# THE INFLUENCE OF MEMBRANE-BOUND PEROXIDASES ON THE DEGRADATION OF IAA BY THE SPECIFIC IAA DEGRADING PROTEIN COMPLEXES IN HOMOGENATES OF PEA ROOTS

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SUMMARY

The amount of peroxidases not able to degrade IAA is crucial in the conversion of this compound by the IAA degrading enzymes. These peroxidases probably serve as a radical trap and are inactivated in the process. Cofactors remedy this situation. Possible reaction schemes are given.

## 1. INTRODUCTION

The degradation of IAA in homogenates of pea roots is performed by two types of enzymes (VAN DER MAST 1969, 1970a). The first type shows peroxidase activity, is resistant to urea, and does not adsorb to polyvinylpyrrolidone (PVP), whereas the second type does not exhibit peroxidase activity, is denaturated by urea, and adsorbs to PVP at low pH. Both types are inseparable with electrophoresis or ion exchangers and possess a common isoelectric point (VAN DER MAST 1970b). It seems probable that both enzyme types are aggregated into protein complexes.

In another paper (VAN DER MAST 1970c) evidence was presented for a membrane-bound state of part of the IAA degrading enzymes in homogenates of pea roots. Various compounds like KCl, urea, acridine orange, biebrich scarlet, and PVP remove the enzymes from their positions on the membranes. Raising the pH towards the isoelectric point gave a similar result. The action of most compounds was found to be reversible, i.e., recoupling of the enzyme complexes to membranes devoid of these enzymes could be accomplished. Only when the membranes were treated with PVP or acridine orange recoupling was impossible. The bonding of the protein complexes to the membranes is probably mediated through an intermediate compound. Changing the configuration of the membraneous proteins by heat also resulted in loss of the binding capacity.

In a previous paper (VAN DER MAST 1970b) the presence of peroxidases not capable of degrading IAA was noted in the same fractions containing the IAA degrading enzymes after gel chromatography on Sephadex G-100. At least three cationic peroxidases cover a range of molecular weights as commonly displayed by the IAA degrading complexes. The possibility was mentioned that these

peroxidases are also bound to the membranes and show spontaneous release from these structures, as was found for the IAA degrading enzymes. The amount of these peroxidases is larger than the peroxidative IAA degrading enzyme fraction, as judged from the intensity of the colour developed with benzidine. The anionic peroxidases were not found due to the method used in these experiments, but it seems plausible to expect membrane-bound enzymes in this category.

The presence of peroxidases in the particulate fraction of a homogenate can be used as a tool for varying the amount of these enzymes in relation to the amount of IAA degrading complexes. In this paper the influence of membrane-bound peroxidases on the reaction mechanism of IAA degradation by the specific enzymes will be shown, and modifications will be proposed of the reaction schemes as described by FOX & PURVES (1968) for the aerobic oxidation of IAA by horse radish peroxidase (HRP).

## 2. MATERIAL AND METHODS

Seeds of *Pisum sativum* L. cv. "Vlijmsche Gele Krombek" were grown as described earlier (VAN DER MAST 1969). For every gram fresh weight of roots, length 2–3 cm, 2 ml of a homogenation buffer were added. A Tris-HCl buffer containing 35 mM Tris, 0.5 mM $CaCl_2$ and 10 mM $MgCl_2$, pH 7.4, was employed for this purpose. Sometimes 50 mM KCl were added to this buffer. The abbreviations TCM and TCMK buffer, respectively, are used.

Cell debris was removed by centrifuging the homogenate during 20 minutes at 27000 g. The resulting solution is here called the 27000 g supernatant. The membranes and ribosomes were collected by a further centrifuging period of 1 hour at 48000 RPM in rotor 50 of a Spinco model L50 ultracentrifuge. The liquid fraction is here called the 180000g TCM or TCMK supernatant. The particulate fraction was resuspended in TCM buffer with the use of a glass cylinder and a tightly fitting teflon piston.

The degradation of IAA was performed in Mc Ilvaine's buffer, pH 5.0, to which IAA and sometimes p-coumaric acid had been added. The end concentrations were 50 ppm and 2 ppm, respectively. The incubation temperature was 25 °C. The amount of residual IAA was determined at 535 nm. In experiments with $H_2O_2$ this compound was added to the incubation mixture.

