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THE INDOLE-3-ACETIC ACID OXIDASE OF LUPINUS LUTEUS L. I. A QUALITATIVE COMPARISON OF THE ACTIVITY OF THIS ENZYME IN ROOT NODULES AND ROOTS

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SUMMARY

Ammonium sulphate precipitation and filtration through Sephadex G-100 were used to purify the IAA-oxidase present in root nodules and roots of *Lupinus luteus* L. During the gel filtration all fractions showing IAA-oxidase activity also showed polyphenoloxidase activity – although this is presumably a different enzyme – and peroxidase activity, the latter being almost strictly in parallel with IAA-oxidase activity, even after ion-exchange chromatography. We conclude that the same IAA-degrading enzyme system is present in root nodules and roots.

1. INTRODUCTION

This study forms part of a physiological research programme being conducted by our laboratory on the several aspects of symbiotic nitrogen fixation in leguminous plants.

It is known that after infection of the roots with *Rhizobium* bacteria, root nodules are formed as a result of the symbiosis of the host plant and the bacteria. The full-grown root nodules of the yellow lupine used in this study were found microscopically to consist almost exclusively of bacteroid tissue. This tissue originates from the root nodule meristem, and the cells are fully elongated. Since it is known from phytopathology that strongly proliferated and hypertrophic tissues show hyperauxiny (SEQUEIRA 1963), we also had reason to expect large amounts of indole-3-acetic acid (IAA) to be present in our root nodules.

By spectrofluorometrical determination of IAA in the acid fraction of methanol extracts of whole root nodules, parental roots, and 14-day-old non-nodulated roots of *Lupinus luteus* L. (the same type of material as used in our study), DULLAART (1967) showed that the root nodules contain about three times more IAA than the roots. This raises the question of the origin of this relatively high amount of IAA in the root nodules. Since it is known that the symbiotic *Rhizobium* bacteria produce IAA from tryptophan (e.g. HARTMANN & GLOMBITZA 1967; DULLAART 1970), this might explain the high level of IAA in the full-grown root nodules. But these levels could also be due to an alteration of the indole metabolism of the tissue itself as a reaction to the infection, the increased IAA level resulting from increased synthesis by the host as well as

from interference with the mechanisms of auxin degradation in the host (SEQUEIRA 1963).

DULLAART (1970) concluded from in vitro experiments that at least a substantial part of the large amount of IAA present in the root nodules was produced via plant enzymes as a result of a metabolic alteration induced by the rhizobial infection. In this connection it seemed interesting to look for possible differences between the IAA-oxidase activity in roots and root nodules. In part I of this report[1] the elution patterns of enzyme activities, obtained after Sephadex filtration and ion-exchange chromatography, are compared qualitatively. The second part[1] deals with the quantitative comparison of the IAA-oxidase activity in root nodules and roots found in vitro.

2. MATERIALS AND METHODS

2.1. Plant materials

Lupinus[2]: Lyophilized nodules, parental roots minus the nodule zones, and young, non-nodulated roots of yellow lupine (*Lupinus luteus* L. 'gele bittere lupine') were used in our experiments. The plants grown in the field were pulled after 38, 56, and 70 days' growth. After the roots and root nodules had been collected, they were thoroughly washed with distilled water, dried on filter paper, lyophilized immediately, and stored at –20 °C.

Young, non-nodulated roots were collected from plants grown in the laboratory. Lupine seeds were soaked for 17 hours in distilled water under aeration, washed several times, and sown in plastic containers containing vermiculite impregnated with distilled water. After a 3-day germination period in the dark at 20 °C, they were grown for 14 days under constant conditions: 16 hours light per day (light source Philips TL 40W/33, distance from light tubes 150 cm, energy on plant level 20,000 erg/sec/cm^2), 8 hours darkness; temperature 20 °C; relative humidity about 70 %. The roots were then pulled, washed with distilled water, dried on filter paper, lyophilized, and stored at –20 °C.

