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

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SUMMARY

The activity was determined of the various enzyme fractions obtained during ammonium sulphate precipitation and gel filtration of the IAA-oxidase present in root nodules and roots of *Lupinus luteus* L. Comparison of these activities on a protein basis showed that IAA degradation was lower in root nodules than in roots.

Several indications were obtained that phenols were present in all of the enzyme fractions and had an inhibitory effect on the enzyme activity. The possible role of phenols in the regulation of IAA-oxidase activity in lupine is discussed.

Rhizobium lupini was isolated from the root nodules, and cell-free enzyme preparations of these bacteria oxidized IAA. The results obtained with cell-free enzyme preparations of bacteroids did not, however, prove conclusively that the bacteroids have this activity.

1. INTRODUCTION

In part I of this report (MENNES 1973) it was shown that the elution pattern of IAA-oxidase activity in root nodules and roots did not differ significantly after filtration on Sephadex G-100. It was therefore assumed that the same IAA-degrading enzyme system was involved. To investigate the possibility that this enzyme plays a role in causing the hyperauxiny found by DULLAART (1967), we made a quantitative study of the activity in vitro. The results of this study are presented here.

2. MATERIALS AND METHODS

2.1. Plant materials

Lupinus: The roots and root nodules were of the same type as for part I (MENNES 1973).

Rhizobium: The bacteria were grown under continuous aeration in a culture medium containing 15 g mannitol, 100 ml yeast extract, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$, 0.2 g NaCl, and 2 g $CaCO_3$ per litre distilled water. The medium was filtered through Whatman No. I filter paper to remove superfluous $CaCO_3$ and the pH was adjusted to 7.0 before sterilization for 40 minutes at 110 °C. After adequate growth in the dark at 28 °C the cultures were centrifuged at 12,000 g for 10 minutes. The bacterial cells were washed once with 0.3 M sucrose by resuspension and centrifugation, and then taken up in phosphate buffer

(Na_2HPO_4/NaH_2PO_4, 0.1 M; pH 5.2).

2.2. Protein measurement

To measure the protein content of the enzyme fractions prepared as described in Part I (MENNES 1973), 2 ml of the solution, in an appropriate dilution, was mixed with 2 ml 10% trichloroacetic acid (TCA) and centrifuged for 15 minutes at 2,000 g. The pellet was resuspended in 5 ml 5% TCA and again centrifuged for 15 minutes at 2,000 g. The pellet was then taken up in 3 ml 0.5 N NaOH, stirred, and incubated in boiling water for 10 minutes, care being taken to avoid evaporation. After restoration to room temperature, 1.0-ml aliquots were used for the determinations according to LOWRY et al. (1951). Colour development took place in the dark, and the optical density (OD) was measured in a Vitatron photometer (filter 742 mμ) between 75 and 120 minutes after the addition of the Folin reagent. The protein content was calculated from a standard curve prepared with bovine albumin. The TCA precipitation was used to avoid the possible influence of phenolic substances.

2.3. IAA-oxidase activity

The activity of IAA-oxidase was measured by two methods, viz. with the Salkowski reagent (cf. MENNES 1973) and the UV method.

With the same reaction system as described for the Salkowski method, the reaction was started by adding the enzyme solution at zero time, and the increase in OD at 253 mμ (HINMAN & LANG 1965) was measured in a Zeiss spectrophotometer against a blank to which 1.0 ml buffer solution had been added instead of the enzyme. Incubation was performed in the dark at 26 °C.

3. RESULTS

3.1. Introduction

Since the aim of this study was to compare the activities of IAA-oxidase deriving from parental roots, young roots, or root nodules, we began by studying the kinetics of the enzyme, using both methods (Salkowski and UV) to measure the activity. The activity of all the purification stages of the enzyme from the plant material under investigation had to be determined to permit comparison with the activity of the same fractions from the other plant sources. The object was to determine which fraction showed good activity at the highest degree of purification, so that this fraction could be used for further kinetic studies.

The protein content was estimated directly before the fractions were frozen and after storage at –20 °C for various periods. Freezing and thawing of the enzyme fractions had no effect on the amount of protein measured by the Lowry method. For root nodule enzyme it had been found that without TCA precipitation the apparent protein content of all enzyme fractions was about twice as high as the values obtained after TCA precipitation. The same was the case for fraction K from parental roots and young roots (the other fractions were not tested). I assume this to be due to phenolic compounds present

