Phytochemistry, 1974, Vol. 13, pp. 2445 to 2453. Pergamon Press. Printed in England.

CYTOKININ CONTROL IN SUBCELLULAR LOCALIZATION OF INDOLEACETIC ACID OXIDASE AND PEROXIDASE

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(*Revised Received* 22 *April* 1974)

Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; tissue culture; IAA oxidase, peroxidase; isoenzymes, *cytokinin*; *kinetin*; *membrane*.

Abstract—IAA oxidase and peroxidase were found in all subcellular fractions of tobacco callus cells. The bound and cytoplasmic fractions differed greatly in IAA oxidase/peroxidase ratio and in isoperoxidase composition. The IAA oxidase/peroxidase ratio was particularly high in the plasma membrane-rich fraction. Kinetin had profound effects on IAA oxidase and peroxidase. The appearance of fast migrating isoperoxidases in response to 0·2 μM kinetin was found only in cytoplasmic, plasma membrane and ribosome-rich fractions; a high concentration of kinetin inhibited their formation. High kinetin concentrations also lowered the specific activity of IAA oxidase and peroxidase in all subcellular fractions, but the effect was much greater on peroxidase than on IAA oxidase, thus resulting in a drastic increase in IAA oxidase/peroxidase ratio. Evidently the activities of IAA oxidase and peroxidase were not equivalent and should be considered separately.

INTRODUCTION

PREVIOUSLY kinetin has been shown to promote certain isoperoxidase in excised tobacco pith.[1] The formation of a group of rapidly migrating, anionic IAA oxidase and peroxidase isoenzymes in tobacco callus cultures was later found to be induced or repressed by cytokinins depending on the concentration used.[2,3] Since the change in the isoenzyme profile occurred before new growth of the tissue, the implication was that the change in these isoenzymes might be related to subsequent growth pattern. In understanding the physiological role of these isoenzymes a necessary first step is to know their subcellular distribution. Plant peroxidase was found not only in the cytoplasm but also associated with cell walls,[4,5,6] membranes,[7] nuclei,[8] mitochondria,[9,10] and ribosomes.[10,11] Although there were some discrepancies in the localization which might be due to variations in the materials and methods used, the existence of bound peroxidase *in vivo* appeared to be of little

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[10] DARIMONT, E. and BAXTER, R. (1973) *Planta* **110,** 205.
[11] PENON, P., CECCHINI, J. P., MIASSOD, R., RICARD, J., TEISSERE, M. and PINNA, M. H. (1970) *Phytochemistry* **9,** 73.

doubt. In the crude extracts of plant tissues the free and bound peroxidases showed differences in isoenzyme composition,[12,13] suggesting that the isoperoxidases might differ in their subcellular distribution. However, a comparison of peroxidase isoenzyme profiles from different subcellular loci of the same tissue was not available.

This report describes the subcellular localization of IAA oxidase and peroxidase, the isoenzyme composition in subcellular fractions, and the effect of kinetin on these enzymes.

RESULTS

Subcellular localization

IAA oxidase activity was found in all subcellular fractions (Table 1). Striking differences in specific activity were apparent when the enzymes isolated from the plasma membrane-rich fraction were compared to those from other fractions. The effect of kinetin was also evident. Previous work has shown that 0·2 μM kinetin is optimum for the formation of the rapidly migrating isoenzymes of IAA oxidase and peroxidase and for the increase of the total activity of both enzymes.[2,3] In the present work therefore, 0·2 μM kinetin was used as a standard treatment. Increasing the kinetin concentration from 0·2 to 5·0 μM caused a decrease in the specific activity of IAA oxidase in all fractions. The most significant change was in the cytoplasmic fractions, in which the IAA oxidase activity decreased by 60% with the increasing concentration of kinetin.

TABLE 1. EFFECT OF KINETIN ON SPECIFIC ACTIVITY OF IAA OXIDASE IN SUBCELLULAR FRACTIONS OF TOBACCO CELLS*

Fraction	IAA oxidase activity μg IAA destroyed/μg protein in 30 min		
	Kinetin 0·2 μM	Kinetin 1·0 μM	Kinetin 5·0 μM
Cell walls	29·4	26·8	22·3
Plasma membranes	8·8	6·0	5·7
Nuclei	28·9	23·2	24·7
Mitochondria	27·4	19·2	17·5
Ribosomes	27·9	27·2	22·4
Cytoplasm	24·9	17·6	10·9

* Grown on Linsmaier and Skoog's medium[14] with 2 μM GA_3, 10 μM IAA and varied concentrations of kinetin.