2.2. Enzyme preparation

Lyophilized nodules or roots (the latter after fragmentation) were powdered in a mortar with some sand (HCl-washed and glowed). For approximately each gram fresh weight, 1.0 ml phosphate buffer (Na_2HPO_4/NaH_2PO_4, 0.1 M; pH 5.5) was then added and the brei homogenized for 30 minutes. (See fractionation scheme on page 696).

All fractions were dialysed for 18 hours (1½ hours against 2 litres of distilled water; 1½ hours against 3 litres of distilled water; 15 hours against 4 litres of buffer solution, 0.01 M). Part of fraction Se 100 was filtered through a Sepha-

[1] These papers formed part of a thesis (MENNES 1972), reprints of which are available on request.

[2] The author is indebted to Mr. G. Bikker of the Rijkskwekerij Drakenburg at Baarn, who kindly supplied the plant material.

The brei was centrifuged at 20,000 g for 10 minutes.

↓ Pellet discarded.

↓ Supernatant fluid centrifuged at 100,000 g for 20 minutes.

↓ Pellet discarded.

↓ Supernatant fluid: FRACTION E

↓ Supernatant fluid 30% saturated with solid $(NH_4)_2SO_4$ (20 minutes under mechanical stirring) and centrifuged at 20,000 g for 20 minutes.

↓ Pellet dissolved in a small volume of buffer: FRACTION Se 30

↓ Supernatant fluid 50% saturated with solid $(NH_4)_2SO_4$ (20 minutes under mechanical stirring) and centrifuged at 20,000 g for 20 minutes.

↓ Pellet dissolved in a small volume of buffer: FRACTION Se 50

↓ Supernatant fluid 100% saturated with solid $(NH_4)_2SO_4$ (at least 2 hours under mechanical stirring) and centrifuged at 20,000 g for 20 minutes.

↓ Pellet dissolved in a small volume of buffer: FRACTION Se 100

↓ Supernatant: FRACTION Su 100

dex G-100 column and the fractions belonging to the activity peak were combined to form fraction K.

After dialysis the fractions were centrifuged at 20,000 g for 30 minutes to remove the precipitates formed during dialysis, and the clear supernatant fluids were stored in small portions at −20 °C when not used immediately.

All procedures were carried out at +2 °C, and the relevant amounts of $(NH_4)_2SO_4$, calculated from the table given by GREEN & HUGHES (1955), were corrected for this temperature.

2.3. Gel filtration

Sephadex G-100 (particle size 40–120 μ, fractionation range 5,000–100,000) was allowed to swell for 3 days in excess buffer solution (Na_2HPO_4/NaH_2PO_4, 0.1 M; pH 5.5) with intermittent stirring and decantation. After swelling the gel was packed into a column measuring 90 × 1.16 cm (inner diameter). A sample of fraction Se 100 was layered on the column and washed out with an identical elution buffer at an operating pressure of 30 cm, using a Mariotte flask as a constant head reservoir. The column was provided with a cooling jacket and kept at a temperature of +4 °C. Aliquots of 2 ml were collected with the aid of a fraction collector (LKB 400 B RadiRac). The optical density (OD)

of the effluent was measured at 280 mμ (protein measurement) in a Uvicord (LKB 8300 A Uvicord II). Test tubes were kept at +4 °C during the fractionation by placing the test-tube racks in a cooling trough. The void volume was determined with Blue Dextran 2000 as a marker.

2.4. Ion-exchange chromatography

SP-Sephadex C-50 cation-exchanger was allowed to swell in a large excess of buffer solution (Na_2HPO_4/NaH_2PO_4, 0.1 M; pH 5.5). Over a period of 24 hours the supernatant liquid was replaced three times by fresh buffer solution. A column measuring 44 × 1.16 cm (inner diameter) and equipped with a cooling jacket was packed with the swollen ion-exchanger. After the sample had been layered, the column was developed by a starting condition procedure under an operating pressure of about 40–45 cm. Aliquots of 2 ml were collected (see above). Upon completion of an experiment the ion-exchanger was regenerated and the column repacked.