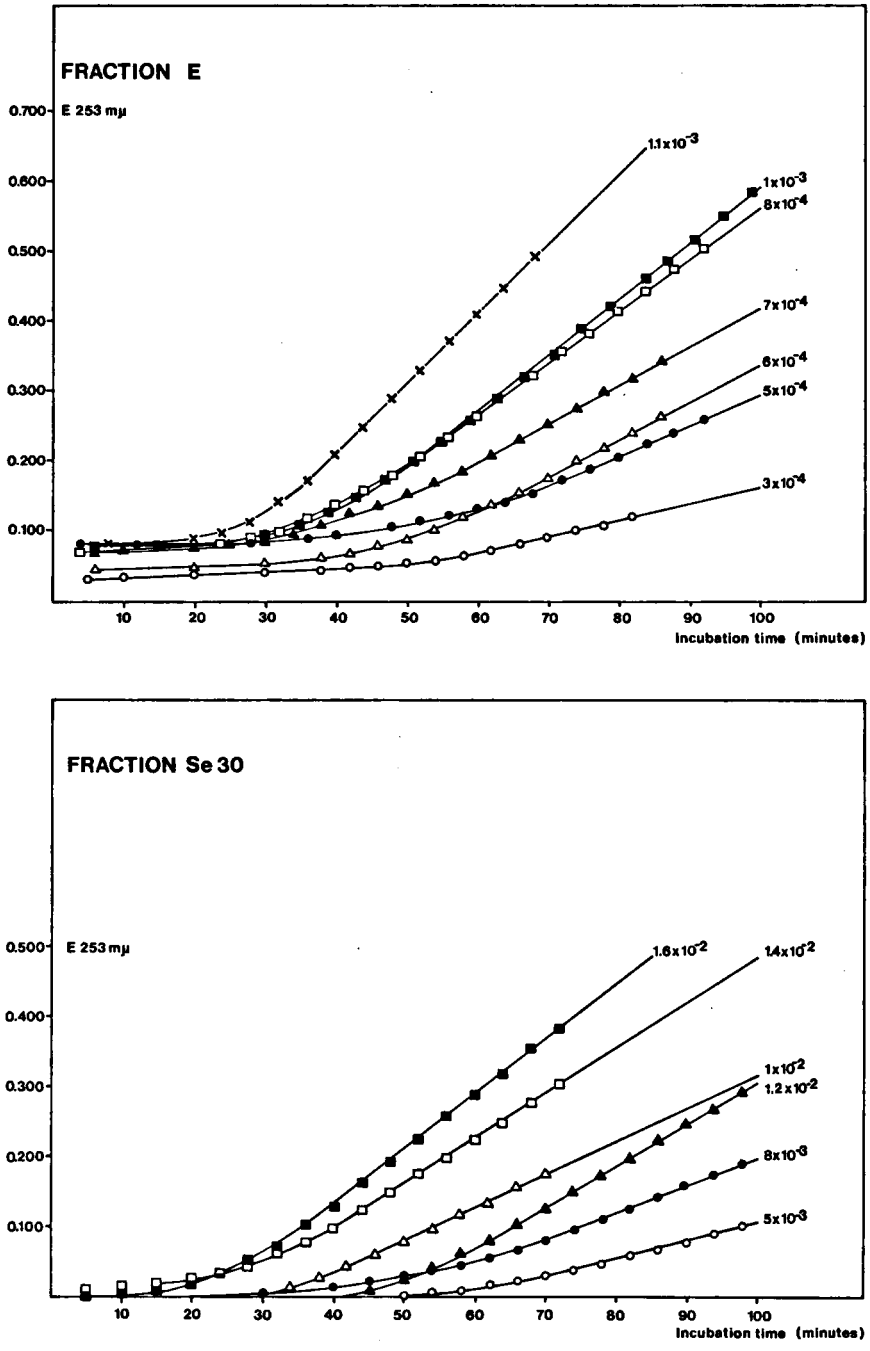
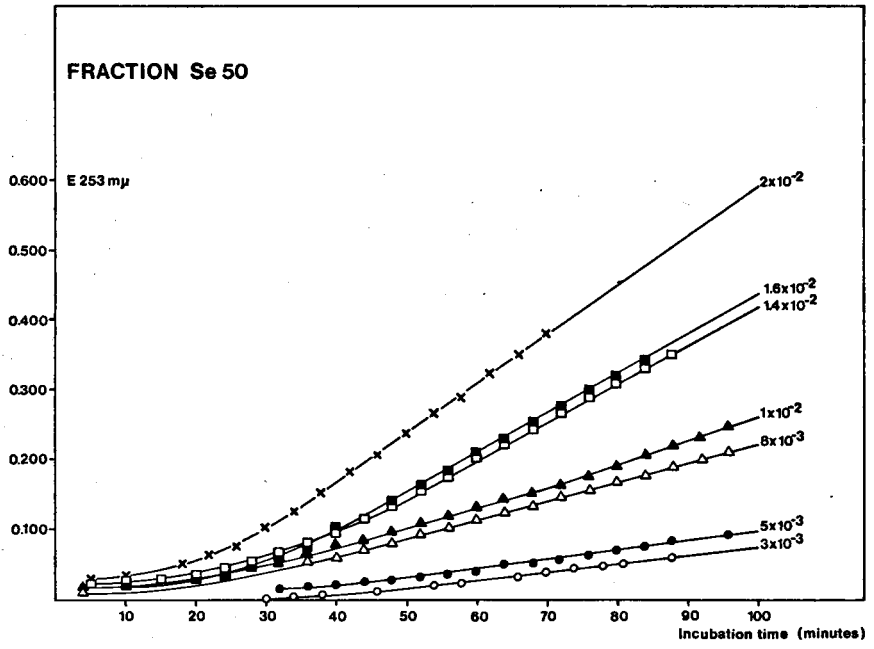
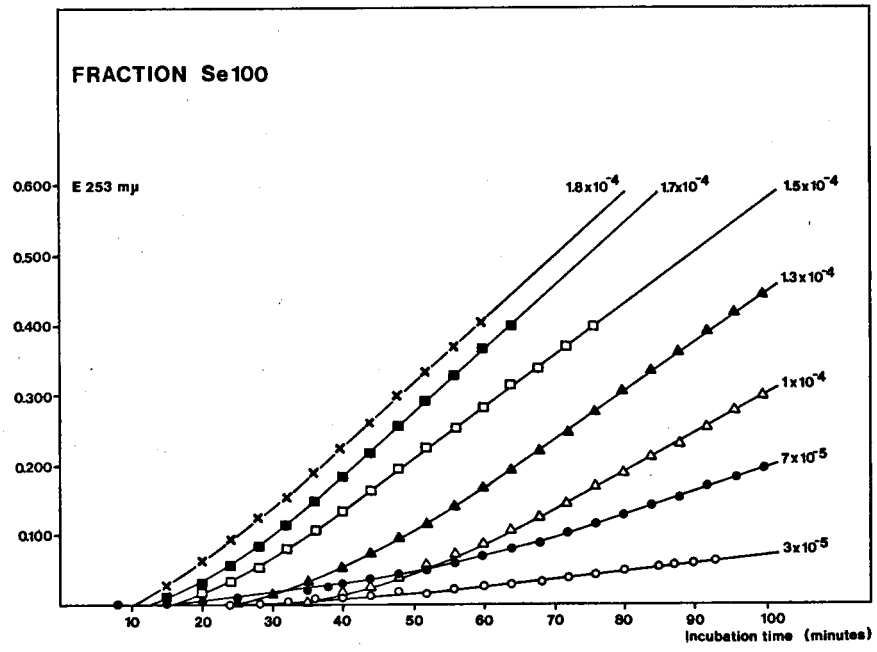


Fig. 1. Time curves of IAA-oxidase activity expressed as increase in optical density at 253 mμ.





The enzyme dilutions tested are indicated for each curve.

in all enzyme fractions and which react well with the Folin reagent.

Ammonium sulphate remaining after dialysis in a final concentration of more than 0.15 per cent can influence the Folin reaction by decreasing colour development (LOWRY et al., 1951). Nevertheless, we found that TCA precipitation of a protein solution to which a high $(NH_4)_2SO_4$ concentration had been added (80% saturation) resulted in the same protein value as when $(NH_4)_2SO_4$ was omitted. Thus it is clear that the influence of this interfering substance is completely eliminated by TCA precipitation, including resuspension in TCA (see Materials and Methods).

According to DIXON & WEBB (1958), the chief factors determining the initial velocity of a particular reaction are enzyme concentration, substrate concentration, pH, temperature, and the presence of activators or inhibitors. Because of the possibility that these factors might vary substantially for the several IAA-oxidase preparations to be tested, the enzyme activity was determined under standard conditions, as described under Materials and Methods. The results were expressed in enzyme units per millilitre (U/ml), the unit being equal to the amount of enzyme which, under standard conditions (cofactors added; temp. 26 °C; pH 5.5) and at an enzyme-saturating substrate concentration (v approximates V max), caused an increase in absorption at 253 mμ or a decrease in absorption at 530 mμ, of 0.01 unit of absorption per minute in the reaction mixture used. The findings are reported in three sections: parental roots (3.2), young roots (3.3), and root nodules (3.4).

3.2. Parental roots

3.2.1. Determination of enzyme activity

A series of varying enzyme dilutions of the crude enzyme fraction and the three $(NH_4)_2SO_4$ precipitation fractions were used to follow the course of IAA-oxidase activity. For each dilution the activity, expressed as an increase in OD at 253 mμ (or as a decrease in OD at 530 mμ where the Salkowski reagent is applied), was plotted against the incubation time. As can be seen from *fig. 1*, a lag phase preceding the linear increase in OD was found consistently. At low enzyme concentrations the lag phase was about one hour, but it was found that the higher the enzyme concentration, the shorter the lag phase, which suggests that the lag phase could not have been due to inhibitors present in the enzyme preparation. Moreover, the lag phase occurred in all stages of purification and was even found to be of the same magnitude.

The addition of H_2O_2 to the reaction system shortened the lag phase, but 'complete' elimination required such high concentrations (36×10^{-5}g) that the Salkowski method could not be used, because of the marked effect on colour formation (cf. PLATT & THIMANN 1956; SIEGEL & WEINTRAUB 1952). Furthermore, RAY (1962) has shown that addition of H_2O_2 increased the steady rate to a higher value than could be obtained without addition of peroxide. Because we were not quite sure whether this rate increase in the steady state would always be of the same magnitude and would occur for all the enzyme fractions to be tested, we decided not to add H_2O_2, especially since unexpected results had

been obtained after the addition of H_2O_2 during kinetic studies with fraction K of several materials (MENNES 1972).