The peroxidase activity was assayed in Mc Ilvaine's buffer, pH 5.0, which contained 0.03 M pyrogallol and $5.5 \times 10^{-3}$ M $H_2O_2$. The amount of the resulting colour was read at 500 nm after exactly 2 minutes.

## 3. RESULTS

The influences of various treatments of the homogenates on the degradation of IAA by these preparations are shown in *fig. 1*.

The reaction rates are nearly identical in all cases where p-coumaric acid was added to the incubation mixture. However, large differences in lag-time are ap-

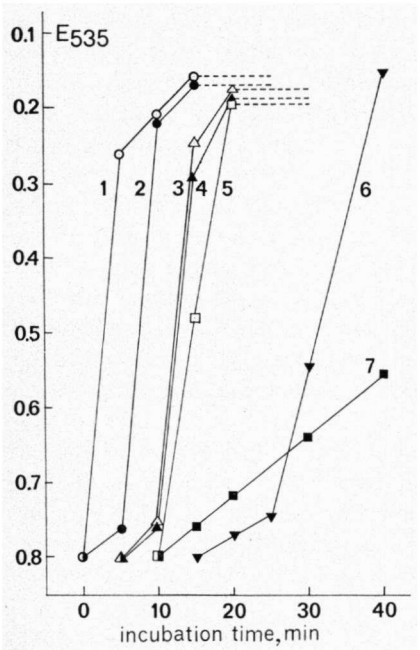


Fig. 1. The influence of membrane-bound peroxidases on the lag-time of the IAA degradative reaction. The addition of membranes devoid of the IAA degrading enzymes to a supernatant without the membraneous fraction or of KCl prior to the ultracentrifuging period progressively reduces this lag-time. The action of KCl is even apparent in the 27000 g supernatants. The addition of $H_2O_2$ to an ultracentrifuged sample in which KCl-treated membranes had been resuspended completely abolished this lag-time. The influence of $H_2O_2$ is only discernible in the presence of a cofactor.
The reaction mixtures consisted of 8 ml Mc Ilvaine's buffer which contained IAA and, except in the case of line 7, p-coumaric acid as a cofactor. $H_2O_2$ was used in a concentration of $5 \times 10^{-3}$ M. From the supernatants 2 ml samples were added to this mixture. When membranes were added to TCM supernatants this was accomplished by resuspending these structures directly in these supernatants. A ratio of 1:1 was employed meaning that every ml of an ultracentrifuged sample contained the normal complement of membranes.

○——○ (line 1) IAA degradation by a 180000 g TCM supernatant + TCMK membranes + $H_2O_2$.
●——● (line 2) IAA degradation by a 27000 g TCMK supernatant.
△——△ (line 3) IAA degradation by a 27000 g TCM supernatant.
▲——▲ (line 4) IAA degradation by a 180000 g TCM supernatant + TCMK membranes.
□——□ (line 5) IAA degradation by a 180000 g TCMK supernatant.
▼——▼ (line 6) IAA degradation by a 180000 g TCM supernatant.
■——■ (line 7) IAA degradation by a 180000 g TCM supernatant + TCMK membranes + $H_2O_2$, but without a cofactor. The same line represents the IAA degradation by a 180000 g TCM supernatant + membranes, but without both $H_2O_2$ and the cofactor.

parent. The removal of the membranes from a TCM preparation (line 6) increases the lag-time of the degradation of IAA by the supernatant to 25 minutes when the starting point of the rapid reaction is taken as a criterion. When, however, the membranes are removed from a TCMK preparation the lag-time displayed by this supernatant is considerably shorter and comes into the order of 10 minutes (line 5).

The addition of TCMK membranes to a 180000 g TCM supernatant also reduces the lag-time from 25 to 10 minutes (line 4). The action of KCl on the lag-time reduction is even apparent in the case of the 27000 g supernatants. This compound reduces their lag-time from 10 minutes (line 3) to 5 minutes (line 2).

The addition of $H_2O_2$ to a 180000 g TCM supernatant into which the membranes from a TCMK preparation had been resuspended completely abolished this lag-time phenomenon, as shown by line 1; without this compound the results are as expressed by line 4.

The lag-time abolishment by $H_2O_2$ is only accomplished in the presence of p-coumaric acid. When this latter compound was omitted from the incubation mixture, which then contained the 180000 g TCM supernatant, TCMK membranes, $H_2O_2$ and IAA, the results found are as shown by line 7. The same results were found when both p-coumaric acid and $H_2O_2$ were omitted from the reaction medium.

