THE PRESENCE OF MEMBRANE-BOUND IAA DEGRADING PROTEIN-COMPLEXES IN HOMOGENATES OF PEA ROOTS AND THE MANNER OF ATTACHMENT TO THESE MEMBRANES

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SUMMARY

Part of the IAA degrading protein complexes from pea roots are bound to the membranes. Various compounds can effect the release while recoupling can also be accomplished. The bonding of these enzymes to the membranes is probably effected by an intermediate compound or compounds, removal of which makes recoupling impossible. This intermediate compound is probably attached to the proteins of the membranes. A model of the bound state is given.

1. INTRODUCTION

The degradation of IAA in homogenates of pea roots is accomplished by two types of enzymes which can be separated by chromatography on polyvinylpyrrolidone (PVP) at low pH (VAN DER MAST 1969, 1970a). Both enzyme types are intimately associated into a protein complex which electrophoretically behaves as one protein with an isoelectric point (I.E.P.) of 9.5 (VAN DER MAST 1970b). In this paper also some evidence was presented to suggest that part of these IAA degrading protein complexes are bound to membranes. It was found that part of the enzymes under study did not adsorb to a cation exchanger at low ionic strength. This non-adsorbing portion could be removed from the homogenate by ultracentrifugation. Also during electrofocusing experiments of short duration part of the IAA degrading enzymes was found at an I.E.P. of 5.6 instead of 9.5, probably due to the presence of the membranes at the former pH. A migration of these enzymes from pH 5.6 to 9.5 was noted due to spontaneous release of the enzyme molecules.

Both the high molecular weight and the free IAA degrading enzymes consist of the two enzyme types aggregated into protein complexes (VAN DER MAST 1970a).

There were already some indications that binding of various types of enzymes to other cell constituents occurs. ADATTHODY & RACUSEN (1967) reported that the recovery of malate dehydrogenase from bean leaves was dependent on the ionic strength of the homogenation medium, whereas catalase was not affected. They also found that a major portion of the total cationic peroxidases in this leaf tissue was sorbed to naturally occuring insoluble polymers at low ionic strength and neutral pH. They presumed that the uronic acid groups in the car-

bohydrate polymers or the acidic amino acids in structural proteink or both, caused ion exchange of these peroxidases when the ionic strength or the pH were raised resulting in a higher recovery of the enzymes.

In the present paper further evidence for a two phase system of the IAA degrading complexes and some deductions of the possible binding forces and intermediate compounds concerned will be given.

2. MATERIAL AND METHODS

Seeds of *Pisum sativum* L. cv. "Vlijmsche Gele Krombek" were grown as described earlier (VAN DER MAST 1969). The following buffers were used for the homogenation of the roots: Mc Ilvaine's buffer, pH 5.0 or Tris-HCl buffer (35 mM Tris, 0.5 mM $CaCl_2$ and 10 mM $MgCl_2$), pH 7.4. For the sake of brevity the latter will be called TCM buffer, while after the addition to this medium of KCl, urea, acridine orange or biebrich scarlet respectively the abbreviations TCMK, TCMU, TCMA and TCMB will be employed. In all cases 2 ml of the various buffers were added for every gram fresh weight of roots.

The pelleting of the membranes and ribosomes was performed in the various buffers by centrifuging the 27,000 g supernatant during 1 hour at 48,000 RPM in rotor 50 of a Spinco model L 50 ultracentrifuge. The pellets were resuspended using a glass cylinder and a tightly fitting teflon piston. Material still aggregated was removed by centrifugation during 5 minutes at 10,000 g.

Density gradient centrifugation was carried out in rotor SW 25. Usually a linear sucrose gradient of 5–20% was used on a cushion of 3 ml 60% sucrose in buffer, but sometimes only a layer of 20% sucrose in buffer was brought on this cushion. In the former case centrifugation was carried out at 24000 RPM during 1 hour, while in the latter 1.5 hour was needed. At the end of a run the solution was removed with the aid of a buffer filled steel tube of small diameter which was lowered smoothly through the gradient untill the split conical inlet rested on the bottom of the centrifugation tube. Draining the tube by puncturing with an injection needle had the disadvantage that part of the membraneous material was not collected but stuck to the bottom. With a peristaltic pump fractions of 1 ml were obtained.