The peroxidase activity, determined with guaiacol and *o*-dianisidine as H-donors, was found associated also with all subcellular fractions but it varied greatly according to fraction (Table 2). For example, the specific activity of the cytoplasmic peroxidase was 10× greater than that of the plasma membrane-bound peroxidase which, similarly with IAA oxidase activity, was the lowest. Increasing the kinetin concentration significantly lowered the specific activity of peroxidase in all subcellular fractions and the extent of the decrease was greater than that for IAA oxidase. In the cytoplasmic fraction, for example, the peroxidase activity decreased by more than 90% when the concentration of kinetin was increased from 0·2 to 5·0 μM.

[12] MACNICOL, P. K. (1973) *Phytochemistry* **12**, 1269.
[13] LEE, T. T. (1973) *Physiol. Plant.* **29**, 198.
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TABLE 2. EFFECT OF KINETIN ON SPECIFIC ACTIVITY OF PEROXIDASE IN SUBCELLULAR FRACTIONS OF TOBACCO CELLS*

Fraction	Peroxidase activity A470 nm/μg protein in 1 min		
	Kinetin 0·2 μM	Kinetin 1·0 μM	Kinetin 5·0 μM
	Guaiacol as H-donor		
Cell walls	0·340	0·285	0·095
Plasma membranes	0·033	0·008	0·004
Nuclei	0·279	0·102	0·078
Mitochondria	0·220	0·060	0·028
Ribosomes	0·164	0·164	0·062
Cytoplasm	0·384	0·101	0·036
	o-Dianisidine as H-donor		
Cell walls	0·510	0·450	0·125
Plasma membranes	0·056	0·015	0·008
Nuclei	0·760	0·159	0·125
Mitochondria	0·365	0·088	0·045
Ribosomes	0·215	0·215	0·066
Cytoplasm	0·600	0·148	0·048

* Grown on Linsmaier and Skoog's medium[14] with 2 μM GA_3, 10 μM IAA and varied concentrations of kinetin.

From previous work with crude extracts, it has been known that a group of anionic IAA oxidase and peroxidase isoenzymes with high electrophoretic mobilities was mostly affected by cytokinins[2,3] and it is of interest to know whether this group of isoenzymes is bound or cytoplasmic. Figures 1–3 show the isoenzyme profiles of peroxidase isolated from different subcellular fractions. For each fraction, two profiles of isoperoxidase isolated from the tissue treated with two concentrations of kinetin are compared. Since the enzyme samples contained known amounts of proteins and since the electrophoresis and the enzyme assay were done under similar conditions, the isoenzyme profiles are directly comparable. Figures 1–3 reveal two major differences: a different subcellular distribution of isoperoxidase and a differential effect of kinetin on certain localized isoperoxidases.

The isoperoxidases thus separated have not been individually characterized. For convenience in description, the isoenzymes may be arbitrarily classified into three groups (see Fig. 1, cytoplasmic). The first is a slow migrating group with R_f from 0 to 0·23, the second is an intermediate group with R_f from 0·23 to 0·58, and the third is a fast migrating group with R_f from 0·65 to 0·90. The most noticeable difference in subcellular localization was the absence of the fast migrating group in the cell wall, nuclei and mitochondria-rich fractions. This group was found predominantly in the cytoplasmic fraction and less so in the ribosome and plasma membrane-rich fractions. In the other two groups, the relative activity of individual isoperoxidase also varied with subcellular fractions. For example, the isoperoxidase in the slow migrating group with an R_f ca 0·2 and corresponding to P_3 in the cytoplasmic fraction was prominent in the nuclei, mitochondria and ribosome-rich fractions but it was negligible in the cell wall and plasma membrane-rich fractions. The isoperoxidase P_7 in the intermediate group with an R_f ca 0·45 showed high activity in the cytoplasmic and ribosome-rich fractions but was missing in the cell wall, plasma membrane, nuclei and mitochondria-rich fractions. Thus significant differences in peroxidase isoenzyme composition among subcellular loci in the same tissue were apparent.