The velocity of the reaction, calculated from the curves and expressed as the increase in OD at 253 mμ (or decrease in OD at 530 mμ in the case of the Salkowski method) per 20 minutes at steady state, was plotted against the enzyme concentration (*fig. 2*). A linear correlation was found to exist only up to a certain enzyme concentration (see *fig. 5*), usually at an OD of about 0.200 (UV method). The lack of proportionality of the velocity to the enzyme concentration cannot have been due to exhaustion of the substrate, because velocity was measured during the steady state. According to DIXON & WEBB (1958), such an activity decrease can be due to a limitation inherent in the method of estimation. If this was the case with our system, then both methods of estimation must have had the same limitation, because the Salkowski reaction gave the same divergence from linearity. Another and in this case perhaps more relevant explanation put forward by these authors concerned the suspected presence in the enzyme preparation of an inhibitor combining reversibly with the enzyme to give a non-active enzyme-inhibitor complex. This possibility will be discussed below.

Another remarkable observation concerned the determinations done with the Salkowski reaction. The curve showing the proportionality between initial velocity and enzyme concentration was consistently displaced from the origin and intersected the abscissa. Such a curve is a very rare phenomenon, although instances occur in the enzyme literature (DIXON & WEBB 1958; KRUPASAGAR & SEQUEIRA 1969), where it is interpreted as being due to some highly toxic impurity present in one of the components of the incubation system other than the enzyme solution itself. This factor will totally combine with a small amount of the enzyme, so that at every enzyme concentration the rate of decrease in the amount of active enzyme always corresponds to the amount of enzyme, which is proportional to the amount of this toxic impurity. This explanation cannot be applicable to our system, because we obtained this type of curve only with the Salkowski reaction and never with the UV method. The explanation of this phenomenon should be sought by comparing the two methods used in the present study.

In our definition of an enzyme unit it was said that the activity of the enzyme has to be tested at an enzyme-saturating substrate concentration. We therefore determined the initial velocity at varying substrate concentrations, keeping the enzyme concentration at a constant value found to be proportional to the velocity. As can be seen from *fig. 3*, the substrate concentration used in our experiments was high enough for complete saturation of the enzyme.

The amount of activity units per millilitre of enzyme preparation were calculated on the basis of the curves in *fig. 2*. The values are given in *table 1* (column 3).

Fig. 4 shows the time curves of the activity of fraction K at various concentrations. Again, a distinct lag phase was found, although somewhat shorter than for the other fractions (see *fig. 1*), and this lag phase, too, decreased with in-

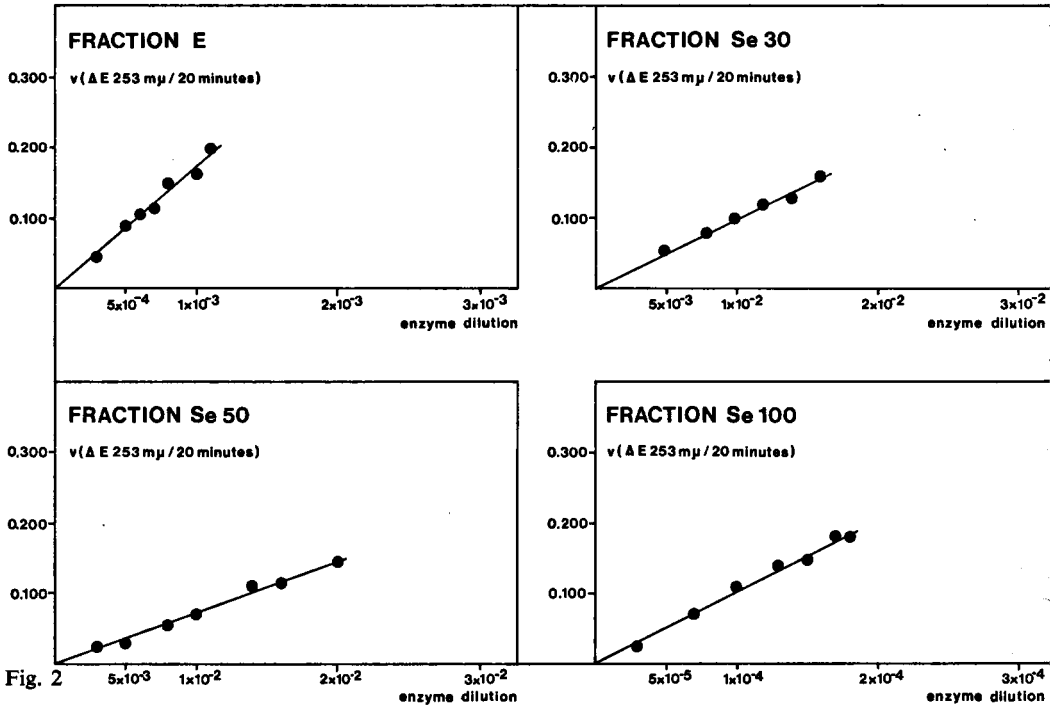

FRACTION E
v(ΔE 253 mμ / 20 minutes)
0.300
0.200
0.100
5x10⁻⁴
1x10⁻³
2x10⁻³
3x10⁻³
enzyme dilution
FRACTION Se 30
v(ΔE 253 mμ / 20 minutes)
0.300
0.200
0.100
5x10⁻³
1x10⁻²
2x10⁻²
3x10⁻²
enzyme dilution
FRACTION Se 50
v(ΔE 253 mμ / 20 minutes)
0.300
0.200
0.100
5x10⁻³
1x10⁻²
2x10⁻²
3x10⁻²
enzyme dilution
FRACTION Se 100
v(ΔE 253 mμ / 20 minutes)
0.300
0.200
0.100
5x10⁻⁵
1x10⁻⁴
2x10⁻⁴
3x10⁻⁴
enzyme dilution

Fig. 2

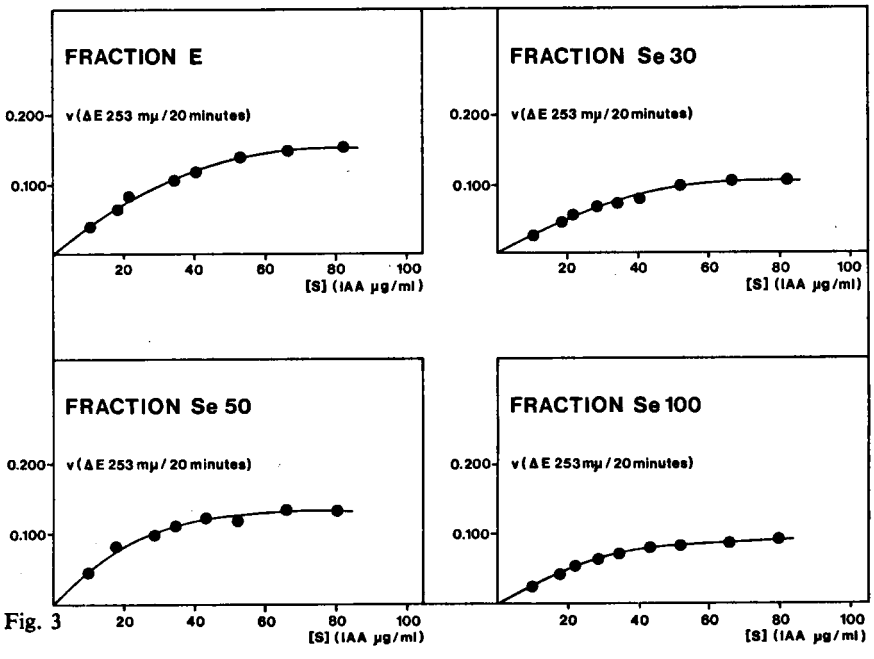

FRACTION E
v(ΔE 253 mμ / 20 minutes)
0.200
0.100
20
40
60
80
100
[S] (IAA μg/ml)
FRACTION Se 30
v(ΔE 253 mμ / 20 minutes)
0.200
0.100
20
40
60
80
100
[S] (IAA μg/ml)
FRACTION Se 50
v(ΔE 253 mμ / 20 minutes)
0.200
0.100
20
40
60
80
100
[S] (IAA μg/ml)
FRACTION Se 100
v(ΔE 253 mμ / 20 minutes)
0.200
0.100
20
40
60
80
100
[S] (IAA μg/ml)