Gel chromatography on Sepharose 2B, an agarose gel from Pharmacia, was carried out in columns with dimensions of 35 × 1.9 cm. Fractions of 5 ml were collected.

Insoluble PVP (Polyclar AT powder) was added in various concentrations as described in the experimental part. The resulting paste was stirred during 30 minutes and the PVP was then removed by centrifugation during 5 minutes at 4,000 g.

The degradation of IAA was carried out by adding 1 ml of Mc Ilvaine's buffer, pH 5.0, containing 100 ppm IAA and 4 ppm p-coumaric acid to 1 ml of the fractions. Incubation was at 25 °C during 25 minutes unless otherwise stated. The amount of residual IAA was determined by adding 1 ml of the incubate to 4 ml Salkowski's reagent and reading the colour at 535 nm after 30

minutes. When the substrate and cofactor had been added to a density gradient the fractions were immediately brought together with this reagent.

The amount of pentoses in 2.5 ml of the fractions was determined with 1.5 ml of the orcinol reagent (COLOWICK & KAPLAN 1957).

Acridine orange and water soluble biebrich scarlet were purchased from the British Drug Houses.

3. RESULTS

The compounds of a pea root homogenate possessing a molecular weight larger than 100,000 were isolated by gel chromatography on Sephadex G-100 and con-

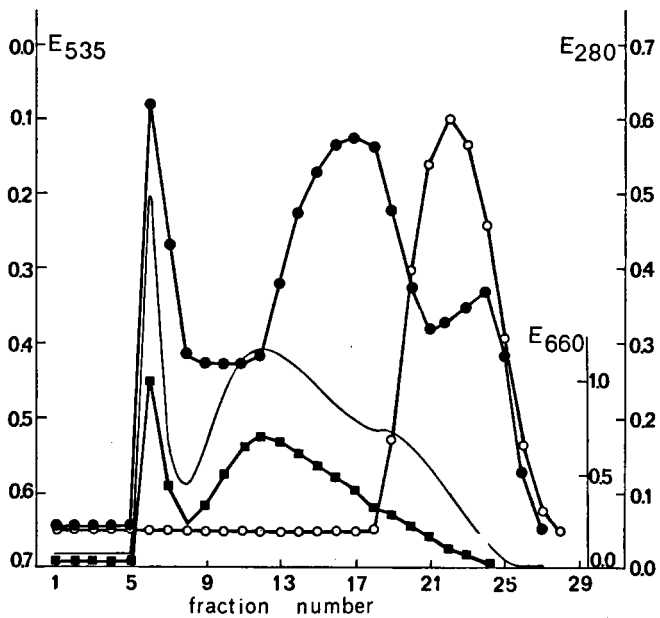

Fig. 1. Elution pattern of high molecular weight substances from homogenates of pea roots and the degradation of IAA by bound enzyme molecules. Part of these enzymes are bonded to large membrane fragments and elute after the passage of the void volume, while molecules bound to smaller fragments and free enzyme molecules are apparent in the second and the third activity peak, respectively. Treatment with PVP eliminates the bound state. The distribution of pentoses is indicated. The high molecular weight substances were obtained by gel chromatography on Sephadex G-100 from 30 ml pea root supernatant, equivalent to 15 gr fresh weight of roots, in TCM buffer. The high molecular weight peak, 60 ml, was concentrated to 10 ml and of this concentrate 3 ml was brought on the Sepharose 2B column. The elution was performed with TCM buffer. PVP was added in amounts of 500 mg pro ml.