2.5. Incubation methods

2.5.1. IAA-oxidase activity

The reaction system contained 1.0 ml buffer solution (Na_2HPO_4/NaH_2PO_4, 0.1 M; pH 5.5), 1.0 ml of a solution of 5×10^{-3} M 2,4-dichlorophenol (DCP) in buffer, 1.0 ml of a solution of 5×10^{-3} M $MnCl_2$ in water, 1.0 ml of a solution of IAA (300 μg/ml) in buffer, and 1.0 ml enzyme solution diluted in buffer. Incubation took place in 5.0-ml tissue culture tubes at 26 °C in diminished light (light source Philips PF 713E). In some cases (indicated in the text) 1.0 ml of a H_2O_2 (36×10^{-7} g/ml) solution in buffer was added instead of the buffer solution. At regular intervals aliquots of 0.5 ml were taken with a pipette and brought into 0.5 ml 1/3 N HCl to stop the reaction, after which 2 ml of the Salkowski reagent (GORDON & WEBER 1951) were added and the mixture thoroughly stirred. Colour development occurred at 26 °C in the dark. Between 60 and 180 minutes after the addition of the Salkowski reagent the OD was measured in a Vitatron photometer (filter 532 mμ) against a blank lacking IAA. A standard curve prepared with a graduated series of IAA concentrations in 1/6 N HCl showed linearity between OD at 530 mμ and the IAA concentration up to 35 μg/ml. The final concentration of the HCl added to stop the reaction, had no influence on the IAA concentration for at least 3 hours. The H_2O_2 concentration used did not influence the colour development of the Salkowski reaction.

2.5.2. Peroxidase activity

The reaction system contained 1.0 ml H_2O_2 0.3% (v/v), 1.0 ml pyrogallol 0.1 M, enzyme solution, and buffer (Na_2HPO_4/NaH_2PO_4, 0.1 M; pH 5.5) to a final volume of 10 ml (KONINGS 1965). The reaction was started by adding 1.0 ml of pyrogallol solution to the mixture of buffer and H_2O_2, followed immediately by the enzyme solution. After 5 minutes' reaction at room temperature OD was measured in a Vitatron photometer (filter 402 mμ) against

distilled water. Corrections were made for a blank lacking the enzyme.

2.5.3. Polyphenoloxidase activity

To a reaction system containing 0.5 ml enzyme solution and 3.0 ml buffer solution (Na_2HPO_4/NaH_2PO_4, 0.1 M; pH 5.5), 0.5 ml of a 5% catechol solution was added (JANSSEN 1970). After 30 minutes' reaction at room temperature OD was measured in a Vitatron photometer (filter 402 mμ) against distilled water. Corrections were made for a blank lacking the enzyme.

3. RESULTS

3.1. General observations

3.1.1. Parental roots

Parental roots were found to contain a large amount of woody tissue. For this reason they were almost impossible to powder, and we therefore first cut the lyophilized roots into small pieces and ground them before powdering them in a mortar with some sand.

The colour of the various enzyme fractions was brown, indicating the presence of oxidized phenols. Fraction Se 100 had the darkest colour as compared with the other fractions, which had been taken up in a similar or even smaller amount of buffer solution; and fraction Su 100 was colourless. After dialysis a dark brown precipitate was found to have formed in all the fractions except Su 100, and the enzyme fractions still showed the brown colour. Precipitate formation was not an effect of dialysis, since it also occurred in fractions left standing for a certain length of time prior to dialysis. Because the volume in which the fractions had been taken up after precipitation with $(NH_4)_2SO_4$ was greater after dialysis, all fractions were brought to a certain volume with buffer solution after completion of the dialysis stage and the precipitates in them were spun down (20,000 g). During storage of the fractions at –20 °C for several months, a new precipitate slowly formed. Such fractions were never used for experiments.

3.1.2. Young roots

Young roots taken from plants which had grown in the laboratory for 14 days in the light never showed any spontaneous nodulation, not even when they were allowed to grow for 4 to 6 weeks. These young roots were also ground before being powdered in a mortar. The various enzyme fractions showed the brown colour, although somewhat lighter than that of the parental roots. The darkest colour was found in fractions Se 100 and Se 50, and fraction Su 100 was colourless. Again a distinct precipitate was found to have formed after dialysis of the fractions, but not in fraction Su 100.