Fig. 3

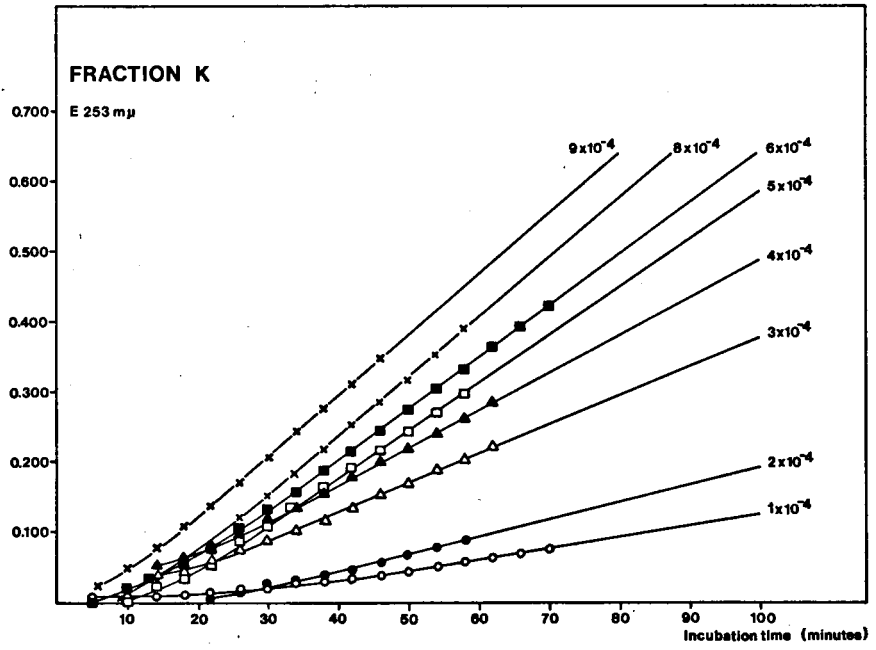


Fig. 4. Time curves of IAA-oxidase activity expressed as increase in optical density at 253 mμ. The enzyme dilutions tested are given for each curve.

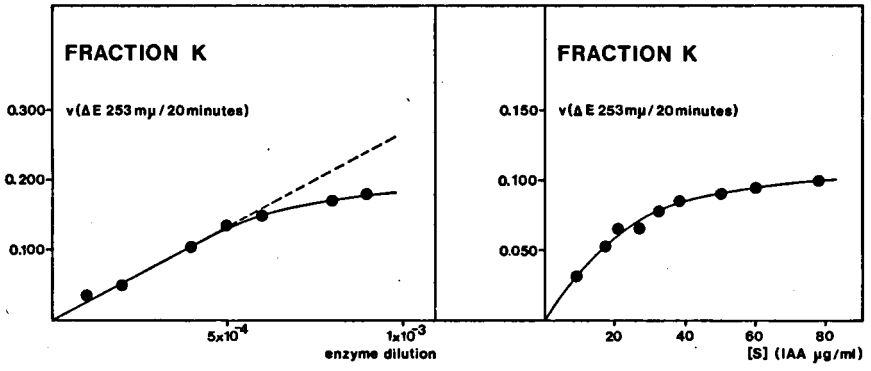


Fig. 5. Relationship between enzyme dilution and IAA oxidation by fraction K of parental roots.

Fig. 6. Effect of substrate concentration on the rate of IAA oxidation by fraction K of parental roots.

Fig. 2. Relationship between enzyme dilution and IAA oxidation by the various fractions of parental roots. (The velocity of the reaction is expressed as Δ OD/20 minutes at 253 mμ obtained during steady-state kinetics.)

Fig. 3. Effect of substrate concentration on the rate of IAA oxidation by the various fractions of parental roots. (The velocity of the reaction is expressed as Δ OD/20 minutes at 253 mμ obtained during steady-state kinetics.)

Table 1. Purification of IAA-oxidase from parental roots (results of several experiments)

1	2	3	4	5	6	7	8	
Procedure	Volume (ml)	Activity (units/ml)	Total units	Protein (mg/ml)	Specific activity or purity (units/mg protein)	Yield (%)	Purification	
								UV method
E	45	1,739.12	78,260.40	1.140	1,525.54	100.00	1.000	first experiment
Se 30	15	49.38	740.70	0.531	92.99	0.94	0.061	
Se 50	15	36.36	545.40	1.323	27.48	0.69	0.018	
Se 100	15	5,063.29	75,949.35	0.276	18,345.25	97.04	12.020	
K	90	1,315.78	118,420.20	0.0225	58,479.11	151.31	38.330	
E	45	2,222.22	99,999.90	1.458	1,524.15	100.00	1.000	second experiment
Se 30	15	68.02	1,020.30	0.756	89.97	1.02	0.059	
Se 50	15	42.19	632.85	1.4985	28.15	0.63	0.018	
Se 100	15	6,153.84	92,307.60	0.339	18,152.92	92.30	11.910	
K[2])	79.41	1,886.79	149,829.99	0.033	57,175.45	149.83	37.510	fifth experiment
E	45	2,197.80	98,901.00	1.44	1,526.25	100.00	1.000	third experiment
Se 30	15	62.11	931.65	0.693	89.62	0.94	0.058	
Se 50	15	75.47	1,132.05	1.665	45.32	1.14	0.029	
Se 100	17	5,050.50	85,858.50	0.273	18,500.00	86.80	12.120	
Se 50	15	97.08		2.055	47.24			fourth experiment
								Salkowski method[1])
E	45	4,651.16	209,302.20	1.458	3,190.09	100.00	1.000	second experiment
Se 30	15	142.85	2,142.75	0.756	188.95	1.02	0.059	
Se 50	15	86.95	1,304.25	1.4985	58.02	0.62	0.018	
Se 100	15	13,333.33	199,999.95	0.339	39,331.35	95.55	12.320	
K[2])	79.41	4,166.66	330,874.47	0.033	126,262.42	158.08	39.570	fifth experiment

[1] Mean values for one experiment.

[2] Except those in column 5, these figures were calculated from a fifth experiment not given here.

creasing enzyme concentration.

The falling-off from the linear relationship between enzyme concentration and velocity of the reaction is clearly shown in *fig. 5*. Although it can be seen from *fig. 6* that an enzyme-saturating substrate concentration was used, it is also clear from *fig. 4* that at enzyme concentrations that are not directly proportional to the reaction velocity (see *fig. 5*) there is no sign of exhaustion of substrate during the 20-minute reaction time required to determine the velocity after the steady state had been reached. The amount of enzyme units per millilitre of fraction K, calculated from *fig. 5*, is given in *table 1* (column 3).

3.2.2. Comparison of the various enzyme activities

Table 1, which shows the results of several experiments with parental roots, is divided into 8 columns as suggested by DIXON & WEBB (1958). The various fractions are listed in column 1, under the heading Procedure; column 2 gives the volumes. The activity, expressed as enzyme units per millilitre, is shown in column 3, and column 4 gives the total number of units (column 2 × column 3). The protein content in milligrams per millilitre enzyme solution is given in column 5. If we divide the values in column 3 by those in column 5, we obtain the purity or specific activity, expressed in enzyme units per milligram of protein, shown in column 6. The yield, given in column 7, was obtained from column 4 by taking the first value in that column as 100 per cent. The degree of purification reached is shown in column 8 and was obtained by taking the first value in column 6 as equivalent to 1. The methods used in determining the IAA-oxidase activity are also indicated in *table 1*. In this table the values listed in column 3 for the first experiment were calculated from *figs. 2* and *5*. This first experiment was carried out with parental roots from plants that had grown in the field for 70 days, the second with material from plants grown for 56 days, and for the third, fourth, and fifth from plants field-grown for 38 days. During the second experiment we had to prepare new stock solutions of the cofactors, but after that all previously recorded activities decreased by a factor of about 2. It was then found that by preparing all working solutions in twice-distilled water instead of in the distilled water used routinely, not only was the original activity restored but there was also a marked decrease in the stability of the various enzyme preparations. This explained why the activity of fraction K in the second experiment was partially lost and had to be calculated from another experiment (nr. 5). When the activity of fraction Se 50 was tested during the second experiment, we found that the lag phase preceding the steady state was much shorter than the lag phase found for the same fraction in the first experiment. The same held for experiments 3 and 4, but here the specific activity of this fraction was found to have approximately doubled. The reason for this increase in specific activity is not clear, and was only found for fraction Se 50 deriving from 38-day-old roots.