●—● degradation of IAA (E_{535}).
○—○ degradation of IAA after PVP treatment (E_{535}).
■— ■ distribution of pentoses (E_{660}).
——— UV-absorption (E_{280}).

centrated as described earlier (VAN DER MAST 1970a). These substances were then chromatographed on Sepharose 2B. The results are depicted in *fig. 1.*

The degradation of IAA occurs in all fractions. Especially the first peak of activity is of interest because the exclusion molecular weight of Sepharose 2B is 20–40 $\times$ 10^6 depending on the shapes of the molecules. This indicates a bound state of the IAA degrading protein complexes to larger structures. The free ribosomes are present in the second UV-absorbing peak as shown by the result of the orcinol test. This is in accord with the results of the approximation of LAURENT (1967) which predicts the presence of compounds with a molecular weight of about 4 $\times$ 10^6 in these fractions for agarose gels with a concentration of 2%. The presence of pentoses in the fractions containing substances with a lower molecular weight is probably due to the various subunits of ribosomes.

The IAA degrading enzymes do not seem to be bound to the ribosomes as the second peak of IAA degrading activity and the free ribosomal peak do not coincide. This was confirmed by purifying the ribosomes by differential centrifugation until the absorbance ratio E_{260}/E_{240} was shown to exceed 1.6, indicating freedom from contaminating proteins (LYTTLETON 1968). No IAA degrading activity by enzymes bound to these organelles could then be detected but only peroxidase activity was apparent as already reported by PENON *et al.*

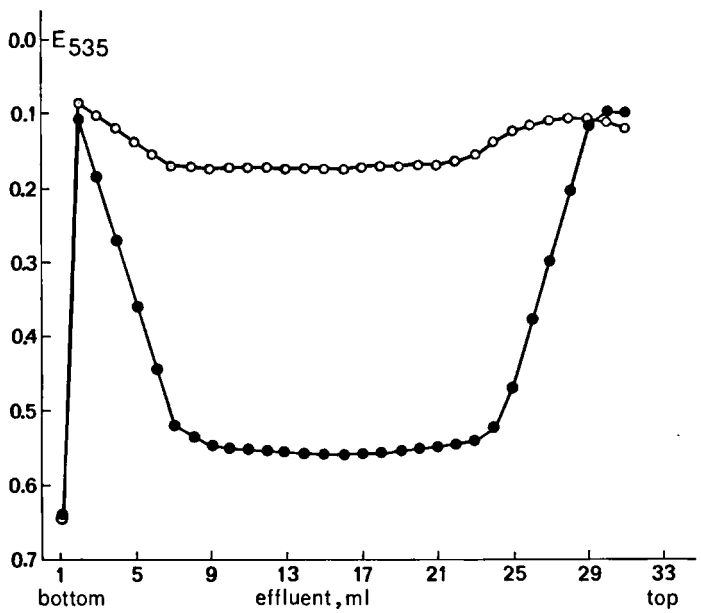

Fig. 2. Ultracentrifugation study of 3 ml samples on density gradients with pH's of 5.0 and 7.4. The release of the enzyme molecules differs in relation with this pH. The type of buffer employed for the homogenation of the roots does not influence the results.
●—● IAA degradation of a sample centrifuged at pH 5.0.
○—○ IAA degradation of a sample centrifuged at pH 7.4.

(1967) for ribosomes from roots of *Lens* and by MATSUSHITA *et al.* (1966) for ribosomes from soybean seedlings.

The first UV-absorbing peak also contains pentoses, probably due to membrane-bound ribosomes and polysomes. The addition of sodium deoxycholate nearly abolished this peak by solubilizing the membranes present here. This treatment also resulted in the removal of IAA degrading activity from all the fractions containing compounds of high molecular weight.

The addition of dry PVP prior to chromatography on the agarose gel was carried out at pH 7.4 to prevent the division of the IAA degrading protein complexes into the two enzyme types with the concomitant loss of activity due to adsorption of one of these types as encountered at low pH (VAN DER MAST 1969). The result is quite similar to that achieved with deoxycholate as all the bound IAA degrading enzymes are released from the membrane fragments to a free state which implies a lower molecular weight. When the first peak, eluted from the Sepharose column, was treated with PVP and rechromatographed, the results were the same as mentioned above, although the loss of activity was considerable.