3.1.3. Root nodules

The root nodules derived from 70-day-old plants harvested just prior to flower-

ing, and after lyophilization, proved very easy to powder. These nodules were full-grown and in microscopic preparations were found to be composed almost exclusively of bacteroid tissue. In the sections the nodules were seen to consist of red (legoglobin) cells, indicating an effective nitrogen-fixing tissue. The enzyme preparations were more darkly coloured (brown) than those of the root enzymes, due to the presence of legoglobin and phenols. The darkest colour was found in fractions E and Se 100; fraction Su 100 was light yellow to colourless. Especially in fractions Se 30 (which was never clear before dialysis) and Se 50, a heavy precipitate formed during dialysis, although here, too, precipitate formation was seen in all fractions except Su 100. After centrifugation each of these precipitates was taken up in a certain volume of buffer solution, in which they were found to be insoluble, and all of them distinctly showed some IAA-oxidase activity. When these solutions containing precipitate were placed in boiling water for two hours, this enzyme activity was totally destroyed. However, addition of these denaturated solutions, either as a suspension or after filtration, to a reaction system containing IAA-oxidase from fraction E or Se 100, clearly showed that these precipitates contained inhibitors of this enzyme.

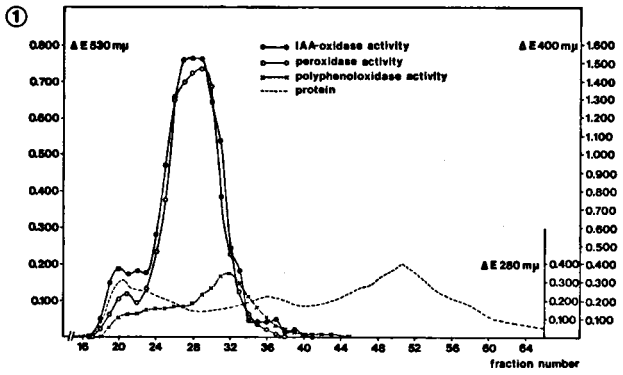
In this connection it should be mentioned that we attempted to remove the phenols present in the plant material by binding to polyvinylpyrrolidone (PVP). Nodule enzyme was prepared by grinding the pulverized, lyophilized nodules in a mortar together with a thin paste of polyclar AT (an insoluble form of PVP) and buffer to which ascorbic acid was added as a reducing agent (LOOMIS & BATTAILLE 1966). Per gram fresh weight of the nodules, we added 1.5 g polyclar AT. Although the final colour of the enzyme after dialysis seemed to be a more reddish brown, the same specific activity was found for fraction E as without the addition of PVP.