On the strength of the data of the various experiments on parental roots shown in *table 1* it can be said that the specific activities of the various fractions showed a high degree of reproducibility. The degree of purification reached

is not very high in spite of the 4 purification steps applied, but compared to the results for nodules and young roots it is still the highest. However, the activity yield for fraction Se 100 and especially for fraction K is very high, for the latter even higher than 100 per cent. The latter finding can only be explained by assuming that, at the very least, the activity found for the crude enzyme is not a real activity but an activity inhibited by contaminating substances. If this is true, then the presence in this enzyme fraction of phenols capable of inhibiting IAA-oxidase activity must be assumed. It is of course also possible that besides such inhibitors, phenolic cofactors are also present. Phenols present in the crude enzyme must have been divided over the various enzyme fractions during the $(NH_4)_2SO_4$ precipitation of the proteins, because these phenols would have been bound to the proteins and were therefore not lost during dialysis. If phenolic cofactors are present together with inhibitors, it cannot be predicted whether the over-all reaction will be inhibitory or stimulatory. However, the role of any phenolic cofactors present in the enzyme fractions cannot have been of great importance, because our experiments were conducted in the presence of added and rather high concentrations of DCP and $MnCl_2$.

The oxidation of phenols to quinones and the reaction of these quinones with reactive groups on proteins can lead to cross-linking, ultimately resulting in the precipitation of proteins. In this connection it is worth mentioning that after being kept for several months at –20°C, the precipitates formed during dialysis of the nodule enzyme fractions looked as though they had undergone some kind of tanning process. It was also found that during the purification of the parental root enzyme there was a great loss of protein, amounting to 37.7, 38.4, and 36 per cent for the first, second, and third experiments, respectively (*table 1*). The possible role of the phenols in regulating IAA-oxidase activity will be discussed below.

Because of the low stability of the enzyme in bi-distilled water, we decided to prepare the enzyme fractions as quickly as possible. Under these conditions some activity was found in fraction Su 100, but it is conceivable that given a longer period for complete saturation with $(NH_4)_2SO_4$, all activity would disappear from this supernatant fraction, as was found under the latter circumstances for fraction Su 100 of root nodules. For this reason, the activity of fraction Su 100 of parental roots was not studied further.

3.3. Young roots

3.3.1. Determination of enzyme activity

For the determination of the specific activities of the various enzyme fractions, experiments were carried out as described for parental roots. By mistake in the first experiment (see *table 2*) only 0.25 ml buffer solution was added for approximately every gram fresh weight, and in all the other experiments 0.5 ml. Compared to the findings in parental roots, the lag phase was now clearly shorter in all fractions except E and K, and proved to be shorter with increasing

Table 2. Purification of IAA-oxidase from young roots (results of 2 experiments.)

1	2	3	4	5	6	7	8	
Procedure	Volume (ml)	Activity (units/ml)	Total units	Protein (mg/ml)	Specific activity or purity (units/mg protein)	Yield (%)	Purification	
								UV method
E	35	1,250.00	43,750.00	1.140	1,096.49	100.00	1.000	first experiment
Se 30	15	20.61	309.15	0.366	56.31	0.70	0.051	
Se 50	15	86.95	1,304.25	1.230	70.69	2.98	0.064	
Se 100	16	2,941.17	47,058.72	0.5475	5,372.00	107.56	4.890	
K	96	476.19	45,714.24	0.055	8,658.00	104.48	7.890	
E	35	2,985.07	104,477.45	2.790	1,069.91	100.00	1.000	second experiment
Se 30	15	77.51	1,162.65	1.350	57.41	1.11	0.053	
Se 50	15	156.25	2,343.75	2.220	70.38	2.24	0.065	
Se 100	16	6,666.66	106,666.56	1.260	5,291.00	102.09	4.940	
K[2])	68.5	1,612.90	110.483.65	0.180	8,960.55	105.74	8.370	third experiment
								Salkowski method[1])
E	35	6,349.20	222,222.00	2.790	2,275.69	100.00	1.000	second experiment
Se 30	15	156.25	2,343.75	1.350	115.74	1.05	0.050	
Se 50	15	327.86	4,917.90	2.220	147.68	2.21	0.064	
Se 100	16	14,285.71	228,571.36	1.260	11,337.86	102.85	4.980	
K[2])	68.5	3,333.33	228,333.10	0.180	18,518.50	102.74	8.130	third experiment

[1] Mean values for one experiment.
[2] Except those in column 5, these figures were calculated from a third experiment not given here.

enzyme concentration. Although the substrate concentration used was found to be high enough to saturate the enzyme, the same declination of the curve representing the relation between enzyme concentration and velocity of the reaction was seen for all enzyme fractions, as well as the displacement of this curve from the origin when the Salkowski method had been used.

3.3.2. Comparison of the various enzyme activities

The results of the specific activity determinations for the various enzyme fractions of young roots are given in *table 2*. It is clear from these data that the specific activities are almost the same in spite of the great differences in protein content between these experiments. This suggests the existence of a direct correlation between the extracted soluble-protein content and the IAA-oxidase activity of the various fractions. The degree of purification reached is very low, and the loss of protein, as determined after purification, amounted to 18 and 24 per cent for the first and second experiments, respectively (*table 2*). Again, fraction Su 100 still showed some activity and the yield was very high for fractions Se 100 and K.

3.4. Root nodules

3.4.1. Determination of enzyme activity

Determination of the specific activities of the various nodule-enzyme fractions revealed the following phenomena, already seen for roots: (i) a lag phase of about the same duration, preceding the steady state and becoming shorter with increasing enzyme concentration; (ii) in spite of the use of an enzyme-saturating substrate concentration, the curve of enzyme concentration versus velocity showed a drop; and (iii) displacement of this curve for the Salkowski method.

3.4.2. Comparison of the various enzyme activities

Table 3 shows the results of the specific activity determinations in the various enzyme fractions of root nodules. It can be seen that in several experiments these specific activities showed good reproducibility. The degree of purification reached is, however, very low, as for young roots, and the loss of protein during purification amounted to 36.4 and 34.2 per cent in the first and second experiments, respectively (*table 3*).

3.5. The presence of IAA-oxidase in Rhizobium[1]

3.5.1. Introduction

Since, as already mentioned, the nodules consisted almost exclusively of bacteroid tissue, it seemed very interesting to find out whether these bacteroids are capable of destroying IAA. If bacteroids with IAA-degrading activity were

[1] I am greatly indebted to Mr. A. A. N. van Brussel of our Department of Microbiology for isolating *Rhizobium lupini* and for his valuable assistance during this part of my work.

Table 3. Purification of IAA-oxidase from root nodules (results of 2 experiments).