As shown in *fig. 2* ultracentrifugation of a pea root homogenate on a density gradient gives results comparable to those achieved with gel chromatography, i.e. a rapidly sedimenting fraction containing part of the IAA degrading enzymes and a slower moving enzyme fraction. When the gradient was buffered with TCM the ribosomes were only displaced for about 2 cm during the centrifugation times employed, when Mc Ilvaine's buffer was included in the gradient these organelles were unfolded into slower sedimenting molecules by the chelating action of the citric acid. Treatment of the homogenate with 0.5% sodium deoxycholate, 2% sodium lauryl sulphate or with an equal volume of butanol resulted in a total removal of the rapidly sedimenting fraction with a concomitant removal of IAA degrading activity from this region, indicating that part of the IAA degrading protein complexes are bound to membranes. It is also shown in this figure that the binding of IAA degrading protein complexes to the mem branes is dependent on pH.

At pH 5.0 two discrete peaks of activity are apparent while at pH 7.4 the release of enzyme complexes occurs in a larger proportion resulting in IAA degradation throughout the whole gradient. This spontaneous release of the enzymes from the membranes imposes certain restrictions on the minimum amount of pea root homogenate to be brought on the gradient solution as shown in *fig. 3.*

In this experiment various amounts of the homogenate were layered on a gradient containing 50 ppm IAA and 2 ppm p-coumaric acid. It can be seen that only when 2 ml of the 27000 g supernatant was brought on the density gradient part of the enzymes were able to reach the bottom of the tube. When the degradation of IAA by 0.5 ml pea root supernatant on a gradient containing the substrate and a cofactor and on a gradient without these substances are compared, it can be seen that the addition of IAA and p-coumaric acid does not influence this spontaneous release at this pH.

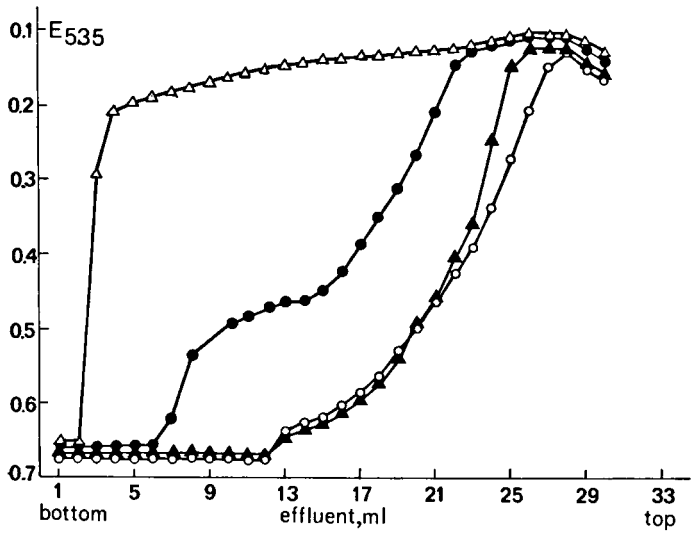

Fig. 3. Ultracentrifugation study of various amounts of the 27,000 g supernatant. To the density gradient 50 ppm IAA and 2 ppm p-coumaric acid were added in three cases. In one case the substrate and cofactor were omitted. There is no difference in the sedimentation pattern of a 0.5 ml sample with or without the additives, indicating that the release of the enzyme complexes is unaffected by these substances at this pH. The gradients were buffered at pH 5.0. Rotor head temperature was maintained at 10 °C when centrifuging a reacting sample. In all other cases this temperature was 4 °C.
○—○ 0.5 ml sample, the gradient contains the substrate and the cofactor.
●—● 1.0 ml sample, the gradient contains the substrate and the cofactor.
△—△ 2.0 ml sample, the gradient contains the substrate and the cofactor.
▲—▲ 0.5 ml sample, gradient without the additives.