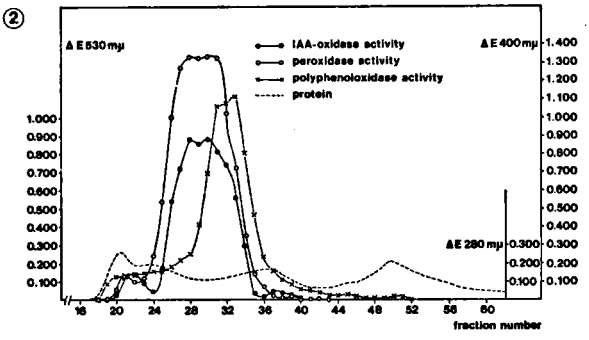
3.2. Gel filtration

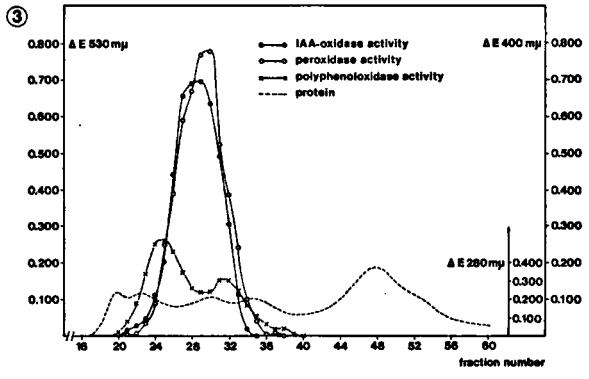
3.2.1. Parental roots

Part of fraction Se 100 was filtered through a Sephadex G-100 column for further purification. Testing of the elution fractions for IAA-oxidase, peroxidase, and polyphenoloxidase activity gave the qualitative elution pattern shown in *fig. 1*. The main peak of IAA-oxidase activity was found after a void volume of 40 ml had passed. It is evident that the fractions which exhibited IAA-oxidase activity contained polyphenoloxidase activity as well as peroxidase activity, the latter being almost strictly in parallel with the IAA-oxidase activity.

A small peak of activity preceding the main peak was found when extracts of roots, parental as well as young roots, were filtered through Sephadex G-100, but not for nodule extracts. The small peak coincided more or less with the peak of Blue Dextran, and therefore the IAA-oxidase in this peak must have a molecular weight of about 100,000 or even more. Determination of the molecular weight of the IAA-oxidase of the main peak by the gel filtration method







Figs. 1–3. Activity of IAA-oxidase, peroxidase, and polyphenoloxidase, and UV absorption of the various elution fractions after filtration of fraction Se 100 from parental roots (5 ml) (*fig.* 1), young roots (5 ml) (*fig.* 2), and root nodules (3 ml) (*fig.* 3) through a Sephadex G-100 column. Fractions (2 ml) were collected from the moment the enzyme was brought onto the gel. The optical density of the effluent was measured continuously at 280 mμ (protein). IAA-oxidase activity was determined by adding 0.1 ml (0.05 ml for parental roots) of each fraction to the incubation system (H_2O_2 present) and is given in ΔE 530 mμ/10 min. Peroxidase activity was determined with 0.1 ml of each fraction, and is given in ΔE 400 mμ/5 min. Polyphenoloxidase activity was determined with 0.5 ml of each fraction, and is given in ΔE 400 mμ/30 min. The curves represent the results of several experiments.

gave a value varying from 71,000 to 87,000 when calculated for the top of the activity peak. Molecular weights were calculated from a standard curve in which the ratio of elution volume (Ve) of reference markers to the elution volume of Blue Dextran 2000 (Vo) were plotted against the molecular weights of the same substances (Ve/Vo = 1.0 for Blue Dextran).

Molecular weight standards were: 1) horse heart myoglobin (16,900), 2) trypsin (23,800), 3) β-lactoglobulin (40,000), and 4) bovine serum albumin (68,000). It is of course possible that the low molecular weight (71,000–87,000) fraction capable of destroying IAA originated from the small activity peak with the high molecular weight.

The elution fractions of the main activity peak (fractions 23–37) were pooled to form fraction K to be used for further kinetic study.

3.2.2. Young roots

The qualitative elution pattern found after filtration of fraction Se 100 through Sephadex G-100 is shown in *Fig. 2*. As for parental roots, the main peak of IAA-oxidase activity, which again appeared after a void volume of 40 ml had passed, was preceded by a small peak. IAA-oxidase and peroxidase activity were found to be almost strictly in parallel, and the polyphenoloxidase activity curve showed about the same elution pattern as was obtained with parental roots. Although the curves in *figs. 1* and *2* represent a qualitative pattern of enzyme activities, it is clear that the young roots really do have a higher polyphenoloxidase activity. The molecular weight of IAA-oxidase, calculated for the top of the main activity peak, varies between 64,000 and 79,000, which is somewhat lower than the values found for parental roots. Presumably the small peak of IAA-degrading activity has a molecular weight of about 100,000. The fractions of the main, low molecular weight, activity peak (fractions 23–37) were pooled to form fraction K and the kinetics of this enzyme fraction were studied.