The influence of the membraneous fraction on the reduction of the lag-times was further studied in experiments in which the membranes from a TCMK preparation were added to a 180000 g TCM supernatant and the incubation with IAA was performed without the presence of an exogenously applied cofactor. The amount of added membranes was varied, leading to the ratios mentioned in the legend of this figure. A ratio of 1:2 implies the presence of an amount of added membranes which is twice the amount originally present in the TCM sample before ultracentrifuging. The results are presented in *fig. 2*. As shown by line c, an amount of added TCMK membranes twice the original complement abolishes the lag-time completely. The quantity of lipoproteins added to the reaction mixture also has a slight influence on the reaction velocity as judged from the slopes of the lines. Further addition of membranes, till four times the original amount, is almost without effect.

Treatment of the membranes with PVP prior to their addition to the TCM supernatants had no influence on the results shown in *fig. 2*. This implies that the phenomena observed here are not due to natural phenolic cofactors bound to the membranes.

However, denaturing the membranes by heating them at 80 °C during 5 minutes completely cancels the promotive effects of the lipoproteins as shown by line a. This is an indication that other, non-IAA degrading enzymes are bound to the membranes, exerting an influence on the IAA degrading reaction by the specific enzymes. Experiments, the results of which are shown in *fig. 3*, confirm this view. Various preparations were incubated with IAA without an added cofactor. During the ensuing IAA conversion the amount of peroxidase activity was determined in samples from these incubation mixtures.

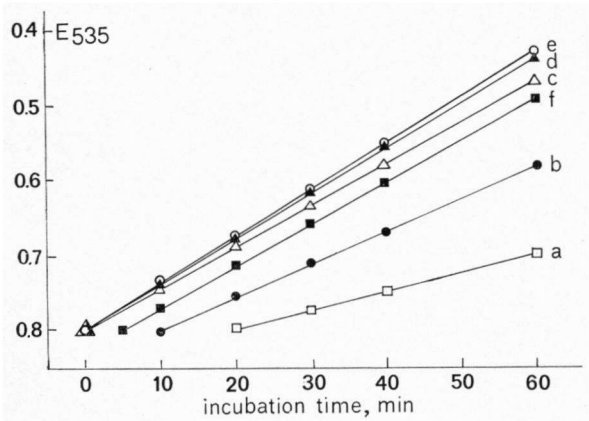


Fig. 2. The influence of membrane-bound peroxidases on the lag-time of the IAA degradative reaction. Membranes treated with KCl to remove the IAA degrading enzymes were resuspended in ultracentrifuged TCM supernatants. The supernatant to membrane ratios varied from 1:0 to 1:4. A ratio of 1:2 gives rise to a degradation of IAA which closely resembles that of a 27000 g TCM supernatant (line c and f). When this ratio is used about 120% of the normal amount of membrane-bound peroxidases is present in the sample due to the partial removal of these enzymes by the action of KCl.
A slight increase in reaction velocity is apparent when membranes are added. Exceedingly high membrane ratios do not further influence this velocity. Heat denaturated membranes are without effect (line a).
The membranes from 20 ml TCMK homogenate were resuspended in 10 ml TCM buffer. To 2 ml samples of an ultracentrifuged TCM preparation 1, 2, 3, or 4 ml of the resuspended membranes were added. All mixtures were made up to 10 ml with Mc Ilvaine's buffer containing IAA. No cofactor was used.

□——□ (line a) IAA degradation by a 180000 g TCM supernatant. Also degradation of IAA by a 180000 g TCM supernatant + denaturated TCMK membranes, ratio 1:1.

●——● (line b) IAA degradation by a 180000 g TCM supernatant + TCMK membranes, ratio 1:1.

△——△ (line c) IAA degradation by a 180000 g TCM supernatant + TCMK membranes, ratio 1:2.

▲——▲ (line d) IAA degradation by a 180000 g TCM supernatant + TCMK membranes, ratio 1:3.

○——○ (line e) IAA degradation by a 180000 g TCM supernatant + TCMK membranes, ratio 1:4.

■——■ (line f) IAA degradation by a 27000 g TCM supernatant.

