrifugation 50  $\mu$ l was applied to the top of the gel. About five units of wild carrot enzymes were applied to the gel. After electrophoresis, the gels were incubated in 50 mM citrate buffer, pH 5.0, for 13 min and in RNA with 50 mM sodium acetate, pH 5.4, for 15 min.

FIG. 7. Effect of EDTA and various buffers on the activity of tobacco nucleases after gel electrophoresis. Approximately 8.4 units were applied to each gel. After electrophoresis the gels were incubated in the following buffers: A: 0.1 M potassium cacodylate, pH 5.8, and RNA in 50 mM cacodylic acid-imidazole, pH 6.2, with 50 mM KCl; B: as in A, except that both buffers contained 4 mM EDTA and no KCl; C: 50 mM sodium citrate, pH 5.0, and RNA in 50 mM cacodylic acid-imidazole, pH 5.0, with 50 mM KCl; and D: as in C, except at pH 6.2.

conveniently set at 12 min for 12 gels. A longer time is required for complete EDTA inhibition (16). Increasing time in the RNA solution increases the background stain, while increasing time in the postincubation buffer increases the effects of the enzymes against a decreasing background stain. A longer postincubation period may reveal a small amount of enzyme. The preincubation buffer may be required to change conditions in the surface layers of the gel to those more compatible with maximum RNase activity. As shown in Figure 7, these conditions differ for different enzymes, as might be expected.

Optimum conditions for pH and ionic concentration are known to vary for the different enzymes (19). The double-banded RNase I preparation has been stable for 4 years when frozen, and it has about equal test tube activity in both sodium citrate and cacodylic acid-imidazole buffers at pH 5. Yet a preincubation in citrate buffer resulted in over 50% greater activity of the slow RNase on a gel, as determined by scanning, compared to cacodylic acid-imidazole; while the fast RNase (the artifact band) required, in addition, 50 mM KCl for high activity.

Specific tests to detect specific enzyme activities after electrophoresis have not been found except for the EDTA inhibition of Nuclease I type enzymes. Dithiothreitol inhibits RNases I and II in test tubes assays (G. Apel, unpublished data). It would be useful if a test for DNase activity could be made, but DNA apparently does not penetrate the gel to any appreciable extent, and thus gives only a very weak stain.

The high sensitivity of the assay allows the use of very small amounts of enzyme, while the short time required for the test does not allow for much diffusion. This makes it possible to assay enzymes without having to concentrate solutions. Not all crude extracts can be used without some treatment. If the gels are overloaded with protein, the top of the column may become clogged so that much of the RNase activity is found in the spacer gel or is smeared throughout the gel. If the bands of RNase are found at different distances when different amounts of solution are placed on the gel, one should suspect either high protein concentrations or high salt concentrations. Dialysis or high speed centrifugation may be sufficient to allow satisfactory resolution.

The separation of corn nucleases into three groups (20) was confirmed by the disc gel electrophoresis. Corn RNase I appears to be a single enzyme and is probably the same in the endosperm and root. RNase II is now distinguished from RNase I by its electrophoretic mobility as well as its location on microsomes. There are two isoenzymes of RNase II, one found in the inbred WF9 and the other in the inbred M14, while both are recovered from the hybrid. There are three Nuclease I type isoenzymes in crude mitochondria which are distinguished from the RNases by EDTA sensitivity. Centrifugation in tris-HCl buffer gives a surprisingly clean separation of RNase II from the Nucleases, especially since a VirTis homogenizer at full speed has very vigorous

action. It is now possible to examine critically questions of the nature of the RNases in different cell organelles and the responses of each type of enzyme to growth and to various treatments.

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