1	2	3	4	5	6	7	8	
Procedure	Volume (ml)	Activity (units/ml)	Total units	Protein (mg/ml)	Specific activity or purity (units/mg protein)	Yield (%)	Purification	
								UV method
E	30	1,346.80	40,404.00	6.360	211.76	100.00	1.00	first experiment
Se 30	15	46.29	694.35	0.984	47.04	1.71	0.22	
Se 50	15	118.20	1,773.00	3.660	32.29	4.38	0.15	
Se 100	20	1,075.26	21,505.20	2.550	421.67	53.22	1.99	
K[2])	339.4	131.23	44,539.46	0.132	994.16	110.23	4.69	third experiment
E	30	1,273.88	38,216.40	6.420	198.42	100.00	1.00	second experiment
Se 30	15	47.61	714.15	1.080	44.08	1.86	0.22	
Se 50	15	123.30	1,849.50	3.960	31.13	4.83	0.15	
Se 100	21	980.39	20,588.19	2.400	408.49	53.87	2.05	
K[2])	279.3	152.20	42,509.46	0.1545	985.11	111.23	4.96	fourth experiment
								Salkowski method[1])
E	30	2,797.20	83,916.00	6.360	439.81	100.00	1.00	first experiment
Se 30	15	97.08	1,456.20	0.984	98.65	1.73	0.22	
Se 50	15	233.64	3,504.60	3.660	63.83	4.17	0.14	
Se 100	20	2,247.19	44,943.80	2.550	881.25	53.55	2.00	
K[2])	339.4	284.09	96,420.14	0.132	2,152.19	114.90	4.89	third experiment

[1] Mean values for one experiment.

[2] Except those in column 5, these figures were calculated from a third and fourth experiment not given here.

even partially disrupted during the grinding of the nodules, any activity found for nodule enzyme would be partially due to this bacteroid enzyme. However, microscopical preparations of the nodule brei showed only undamaged bacteroids.

In this connection the contradictory reports on the presence of IAA-oxidase in *Rhizobia* are also of special interest. HARTMANN & GLOMBITZA (1967) found no degradation of IAA by *Rhizobium leguminosarum*, whereas RIGAUD (1969) reported a distinct activity for *Rhizobium meliloti*. DULLAART (1970) showed IAA-oxidase activity to be present in *Rhizobium lupini*, although he also mentioned that in one case strain A 98–1965 lacked such activity and he therefore suggested that IAA destruction by *Rhizobium* may be an easily modifiable property. We consequently attempted to find IAA-oxidase activity in bacteria and bacteroids of *Rhizobium lupini* isolated from the nodules used in our experiments.

3.5.2. Bacteria

Rhizobium lupini was isolated from fresh nodule material harvested in the field in 1971 at an age of 90 days, just prior to flowering. The root-nodule bacteria were isolated according to VINCENT (1970). The *Rhizobium lupini* strain thus obtained was first checked with respect to its nodulation capacity. Lupine seeds sterilized with sulphuric acid and bromine were inoculated with this strain, held for 5 days in the dark (cf. LIBBENGA 1970) on sterilized gravel impregnated with N-free medium (RAGGIO & RAGGIO 1956) and subsequently grown under the standard conditions described in part I for young roots (MENNES 1973). Since nodules formed directly below the hypocotyl, infection must have taken place very quickly. After 4 weeks the whole root system was cut off and incubated in a gas mixture (air and 10% C_2H_2) for the measurement of acetylene reduction according to AKKERMANS (1971). When the nodules were known to be effective, the bacteria were grown and collected as already described (2.1). This strain proved to grow very slowly on this medium and showed only a short logarithmic phase. The medium in which they had grown showed no IAA-oxidase activity when tested in a standard system to which H_2O_2 (36×10^{-5} g), DCP (5×10^{-3} M), and $MnCl_2$ (5×10^{-3} M) were added in a final volume of 5 ml. The bacteria taken up in 8 ml buffer solution and mixed with an equal volume of Superbrite glass beads (Type 120–5005) were disrupted in a Mickle disintegrator until microscopical examination showed all cells to be fractured. After centrifugation of this suspension for 10 minutes at 3,000 g and analysis of the supernatant fluid for IAA-oxidase activity, this cell-free preparation showed a small but measurable IAA-oxidase activity (UV method).

3.5.3. Bacteroids

For the isolation of bacteroids 378.6 g (fresh weight) nodules deriving from plants harvested in 1971 at an age of 90 days was mixed in small portions with 0.3 M ice-cold sucrose in a Waring Blendor for one minute and the brei filtered

through a Jena D 1 glass filter. The filtrate was centrifuged at 1,100 g for 10 minutes and the bacteroids forming the outer shell of the pellet were resuspended in 0.3 M sucrose and again centrifuged at 1,100 g for 10 minutes. The cells were then washed twice with 0.3 M sucrose, centrifuged at 4,300 g for 10 minutes washed 3 times with 0.3 M sucrose, and centrifuged at 12,000 g for 10 minutes, the cells finally being resuspended in distilled water. After centrifugation at 3,000 g for 10 minutes the washing procedure with water was repeated twice and the bacteroids were then lyophilized to a final dry weight of 3.8 g. Microscopic examination showed that the bacteroids were intact, notwithstanding the osmotic changes during preparation. All supernatant fluids obtained during this washing procedure were checked for IAA-oxidase activity in the same standard as used for bacteria, and all of them proved to be active. During these washings, however, the IAA-degrading activity in the supernatant fluids gradually decreased. We found it rather unusual that the supernatant fluid obtained after centrifugation of a suspension of bacteroids washed 10 times still showed a definite IAA-oxidase activity. I assume that this can only be explained by a slow leakage of the enzyme from the bacteroids or from other cell fragments, precipitating almost simultaneously with the bacteroids. The latter possibility is strongly supported by the following observation. After the second washing with water and centrifugation at 1,100 g, the supernatant fluid proved under the microscope to be free of bacteroids. The IAA-oxidase activity of this supernatant was decreased, however, by the next centrifugation at 3,000 g, where a pellet was formed, but was not lost even at the higher speeds of 48,000 g or even 100,000 g. This means that the enzyme activity was present in a small particulate fraction as well as in the soluble phase.

To find out whether bacteroids really have an IAA-degrading enzyme, the lyophilized cells were disrupted in either a Mickle disintegrator or a Sonifier B-12. Microscopical examination showed that the cells were fractured. The IAA-oxidase activity present in the supernatant fluid after the centrifugation of a suspension of lyophilized bacteroids in buffer was increased by a factor of between 2 and 3 when this suspension was subjected to disintegration by one of these methods before the centrifugation. However, the same effect was observed with young, non-nodulated roots. These young roots were mixed with 0.3 M sucrose in a Waring Blendor and filtered through a Jena D 1 glass filter; the filtrate was centrifuged at 1,100 g for 10 minutes. The pellet was taken up in buffer and subjected to one of the disintegration methods. The supernatant fluid again showed 2 or 3 times more IAA-oxidase activity than was present before disintegration, and again the enzyme was present in the soluble phase as well as bound to a particulate fraction sedimenting at 3,000 g, which means that this effect cannot be due to the disruption of the bacteroids. Since in this case the homogenized roots were extracted only once, whereas the bacteroid suspension in the first experiment was disrupted after 10 successive washings, it was possible that all of the bacteroid enzyme had already leaked out. We therefore repeated the experiment with bacteroids isolated from nodules which were not washed after grinding, filtration, and centrifugation, but instead were

immediately disintegrated by one of the methods. This experiment was carried out with nodules deriving from both field plant material and plants grown in the laboratory and inoculated with our own isolated *Rhizobium* strain. Again the IAA-oxidase activity rose 2 or 3 times after the disruption of the suspended bacteroids. Since this bacteroid suspension had certainly held the IAA-oxidase-containing particulate fraction present in the young roots, this result suggests that either the bacteroids are devoid of IAA-oxidase activity or the activity present was so low that its liberation after disruption of the bacteroids did not substantially increase the amount originating from this particulate fraction. Another possibility to be considered is that the enzyme has already leaked out of the bacteroids during the grinding of the nodules, and in that case the IAA-oxidase activity found for nodules would be at least partially due to the bacteroids. Such leakage must take place through the cell envelope of the bacteroids, because we did not find any microscopical indications that the bacteroids were disrupted by grinding in a mortar with some sand.