As shown by lines B and D the peroxidase activity declines steadily after an initial lag-time. The lag-time previous to this decline coincides with the lag-time found in the IAA degradative reaction. The loss of peroxidases activity is about 50% in the last sample drawn from the incubation mixture as expressed by the diminished extinction. The peroxidase activity of isolated TCM membranes is considerable, as shown by the high extinction of line C. The peroxidase activity of TCMK membranes is lower, as depicted by line A, and amounts to about 60% of the activity displayed by the TCM membranes when the extinctions attained by both preparations are compared. When a cofactor, p-coumaric acid or 2,4-

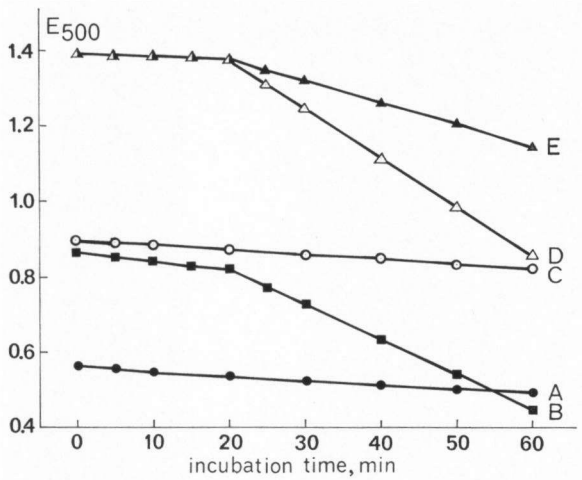


Fig. 3. The decrease of peroxidase activity during the degradation of IAA by the specific enzymes and the amount of peroxidase activity due to peroxidases bound to membranes isolated in TCM and TCMK buffer. The latter possess about 60% of the normal complement of membrane-bound peroxidases. During the degradation of IAA the peroxidase activity decreases by about 50% (line B and D) when no cofactor is added. There also is a lag-time in this decrease which coincides with the lag-time of the IAA degradative reaction. After the addition of a cofactor the decrease of peroxidase activity is about 50% of the decrease without this cofactor (line E).

●——● (line A) Peroxidase activity of isolated TCMK membranes

■ —— ■ (line B) Peroxidase activity of a 180000 g TCM supernatant while degrading IAA without an added cofactor.

○——○ (line C) Peroxidase activity of isolated TCM membranes.

△——△ (line D) Peroxidase activity of a 180000 g TCM supernatant + TCMK membranes while degrading IAA without an added cofactor.

▲——▲ (line E) Peroxidase activity of a 180000 g TCM supernatant + TCMK membranes while degrading IAA in the presence of an added cofactor.

dichlorophenol, was added to the incubation medium this decrease was about half the amount found without these substances (line E), although in the former case the amount of converted IAA is several times larger than the quantity degraded without addition of cofactors.

## 4. DISCUSSION

It is apparent from *fig. 1* that the reaction rates are nearly the same in all cases where p-coumaric acid was added to the incubation mediums (lines 1 to 7), although the TCM preparations actually contain less IAA degrading enzymes than the other preparations, due to the removal of the membrane-bound IAA converting complexes (VAN DER MAST 1970c). The addition of the membranes from a TCMK preparation to a 180000 g pea root supernatant in TCM buffer does not alter this situation as the TCMK membranes are denuded of the IAA degrading enzymes by the action of KCl (VAN DER MAST 1970c).

The resulting reduction of the lag-time attained by TCMK membranes can then only be explained by the presence of other compounds adhering to these membranes. PVP-treatment of the TCMK membranes had no influence on the results indicating that the effect caused by the membraneous fraction is not due to natural cofactors adsorbed to these lipoproteins. Heat denaturation of TCMK membranes prior to mixing them with a 180000 g TCM supernatant abolished the stimulatory effect as shown by line a in *fig. 2*. Therefore the reduction of the lag-time is very probably mediated by enzymes bound to the membranes.

In *fig. 3* the influence of KCl on the peroxidase content of the membraneous fraction is shown. Membranes isolated in TCM buffer display a peroxidase activity as depicted by line C, while membranes isolated in TCMK buffer have a lower activity, as shown by line A. When the extinctions of both preparations are compared it can be seen that the peroxidase activity of TCMK membranes is about 60% of the activity displayed by TCM membranes. This means that the addition of KCl to a 27000 g supernatant liberates about 40% of the membrane-bound peroxidases which are subsequently transferred to the soluble phase. Ultracentrifuging such a preparation will thus leave a supernatant containing more peroxidases than found without this KCl treatment. The lag-time displayed by such a preparation is considerably shorter, as shown by line 5 in *fig. 1*.