The effect of kinetin on peroxidase isoenzyme composition at different loci was also evident (Figs. 1–3). The most striking change associated with a high concentration of kinetin

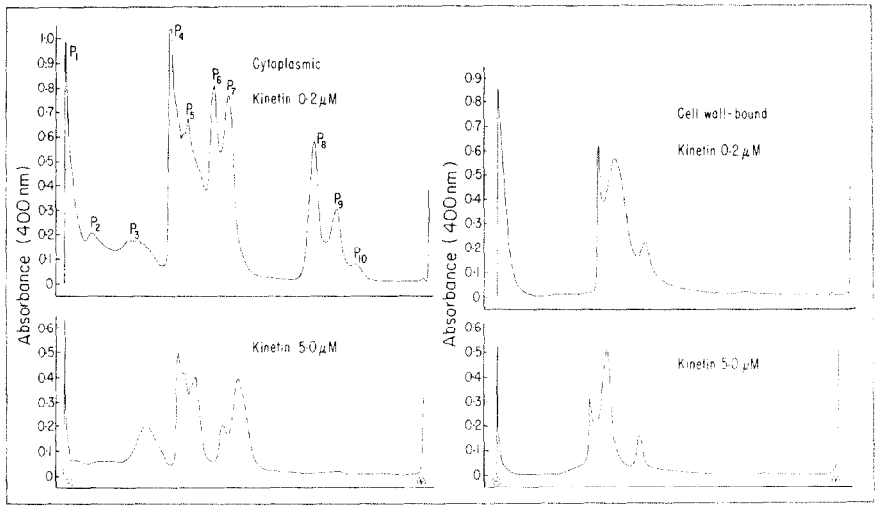

FIG. 1. ISOPEROXIDASE PROFILES OF CYTOPLASMIC AND CELL-WALL FRACTIONS OF TOBACCO CELLS GROWN WITH DIFFERENT CONCENTRATIONS OF KINETIN.
2 μg Protein per gel and 30 min staining.

(5 μM) was the absence of the fast migrating isoenzymes in the cytoplasmic, ribosome and plasma membrane-rich fractions. In these three fractions the development of certain isoenzymes in the slow migrating group was also inhibited by 5·0 μM kinetin. The least affected was the intermediate group in which the isoperoxidase composition was not changed; a high concentration of kinetin lowered only the specific activity. The isoperoxidases associated with the cell wall, nuclei and mitochondria-rich fractions were also less affected.

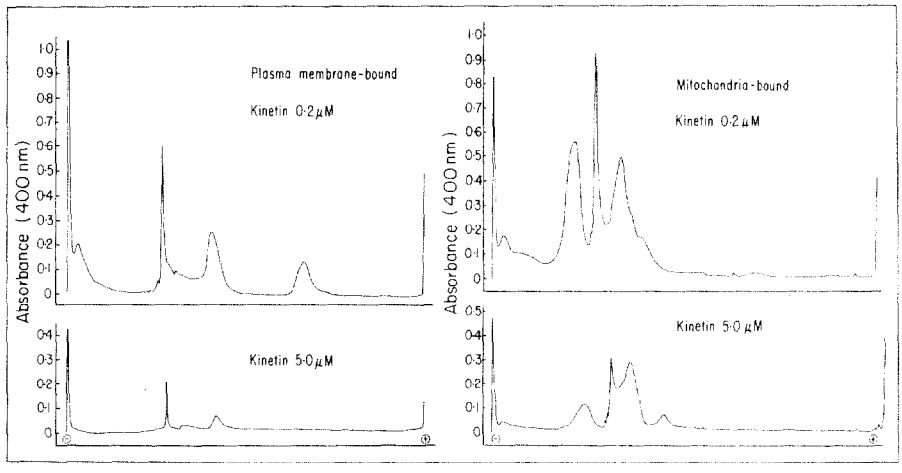

FIG. 2. ISOPEROXIDASE PROFILES OF PLASMA MEMBRANE AND MITOCHONDRIA FRACTIONS OF TOBACCO CELLS GROWN WITH DIFFERENT CONCENTRATIONS OF KINETIN.
2 μg Protein per gel and 30 min staining for mitochondria fraction and 4 μg protein per gel and 60 min staining for plasma membrane fraction.