3.2.3. Root nodules

Fig. 3 shows the qualitative elution pattern after filtration of fraction Se 100 through a Sephadex G-100 column. After a void volume of 40 ml an IAA-oxidase activity peak appeared, although it was not preceded by a smaller peak of a higher molecular weight as has been observed for the root enzymes (cf. *figs. 1* and *2*). Again it was found that the IAA-oxidase and peroxidase activity curves were almost strictly in parallel. The polyphenoloxidase activity curve showed a different elution pattern from that for root enzymes, but still within the range of IAA-oxidase activity. As for parental roots, the polyphenoloxidase activity was low in comparison with the activity of young roots. The molecular weight of the IAA-oxidase of nodules varies between 64,000 and 79,000 when the top of the activity peak is taken for the calculation, and is thus the same as was found for young roots. The fractions of the activity peak (fractions 20–38) were pooled to form fraction K, and the kinetics were studied.

3.3. Ion-exchange chromatography

3.3.1. Parental roots

It can be seen from *fig. 1* that during gel filtration of fraction Se 100 the activities of IAA-oxidase and peroxidase could not be separated, and that the enzymes in the extract showed a high molecular weight. It therefore seems possible that the two enzymes exist as aggregates, as suggested by SEQUEIRA & MINEO (1966). These investigators were able to separate IAA-oxidase from peroxidase in extracts from roots of *Nicotiana tabacum* by using a Sephadex cation-exchanger. We therefore concentrated fraction K to a small volume by dialysing it first against polyethylene glycol 6000 and then against 0.1 M phosphate buffer (pH 5.5), followed by elution through a column of SP-Sephadex C-50 cation-exchanger, as described under Materials and Methods (2.4). The results, given in *fig. 4* indicate that after chromatography of fraction K, IAA-oxidase and peroxidase activities are still closely related. The elution patterns of both enzyme activities, showing peaks and shoulders, suggest the presence of several isoenzymes that cannot be separated by this method. Comparison of *figs. 1* and *4* suggests that IAA-oxidase from parental roots consists of several isoenzymes, intimately associated to a polymeric form with a high molecular weight. Whether IAA-oxidase is really a unique enzyme or is identical with peroxidase, cannot be conclusively determined from these experiments.

3.3.2. Young roots

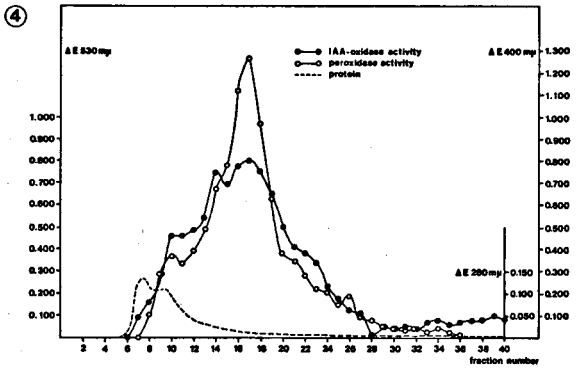
The elution pattern obtained after chromatography of fraction K from young roots is presented in *fig. 5*, from which it can be seen that the curve representing IAA-oxidase activity has almost the same shape as the activity curve for the peroxidase enzyme. Both curves, showing peaks and shoulders, suggest the presence of several isoenzymes.