When isolated TCMK membranes are added to a 180000 g TCM supernatant the remaining 60% of the bound peroxidases are brought into the reaction medium and again a reduction of the lag-time is apparent, as shown by line 4. A comparison between lines 4 and 5 shows that the reduction of the lag-time is of the same order in both preparations, probably due to about the same amount of peroxidases present in the reaction mediums. Thus it can be assumed that the peroxidases not capable of degrading IAA themselves play a role in the enzymatic degradation of this compound as performed by the specific IAA converting protein complexes.

Beside the reduction of the lag-time, the amount of membranes added to a 180000 g TCM supernatant also has some influence on the reaction rate of the IAA degradation when no exogenous cofactor is present in the incubation mixture as shown by *fig. 2*. That further addition of membranes, till 3 or 4 times the original amount, is without effect is probably due to the limiting amount of a natural cofactor, or cofactors, present in the TCM supernatant.

The action of KCl is even discernible in the 27000 g supernatants, as shown by lines 2 and 3 in *fig. 1*. In both cases the preparations contain the same amount of peroxidases. It seems likely that the peroxidases have to be in the free state to exert their action on the IAA degradative system. KCl removes part of the peroxidases from the membranes which results in a shorter lag-time (line 2). Removal from the lipoproteins also proceeds spontaneously (VAN DER MAST 1970b), but probably at a slower rate.

As shown by line 1 and 7 in *fig. 1* the abolishment of the lag-time by $H_2O_2$ is only achieved in the presence of p-coumaric acid, but this compound is without effect when the cofactor is omitted.

*Fig. 3* shows that the amount of peroxidase activity decreases during the conversion of IAA. In the case of a 180000 g TCM supernatant converting IAA without an added cofactor this decrease is about 50% (line B). When TCMK membranes were added to such a TCM supernatant, the amount of initially present peroxidases is the same as obtained by adding the extinctions of both preparations alone (line A + B). In this case the loss of peroxidase activity during the conversion of IAA is about 60%. When, however, p-coumaric acid or 2,4-dichlorophenol were added to the incubation medium, the loss of peroxidase activity was only 50% of the loss without these cofactors, as shown by comparing lines D and E, although in the former case the amount of degraded IAA is higher than without these compounds.

The amount of peroxidase activity displayed by the peroxidative IAA degrading enzyme moiety (VAN DER MAST 1969, 1970a) is only a minor fraction of the total peroxidase activity in a pea root homogenate as judged from the intensity of the colour obtained with benzidine after electrophoresis of these peroxidases (VAN DER MAST 1970b).

Thus it seems very probable that the loss of peroxidase activity during the conversion of IAA as shown in *fig. 3* is due to the inactivation of peroxidases not directly involved in the degradation of this plant hormone.

FOX & PURVES (1968) presented the following reaction schemes to explain the aerobic degradation of IAA by horse radish peroxidase (HRP):

$$O_2 + HRP \rightarrow HRP\text{-}O_2 \quad (1)$$
$$HRP\text{-}O_2 + IAA \rightarrow Per\ I \quad (2)$$
$$Per\ I + e \rightarrow Per\ II + HO\text{-}IAA \quad (3)$$
$$Per\ II + IAA \rightarrow HRP + IAA\cdot \quad (4)$$

(HRP-$O_2$: a complex between ferriperoxidase and the biradical form of oxygen; Per I and Per II: intermediate peroxidase compounds I and II; HO-IAA: hydroxyindolenineacetic acid; IAA·: an IAA free radical).

To explain the promotive effect of 2,4-dichlorophenol (DCP) on the oxidation of IAA they further proposed:

$$Per\ I + DCP \rightarrow Per\ II + DCP\cdot \quad (5)$$
$$Per\ II + DCP \rightarrow HRP + DCP\cdot \quad (6)$$
$$Per\ II + IAA \rightarrow HRP + IAA\cdot \quad (7)$$
$$DCP\cdot + IAA \rightarrow DCP + IAA\cdot \quad (8)$$
$$IAA\cdot + O_2 \rightarrow IAA\text{-}O\cdot_2 \quad (9)$$
$$IAA\text{-}O\cdot_2 \rightarrow p\cdot + CO_2 + H_2O \quad (10)$$
$$DCP + p\cdot \rightarrow DCP\cdot + p \quad (11)$$

They presumed that the IAA free radicals or p· (radical of a product) react with DCP rather than with HRP, thus preventing enzyme inactivation.