Thus, clearly a high concentration of kinetin not only lowered the specific activity of isoperoxidases in general but specifically inhibited the formation of certain isoperoxidases associated with cytoplasm, ribosomes and plasma membranes.

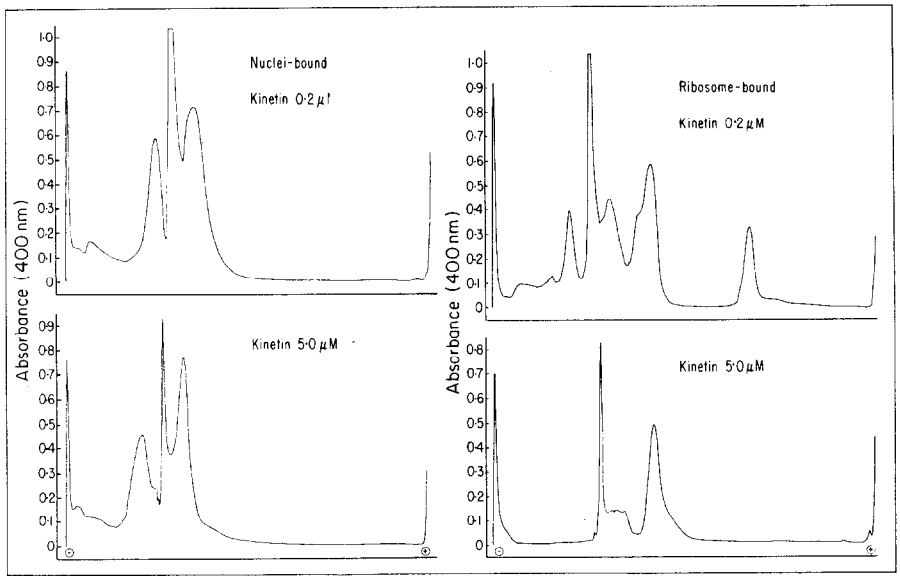

FIG. 3. ISOPEROXIDASE PROFILES OF NUCLEI AND RIBOSOME FRACTIONS OF TOBACCO CELLS GROWN WITH DIFFERENT CONCENTRATIONS OF KINETIN.
2 μg Protein per gel and 30 min staining.

IAA oxidase/peroxidase ratio

The relative activity of IAA oxidase and peroxidase in the same enzyme preparation was used as one of the criteria to differentiate whether the peroxidases isolated from the subcellular fractions were the same proteins. The data show that the enzymes extracted from different subcellular fractions varied greatly in the IAA oxidase/peroxidase ratio (Table 3).

TABLE 3. EFFECT OF KINETIN ON RELATIVE ACTIVITY OF IAA OXIDASE AND PEROXIDASE IN SUBCELLULAR FRACTIONS OF TOBACCO CELLS*

Fraction	IAA oxidase/peroxidase ratio†		
	Kinetin 0·2 μM	Kinetin 1·0 μM	Kinetin 5·0 μM
Cell walls	2·6	3·1	8·0
Plasma membranes	8·8	29·8	56·6
Nuclei	3·4	7·5	10·8
Mitochondria	4·1	10·6	21·8
Ribosomes	5·7	5·6	11·8
Cytoplasm	2·2	5·9	9·9

* Grown on Linsmaier and Skoog's medium[14] with 2 μM GA_3, 10 μM IAA and varied concentrations of kinetin.

† Guaiacol as H-donor for peroxidase. Compared on the basis of 30 min reaction.

Particularly significant was the high relative IAA oxidase activity in the plasma membrane-rich fraction in which the IAA oxidase/peroxidase ratio was 4–5× that in the cytoplasmic fraction and 3–9× that in the cell wall-rich fraction. Increasing the kinetin concentration raised to a varied degree the IAA oxidase/peroxidase ratio; a more than 5× increase was found in the plasma membrane-rich fraction. As a result, the differences in IAA oxidase/peroxidase ratios among subcellular fractions were even more significant in the tissue treated with the higher concentrations of kinetin.