The results of these experiments do not show whether bacteroids are really devoid of IAA-oxidase activity or whether the enzyme only occurs bound to cell fragments from which it can be released. Nothing can be said with certainty about the nature of the particulate fraction or in what form the enzyme is present in it. The fact that this particulate fraction is found in young roots as well as in the bacteroid suspension (even after 10 washings and centrifugations, which suggests that these particles are bound to the bacteroids) and that the released particles themselves are precipitated at a somewhat higher speed than the bacteroids, might be an indication that this fraction consists of the cell membranes (cf. GOODCHILD & BERGERSEN 1966) which surround the bacteroids in the bacteroid tissue. It is known from the work of KIDBY & GOODCHILD (1966) that in *Lupinus luteus* the membranes enclose only one nodule bacterium.

4. DISCUSSION

4.1. The method of enzyme preparation

During this study of the IAA-degrading enzyme we consistently used the soluble protein fraction (E) obtained after centrifugation of the brei of homogenized plant tissue, the pellets being discarded. However, as shown in 3.5.3., these pellets still contain cell fragments to which this enzyme is bound and from which it can be released. After disruption of these cell fragments, the activity of the enzyme in the soluble phase was low compared to that in fraction E. Earlier work in this laboratory with non-nodulated young roots has shown that after two washings of the pellet normally discarded when preparing fraction E, the resulting supernatant fraction contained only a few per cent of the total enzyme activity found in the fraction itself. Moreover, within one kind of plant material we found a clear correlation between the IAA-oxidase activity and the extracted soluble-protein content of the supernatant fraction. This was convincingly shown for young roots (cf. *table 2*).

4.2. The specific activity

We have expressed the specific activity as the enzyme activity per milligram of soluble protein in the various enzyme preparations rather than on the basis of weight, because the latter implies a priori that the total amount of IAA degraded as a consequence of the enzyme activity is directly correlated with the total weight of the plant (PILET & GASPAR 1964). In this connection it must be kept in mind that parental roots contain a large amount of woody tissue, whereas the root nodules used in this study consisted almost exclusively of bacteroid tissue, and that it is not yet known whether the bacteroids have an IAA-oxidase enzyme. However, great differences in contamination with soluble proteins other than IAA-oxidase could have strongly influenced the specific activities found in this work.

4.3. Comparison of IAA-oxidase activity in roots and root nodules

The present experiments have demonstrated that with the possible exception of bacteroids, IAA-oxidase is present in all of the systems investigated. *Table 4* shows the results of the comparison of specific activities found after the kinetics of IAA degradation were studied for all the stages of purification of the enzyme deriving from roots and root nodules. These results clearly show that the IAA-oxidase present in parental root extract is the most active as compared with the activity in extracts of young roots and nodules. However, young root enzyme shows a higher activity than nodule enzyme but a lower activity than parental root enzyme, except in fraction Se 50.

As already mentioned, specific activity was based on the soluble-protein content. On the basis of fresh weight, however, the enzyme extracts of nodules proved to have about 10 times more protein than the enzyme extracts of roots. Comparison of the UV-absorption patterns obtained during the elution of fraction Se 100 of nodule, young root, and parental root material (*fig. 7*) shows that these patterns did not differ very much. Of special interest in this context is the part of the patterns between the two arrows in *fig. 7* indicating the beginning and end of the main activity peak whose fractions were combined to form fraction K. This part of the curve consists of 3 peaks, the second of which was always present in the nodule pattern but was usually scarcely detectable in the patterns of young and parental roots. If this peak really represents a protein, then there must be more of this protein in nodules than in either of the root enzyme fractions, and the low specific activity found for fraction K of nodules must be partly due to the presence of this protein. The other 2 peaks show about the same extinction value in all 3 of the examples in *fig. 7*. In this connection it must be said that in this case the UV-absorption pattern of the nodule enzyme was monitored with a cuvet having a light path half the width of that used for the root enzymes, but 3 ml of fraction Se 100 was brought onto the column instead of the 5 ml used for root enzymes. These differences are virtually compensatory (although favouring the nodule enzyme), so that the

patterns in *fig. 7* are directly comparable. If the outer peaks are taken to be the same in all 3 cases, the high protein content of the nodule must be due to the extra protein present in the second peak. If, however, the same protein value as that found for nodule enzyme in fraction K were taken for the calculation of the specific activity of fraction K of roots, there is still a significant difference between the specific activities of nodules and roots, the activity of roots always being higher. Of course this reasoning holds only if the UV-absorption patterns actually represent only absorption due to protein.

There are, it is true, better methods for comparing the protein pattern of fraction K of roots and root nodules. Electrophoretic experiments are now in progress and will be dealt with in another paper. The present data do indicate, however, that the low specific activity of fraction K of nodules is partially due to a lower IAA-oxidase activity. This was also shown by another series of experiments in which we used nodule and parental root material harvested in 1970 instead of the 1968 material used for the determination of the specific activities given in *Table 4*. In these experiments only the specific activity of fraction K was determined, and this proved to be highest for young roots. The results also showed that except for the pH the conditions used in our standard reaction system (2.3) had been optimal for the nodule enzyme as well as for parental root enzyme. Furthermore, the difference with respect to the value found at optimal pH was greatest for the parental root enzyme. But since, except

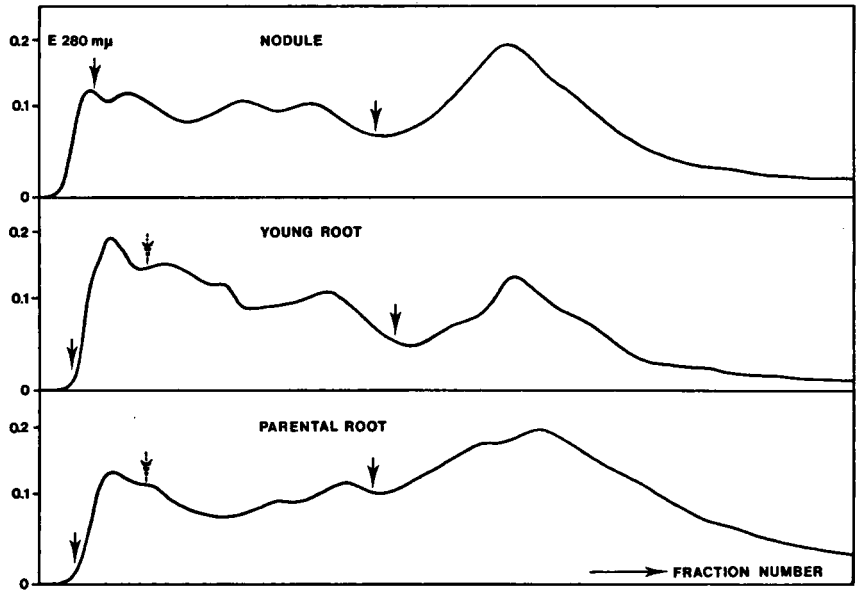


Fig. 7. UV-absorption patterns monitored during filtration of the various Se 100 fractions through a Sephadex G-100 column. Solid arrows indicate the first and last elution fraction showing IAA-oxidase activity, dotted arrows the end of the small high-molecular-weight activity peak preceding the main peak.

Table 4. Specific activities (SA) found for the various purification stages of IAA-oxidase from root nodules, parental roots, and young roots (mean values).