Although the present paper does not deal with the actual mechanism of IAA degradation by the enzyme complexes present in pea root homogenates, the

genesis of IAA free radicals in this raction is important to explain the presented results.

It is clearly apparent from *fig. 3* that the interaction of IAA free radicals is not limited to the active IAA degrading enzymes but that this interaction is much more likely to occur with other peroxidases present in the preparations.

The following pathways of the free radicals are proposed:

*Situation I.*

(The naturally present cofactors in the homogenate are extracted with PVP or removed by gel chromatography. No other cofactor is supplied to the reaction mixture.)

n IAA· + peroxidase → n IAA + inactivated peroxidase (n is an abstract number).

This reaction will quickly come to a stop as reactivation of the peroxidase molecules does not take place.

*Situation II.*

(Natural cofactors or added ones are present in the homogenate.)

n IAA· + peroxidase → n IAA + inactivated peroxidase

inactivated peroxidase + cofactor → active peroxidase + cofactor·.

The IAA free radicals will not interact directly with the cofactor, as proposed by FOX & PURVES (1968), because the lag-time is dependent on the amount of peroxidases and not on the cofactor, as shown in *fig. 1*. The reaction will stop after depletion of the cofactors.

*Situation III.*

($H_2O_2$ is added to an incubate containing an exogenous cofactor.)

$H_2O_2$ + peroxidase → peroxidase + $H_2O$ + O

IAA· + 2 O → IAA-$O\cdot_2$

IAA-$O\cdot_2$ → p· + $CO_2$ + $H_2O$

p· + cofactor → p + cofactor·

The last three reactions are reactions 9, 10 and 11 mentioned by FOX & PURVES (1968).

The action of $H_2O_2$ is only exerted in the presence of an added cofactor which functions as a final radical trap. Omission of such a compound results in a normal lag-time notwithstanding the presence of $H_2O_2$ in the incubate (line 7 in *fig. 1*). It is apparent from the scheme explaining sitation III that the free radicals never interact directly with a peroxidase molecule in the presence of $H_2O_2$, implying that the degradation of IAA is now independent of the amount of peroxidases in the homogenate thus abolishing lag-time phenomena.

JANSSEN (1969) reported different pH-optima for IAA oxidase from pea roots due to the kind of cofactor added. It seems very likely that this result can be explained by a pH-dependent affinity of these compounds for the radicals.

It was already shown by the present author that a cofactor like p-coumaric

acid or an inhibitor like caffeic acid probably do not adhere to the IAA degrading enzymes and thus do not exert their action by modifying the catalytic site present on these proteins for IAA (VAN DER MAST 1970c). At this moment it seems very likely that the inhibitors of the conversion of IAA exhibit their action via the non-IAA degrading peroxidases by hindering the trapping of the IAA free radicals by these enzymes (to be communicated).

The bonds between the membranes and the various peroxidases are probably mediated in another way than exists for the IAA degrading enzymes (VAN DER MAST 1970c) because KCl only displaces these peroxidases partially and PVP has no influence on the binding sites.

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REFERENCES

FOX, R. & W. K. PURVES (1968): Mechanism of enhancement of IAA oxidation by 2,4-dichlorophenol. *Plant Physiol.* **43**: 454–456.

JANSSEN, M. G. H. (1969): The influence of some phenolic cofactors on the pH optimum of indoleacetic acid oxidase from pea roots. *Acta Bot. Neerl.* **18**: 634–638.

MAST, C. A. VAN DER (1969): Separation of IAA degrading enzymes from pea roots on columns of polyvinylpyrrolidone. *Acta Bot. Neerl.* **18**: 620–626.

— (1970a): Separation of IAA degrading enzymes from pea roots on columns of polyvinylpyrrolidone. II. *Acta Bot. Neerl.* **19**: 141–146.

— (1970b): Isoelectric focusing of indoleacetic acid degrading enzymes from pea roots. *Acta Bot. Neerl.* **19**: 363–372.

— (1970c): The presence of membrane-bound IAA degrading protein complexes in homogenates of pea roots and the manner of attachment to these membranes. *Acta Bot. Neerl.* **19**: 546–559.