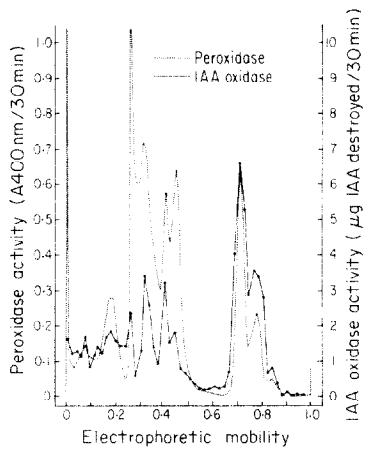

FIG. 4. COMPARISON OF IAA OXIDASE AND PEROXIDASE PROFILES OF THE CYTOPLASMIC FRACTION OF TOBACCO CELLS.
The tissue was grown with 0·2 μM kinetin, 10 μM IAA and 2 μM GA_3. An extract containing 2 μg protein was applied to each gel.

The relative activities of IAA oxidase and peroxidase in isoenzyme fractions were also compared and one example is shown in Fig. 4. In this case after electrophoresis, the IAA oxidase was extracted from the gel slices by an overnight incubation with a phosphate buffer and then assayed while the peroxidase was assayed by incubating the intact gel with benzidine as the H-donor. Because of differences in the methods for enzyme preparation and assay, the relative enzyme activities shown in Fig. 4 are not directly comparable to those presented in Table 3, but there is a clear indication of differences in relative activities of IAA oxidase and peroxidase among isoenzyme fractions. The peaks of peroxidase matched very well with those of IAA oxidase but their relative enzyme activities were different. Apparently the isoenzymes in the fast migrating group had higher IAA oxidase/peroxidase ratios than those in the intermediate group.

Thus the IAA oxidase/peroxidase ratio was different not only among the crude enzyme preparations from different subcellular fractions but also among isoenzymes of a single fraction. A treatment with a high concentration of kinetin increased the IAA oxidase/peroxidase ratio.

Plasma membrane-bound IAA oxidase

The IAA oxidase associated with the plasma membrane-rich fraction appears to be of special interest because of the high IAA oxidase/peroxidase ratio in this fraction. Preliminary results showed it had a cofactor requirement *in vitro* similar to that of the cytoplasmic

IAA oxidase; in both cases, 2,4-dichlorophenol and Mn^{2+} ions were required. The destruction of IAA with the formation of intermediates and end-products were followed by recording the UV spectrum of the reaction mixture. The change in UV spectrum, during IAA oxidation *in vitro*, catalyzed by the enzyme solubilized from the plasma membrane-rich fraction, was similar to that seen when catalyzed by the cytoplasmic enzyme. In both cases, the prominent product absorbed strongly at 248 and 254 nm. The product is likely to be 3-methyleneoxindole according to Hinman and Bauman.[15]

DISCUSSION

Although the enzyme activity associated with the insoluble particulate fractions after maceration of the tissue may not be due to particulate-bound enzyme *in vivo*, the data presented here suggest that the bound IAA oxidase and peroxidase isolated were different from those readily extractable by an acetate or phosphate buffer of low ionic strength. The match of the isoperoxidase peaks with those of IAA oxidase (Fig. 4) suggests the major isoperoxidases and IAA oxidase isoenzymes were closely associated with each other. If the bound enzymes were cytoplasmic *in vivo* and became bound as a result of maceration of the cells, then the IAA oxidase/peroxidase ratio should remain the same as the cytoplasmic fraction. On the contrary, the ratios were significantly different (Table 3).