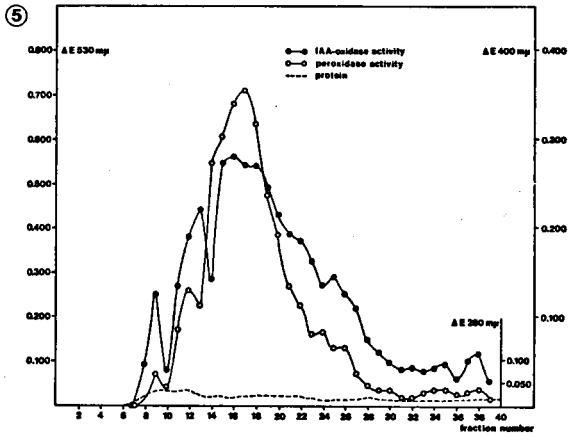
3.3.3. Root nodules

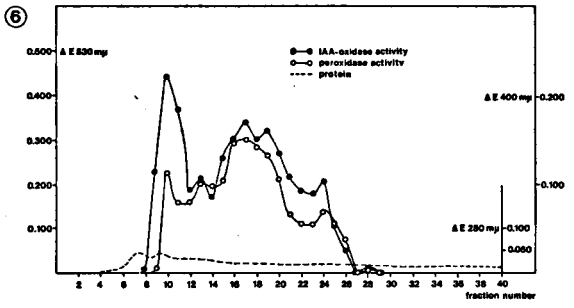
The curves representing the IAA-oxidase and peroxidase activity after ion-exchange chromatography of fraction K from root nodules show almost the same pattern (*fig. 6*). Here, too, the presence of several isoenzymes is suggested.

4. DISCUSSION

The present results do not provide any grounds for assuming the presence of different IAA-degrading enzyme systems in roots and root nodules. The same general impression was obtained in our study of the kinetics of fraction K from roots and root nodules, where the only striking differences were the higher optimum values of $MnCl_2$ and DCP found for young root enzyme (MENNES 1972). Moreover, as will be shown in part II of this report, the kinetics of the enzyme are virtually the same in roots and root nodules, which means that there is also no reason to attribute any influence to the presence of a specific IAA-oxidase in bacteroids.







Figs. 4–6. Activity of IAA-oxidase and peroxidase, and UV absorption of the various elution fractions after ion-exchange chromatography of fraction K from parental roots (*fig.* 4), young roots (*fig.* 5), and root nodules (*fig.* 6.) on SP-Sephadex C-50. Fractions (2 ml) were collected from the moment the enzyme was brought onto the gel. The relative protein content in each fraction was monitored by continuously measuring absorption at 280 mμ. IAA-oxidase activity was determined with 0.5 ml (0.3 ml for young roots) in a reaction system to which H_2O_2 was added, and is given in ΔE 530 mμ/20 min. (ΔE 530 mμ/10 min. for parental roots). Peroxidase activity was determined with 0.5 ml (0.3 ml for young roots) and is given in ΔE 400 mμ/5 min.

The lupine enzyme always shows a lag phase before the steady rate of IAA degradation is reached, but this lag phase becomes much shorter after the addition of H_2O_2 to the reaction system (MENNES 1973). In none of our experiments, however, was the IAA degradation by the lupine enzyme a simple peroxidation; it required the presence of a phenolic cofactor, and its action, as shown for DCP, was strongly increased by the addition of $MnCl_2$ (MENNES 1972). Filtration of the enzyme through a Sephadex G-100 column gave almost the same elution pattern for all of the three plant materials tested, the only difference being a small activity peak with a high molecular weight, seen only in root enzymes, not in root nodule enzyme.

The results of the enzyme activity determinations strongly suggest that polyphenoloxidase is a different enzyme, and also that there is a strong resemblance between peroxidase and IAA-oxidase, even on the basis of ion-exchange chromatography. The high molecular weight of IAA-oxidase in fraction K, together with its elution pattern found after ion-exchange chromatography, led us to believe that the IAA-degrading enzyme from roots and root nodules consists of a number of isoenzymes, closely associated to form a polymer with a high molecular weight. The differences found during the kinetic study of fraction K of the various materials (MENNES 1972) might be explained by possible differences in the isoenzymic pattern of IAA-oxidase from roots and root nodules. Peroxidase isoenzymes are known to increase in number and activity as a result of infection with, for instance, *Agrobacterium tumefaciens* (CURTIS 1971) which is closely related to our *Rhizobium*. From the work of LEE (1971), differences in activity of IAA-oxidase isoenzymes are also to be expected as a consequence of the difference in the IAA content of roots and root nodules found by DULLAART (1967). A study of the isoenzymic patterns of IAA-oxidase, peroxidase, and polyphenoloxidase present in fraction K of roots and root nodules is in progress.

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