Procedure	(UV method) Root nodule	Parental root	Young root	$\frac{\overline{SA}\text{ Parental root}}{\overline{SA}\text{ Nodule}}$	$\frac{\overline{SA}\text{ Young root}}{\overline{SA}\text{ Nodule}}$	$\frac{\overline{SA}\text{ Parental root}}{\overline{SA}\text{ Young root}}$
E	205.09	1,524.84	1,083.20	7.43	5.28	1.40
Se 30	45.56	91.48	56.86	2.00	1.24	1.60
Se 50	31.71	or 27.81 / 46.28	70.53	or 0.87 / 1.45	2.22	or 0.39 / 0.65
Se 100	415.08	18,249.08	5,331.50	43.96	12.84	3.42
K	989.63	57,827.28	8,809.27	58.43	8.90	6.56

Procedure	(Salkowski method) Root nodule	Parental root	Young root	$\frac{\overline{SA}\text{ Parental root}}{\overline{SA}\text{ Nodule}}$	$\frac{\overline{SA}\text{ Young root}}{\overline{SA}\text{ Nodule}}$	$\frac{\overline{SA}\text{ Parental root}}{\overline{SA}\text{ Young root}}$
E	439.81	3,190.09	2,275.69	7.25	5.17	1.40
Se 30	98.65	188.95	115.74	1.91	1.17	1.63
Se 50	63.83	58.02	147.68	0.90	2.31	0.39
Se 100	881.25	39,331.35	11,337.86	44.63	12.86	3.46
K	2,152.19	126,262.42	18,518.50	58.66	8.60	6.81

for the pH, the optimal conditions found for young root enzyme differed widely from those of the standard system, the values found for the ratio of the 3 K fractions must be even greater than those given in *table 4* (cf. MENNES 1972).

It was shown by DULLAART (1967) that root nodules of yellow lupine contain substantially more IAA than the roots, and this of course is borne out by our results, assuming that the results obtained in vitro are representative of the situation in vivo. Moreover, DULLAART (1970) concluded from some preliminary experiments on IAA degradation that the activity of this enzyme is lowest in cell-free enzyme extracts of mature (parental) roots. It is not clear, however, on what basis he compared the values of this enzyme activity. On the basis of the protein content, as indicated in his tables, the mature root enzyme extracts, both dialysed and non-dialysed, show a higher and not a lower level of IAA breakdown compared with the values found for the (dialysed and non-dialysed) young root enzyme extracts. Moreover, our kinetic study showed that a lag phase preceded the IAA-oxidase activity, and when IAA, for instance, was incubated with a crude, dialysed, highly concentrated enzyme preparation of root nodules, with or without the addition of the cofactors $MnCl_2$ and DCP, no degradation of IAA occurred for at least 3 hours. A crude, dialysed, and highly concentrated enzyme preparation of young roots incubated with IAA in the presence of H_2O_2 and $MnCl_2$ but without DCP, also showed no activity at all, at least for 20 minutes, and addition of DCP resulted in an almost complete loss of the IAA within the same period of incubation. In this connection it should be emphasized that it is wrong to draw quantitative conclusions from enzyme activity values found in experiments done in vitro and based on only one determination after a long period of incubation, without taking into consideration what has occurred during this time. Such results will not give a true picture.

4.4. The possible role of phenols

The differences found between the activity of IAA-oxidase from roots and root nodules may reflect differences in amounts of enzyme or a possible regulation of the enzyme activity by phenols present in the enzyme solutions. This study yielded several indications of the presence of naturally occurring phenolic inhibitors and perhaps also of cofactors present in all the enzyme fractions obtained during the purification of the IAA-oxidase of roots and root nodules. Mention should be made first of all of the brown colour of all the enzyme fractions, indicating the presence of oxidation products of certain monohydric and *o*-dihydric phenols. It is known that unless sterically hindered, all phenols take part in hydrogen bonding and that the bond formed between the phenolic hydroxyl group and N-substituted amides is one of the strongest types of hydrogen bond (cf. LOOMIS & BATTAILLE 1966). In this way phenols can form complexes with proteins, but they also form intramolecular as well as intermolecular H-bonds, resulting in the last case in increasing molecular complexity (THOMSON 1964). Phenols are, however, also susceptible to oxidation, which can occur non-enzymatically or be catalysed by phenoloxidases or peroxidases.

Many phenols are readily oxidized to quinones or semiquinones, both of which are highly reactive and easily polymerized or form covalent bonds with any suitable group present in proteins. The discovery that the precipitate formed during dialysis showed a tanning effect (3.2.2; see also below) suggests that the enzyme preparations also contained more complex phenols, such as tannins. The tannins are also known to form effective links with proteins by means of H-bond formation, covalent bonds, and ionic bonds between suitably charged anionic groups on the tannin and cationic groups on the protein (SWAIN 1965).

During the preparation of the various enzyme fractions the phenols must have remained in their protein-bound form during the $(NH_4)_2SO_4$ precipitation and after dialysis. As a consequence of the TCA precipitation procedure used in the protein determination (2.2), however, the phenols or even phenol complexes coupled via H-bonds must have lost their protein binding, resulting in a lower value of Folin-positive material than was seen when no TCA had been applied (cf. 3.1). The tannin-protein complex we assume to have formed during dialysis, was found to contain inhibitors of IAA-oxidase (cf. 3.1.3 in: MENNES 1973). It must, however, be mentioned that we studied these effects only for root nodules, and therefore we do not know whether the tanning effect and its inhibitory action on the enzyme activity would also occur with root material. In all of the enzyme fractions tested during this study, a deviation from linearity was found at the high concentrations when the velocity of the reaction was plotted against enzyme concentration. If this was due to the presence of a dissociable phenolic inhibitor present in the enzyme preparations (3.2.1), it must be a high-molecular inhibitor because it was not removed by dialysis. In addition, the unusual results of the determination of the total activity (given as yield in column 7 of *tables 1*, *2* and *3*) can in my opinion only be explained by assuming the presence of phenolic inhibitors, perhaps together with cofactors. In the latter case, however, the protein-bound phenol would have had to have retained its capacity to act as a redox system. Finally, it is known that inhibitors can also protect an enzyme from denaturation, and therefore the presence of phenolic inhibitors in the IAA-oxidase preparations would easily explain their rather high degree of thermostability (cf. MENNES 1972).

It should also be emphasized here that the conditions prevailing for our enzyme preparations were presumably very different from the situation in vivo, but it cannot be ruled out that in vivo, too, more complex phenols than those usually taken into account in model systems might have a regulatory effect on IAA-oxidase activity. In this connection the report of STONIER et al. (1970) is of special interest, because of the suggestion that the auxin protectors present in the Japanese morning glory may be polymers or oligomers of phenolic substances. In further studies on the IAA-oxidase of yellow lupine special precautions should be applied for the removal of the phenols from the plant extracts during the isolation of the enzyme. It is known, for instance, that the formation of a complex between peroxidase and tannins can be reversed by a number of reagents (GOLDSTEIN & SWAIN 1965), and there are more recent

reports of the removal of phenolics from plant extracts with anion-exchange resin (LAM & SHAW 1970) or carbowax (VAN JAARSVELD & MEYNHARDT 1967), the latter normally being used as the stationary phase in gas-chromatographic separation of phenols.

Our in vitro experiments have clearly demonstrated that, on the basis of protein content, the root nodule enzyme shows the lowest IAA-oxidase activity, but these results cannot be said with certainty to represent the situation in vivo. But if they do, then the present results might also explain the hyperauxiny found by DULLAART (1967) in root nodules.

Since, however, the present study has shown that the roots and root nodules of the yellow lupine possess the capacity to degrade large amounts of IAA, it is surprising that any IAA could be extracted from these tissues. That IAA was extracted may reflect compartmentation or indicate that the enzyme is carefully regulated in vivo by phenols. A study of these phenols would undoubtedly be of great interest.

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