One other possibility is that the difference in IAA oxidase/peroxidase ratio could be due to different affinity of the isoenzymes for the binding site in the cell walls, membranes and ribosomes. Because the isoenzymes had different relative activity of IAA oxidase and peroxidase (Fig. 4), the overall IAA oxidase/peroxidase ratio would be different if the isoenzymes were differentially adsorbed to the particulate fractions. If this were the case, in order to have a high IAA oxidase/peroxidase ratio the level of the fast migrating group should be increased and the level of the intermediate group should be decreased. It is difficult to calculate precisely the contribution of different isoenzymes to the overall relative IAA oxidase activity because the relative enzyme activity shown in Fig. 4 and Table 3 are not directly comparable. By an approximation, however, it appears that the high IAA oxidase/peroxidase ratio in the plasma membrane and mitochondria-rich fraction cannot be interpreted solely on the basis of differential adsorption of cytoplasmic isoenzymes. Taking the sample treated with 0·2 μM kinetin as an example, the low level of the fast migrating group in the plasma membrane-rich fraction and the absence of this group in the mitochondria-rich fraction (Fig. 2) would decrease the overall IAA oxidase/peroxidase ratio; but in fact the ratio increased significantly (Table 3). The change in the relative proportion of the intermediate isoenzyme group alone appears to be insufficient to account for the 1–3 × increase in IAA oxidase/peroxidase ratio. Differences in relative IAA oxidase activity have been noted between the bound and water-soluble peroxidase in different plant tissues.[12,16] The indications therefore are that the bound peroxidases thus isolated were unlikely to be an extraction artefact.

From the isoperoxidase profile (Figs. 1–3) and from the crude enzyme activity (Tables 1–3), the plasma membrane rich fraction showed its own identity. It had five distinct isoperoxidase peaks corresponding to P_1, P_2, P_4, P_6 and P_8 in the cytoplasmic fraction. It differed from the mitochondria and nuclei-rich fractions which had prominent peaks of P_3 and P_5 but had none of P_8. It differed from the ribosome-rich fraction which had distinct peaks of P_3 and P_5 but very little P_2. It differed also greatly from other fractions

[15] Hinman, R. L. and Bauman, C. P. (1964) *J. Org. Chem.* **29**, 2431.

[16] Raa, J. (1973) *Physiol. Plant.* **29**, 247.

in specific and relative IAA oxidase and peroxidase activities. Therefore, the plasma membrane-rich fraction was not identical to any other fraction. As far as IAA oxidase and peroxidase are concerned, the possible inclusion of nuclei, mitochondria and ribosomes in this fraction as impurities was negligible.

The enzyme associated with the plasma membrane-rich fraction is of particular interest because of the high relative IAA oxidase activity (Table 3). The striking difference in IAA oxidase/peroxidase ratios between the plasma membrane-bound and the cytoplasmic enzymes suggests a different physiological role. Although the cofactor requirement and the UV spectrum of the product of IAA oxidation *in vitro* were similar for the solubilized bound enzyme and the cytoplasmic enzyme, the behaviour of the plasma membrane-bound enzyme *in vivo* may be different.

Too often, peroxidase activity has been used to represent IAA oxidase activity and the results could be misleading. From the present work, evidently the activities of IAA oxidase and peroxidase are not equivalent (Table 3 and Fig. 4). The relative IAA oxidase activity, per unit peroxidase, varied with the subcellular localization and the isoenzyme fraction in the same tissue and with the tissue treated with kinetin at different concentrations.

Profound effects of kinetin on subcellular localization of the enzymes are apparent. The appearance of the fast migrating isoperoxidases in response to a low concentration of kinetin was found only in the cytoplasmic, ribosome and plasma membrane-rich fractions (Fig. 1–3). Earlier work has shown that the formation of this group of isoenzymes was inhibited by cycloheximide and actinomycin-D suggesting a requirement of protein and RNA synthesis.[2,3] The present finding that the fast migrating isoperoxidase is associated with ribosomes supports the previous conclusion. A high kinetin concentration inhibited their formation.

The differential effect of kinetin on IAA oxidase and peroxidase may be of particular significance. From Tables 1 and 2, apparently the decrease in peroxidase activity in response to a high concentration of kinetin was more pronounced than the decrease in IAA oxidase activity. This difference is reflected in the drastic increase of the IAA oxidase/peroxidase ratio (Table 3). Thus a high concentration of kinetin not only reduced the total and specific activities of IAA oxidase and peroxidase in the tissue[2,3] and effectively inhibited the synthesis of the fast migrating isoenzymes,[2,3] but also it altered the characteristics of the enzyme as an oxidase or peroxidase. The drastic decrease in peroxidase activity and the increase in relative IAA oxidase activity could mean a change in physiological function of the enzyme; this change should be further studied together with the change in plant growth and morphogenesis.

EXPERIMENTAL

Plant material. Tobacco callus tissues (*Nicotiana tabacum* cv. White Gold) were grown as previously described.[2] The tissue was chosen because it required an exogenous supply of cytokinin for growth and was sensitive to low concentrations of kinetin. Five slices of stock tissue were used in each 125-ml conical flask containing 50 ml of medium and 10 or more replicate flasks were used in each experiment. Four-week-old tissue was used for enzyme preparation.

Enzyme preparation. Ca. 50–100 g of tissue was ground in a mortar with an acetate buffer (25 mM, pH 5·5) containing 0·45 M sucrose. The slurry was filtered through 1 layer of miracloth under suction. The particles retained in the miracloth were used as the starting material for the cell-wall fraction, as described below. The filtrate was subjected to stepwise differential centrifugations at 100, 2000 and 20000 *g* for 10 min each and at 105000 *g* for 2 hr. The supernatant of the last-step centrifugation was used as the cytoplasmic fraction. The pellet collected at each step was re-suspended in fresh medium and centrifuged at the appropriate speed; the washing was repeated 5 × for each fraction. The washing removed soluble enzymes and improved the homogeneity of

the fractions. The washed pellets of 100, 20000 and 105000 *g* were regarded as the nuclei, mitochondria and ribosome-rich fractions, respectively.

The pellet centrifuged at 2000 *g* for 10 min, after being washed 5×, was re-suspended in 3 ml of the extraction medium and loaded on a discontinuous sucrose gradient of the following composition: 5 ml of 1·25 M, 9 ml of 1·50 M, 9 ml of 1·75 M and 5 ml of 2 M sucrose. It was centrifuged in a Beckman L-2 ultracentrifuge with SW-25 rotor at 63000 *g* for 2 hr. After centrifugation, the material was collected at each interface and washed with the acetate buffer. By means of electron microscopic examination the material collected at the 1·5 M/1·75 M interface was chosen as the plasma membrane-rich fraction.

The particles remaining on the miracloth after the first filtration was further thoroughly homogenized in a motor-driven Potter–Elvehjem homogenizer. The insoluble matter was washed with several portions of large vol. of dilute acetate buffer on miracloth which was changed frequently to facilitate the washing. The exhaustively washed insoluble material was used as the cell wall-rich fraction.

All particulate fractions were then suspended in 3–4 ml of second extraction medium containing 0·8 M KCl, 10 mM EDTA and 25 mM acetate buffer (pH 5·5) and sonicated; the suspensions were centrifuged 2 hr later. All extracts were dialyzed overnight against a large vol. of cold de-ionized H_2O. Except for the ribosome fraction, which was centrifuged at 105000 *g* for 2 hr, the dialyzed extracts were clarified by centrifuging at 20000 *g* for 10 min. All operations were done at 2–4°. The clarified extracts were assayed for protein content[17] and diluted on the basis of unit weight of protein.

Electrophoresis. Electrophoresis was accomplished using the method of Davis.[18] The gel column was composed of 2 sections, 0·4 ml of the stacking gel at the top and 1 ml of 5·5% polyacrylamide separation gel at the bottom. The clarified extracts containing 2–4 μg protein were applied to each column. Other details were the same as previously described.[3]

Enzyme assay. The IAA oxidase and peroxidase activities in the crude enzyme preparation and in the isoenzyme fractions after electrophoretic separation were determined by the same methods as previously described.[2,3] The peroxidase activity was also determined with *o*-dianisidine (6 mM) as the H-donor. The IAA oxidase activity was also studied by following the change in UV spectrum. In this case, the reaction mixture consisted of 0·05 M phosphate buffer, 0·05 mM 2,4-dichlorophenol, 0·1 mM $MnCl_2$ and 0·13 mM IAA at pH 5·9. The reaction was started by adding the enzyme with 3 μg protein and the reaction mixture was incubated in a shaking water bath at 37°. The UV spectrum was scanned every 10 min with a Unicam SP 8000 spectrophotometer.

Acknowledgements—I thank Mrs. C. E. Wilkinson and Mr. W. G. Graham for technical assistance.

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[18] DAVIS, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404.