When a pH gradient was established in the sucrose gradient the membranes reached the bottom of the tube, but the IAA degrading complexes were totally released at a level with a pH of 9 as shown in *fig. 4*. When, however, IAA and p-coumaric acid, or the cofactor alone, were also present in this gradient system this release was not apparent.

The enzyme complexes could also be removed from the membranes without loss of the sedimentation characteristics of these latter structures by 50 mM KCl or $CaCl_2$, 6 M urea, PVP, acridine orange and biebrich scarlet.

Acridine orange, a cationic dye with metachromatic properties, possesses a remarkable affinity for RNA-phosphates and other polyanions (FURANO *et al.* 1966). Due to this property it is possible to make visible the various cell organelles like ribosomes and membranes in a density gradient in normal light, while another yellow band is present in the original sample. As shown by *fig. 5* the amount of acridine orange needed to remove the enzyme complexes from the membranes is 3.3×10^{-3} M. At lower dye concentrations this removal was only partly effected.

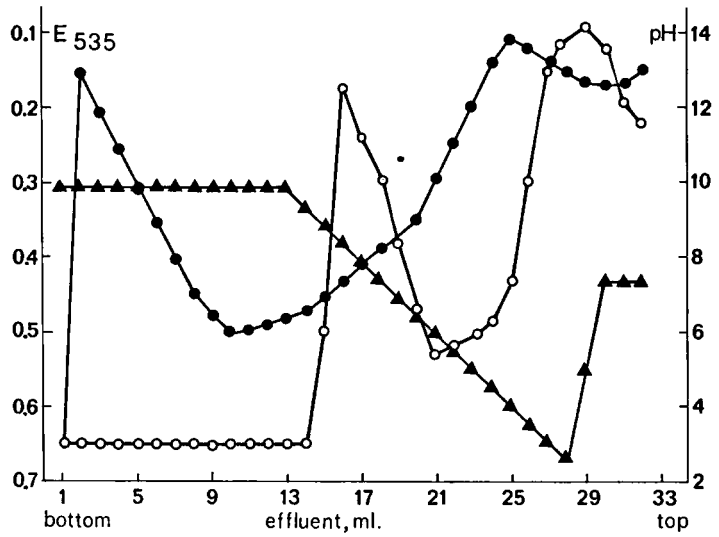

Fig. 4. pH-dependent release of membrane-bound IAA degrading enzymes. A pH gradient was established in the density gradient with citric acid and Na_2HPO_4. On the gradient solution a 3 ml sample in TCM buffer was layered. Total release of the enzyme complexes is effected near pH 9 which is prevented by the addition of the substrate and p-coumaric acid to this gradient system, but not by p-coumaric acid alone. Degradation of IAA does not take place in the gradient at the higher pH's. When these substances had been added the conversion of the substrate was measured after the centrifugation run by lowering the pH in the fractions with citric acid and incubating normally.

▲—▲ pH gradient.

○—○ IAA degradation of ultracentrifuged sample.

●—● IAA degradation of ultracentrifuged sample, substrate and cofactor added to the gradient.

Biebrich scarlet is an anionic dye with a concomitant affinity for basic compounds, i.e. for the IAA degrading protein complexes themselves. A dye concentration of 1.6×10^{-3} M was used to effect the release of these enzymes.

In the following experiments one batch of pea roots was homogenized in TCMK buffer, while another batch was treated in TCM buffer. After a preliminary centrifuging period at 27,000 g to remove the cell debris the membranes of both batches were collected by ultracentrifuging. The pelleted TCMK membranes were washed with TCM buffer and then resuspended in the buffer. The resuspended membranes were then added to the TCM supernatant of the second batch. After 30 minutes incubation time this solution was layered on a sucrose gradient and centrifuged in rotor SW 25. The results are depicted in *fig. 6.*

It is apparent from this figure that membranes which are devoid of IAA degrading enzymes due to the action of KCl can, after removal of this compound, bind free IAA degrading enzymes from another preparation.

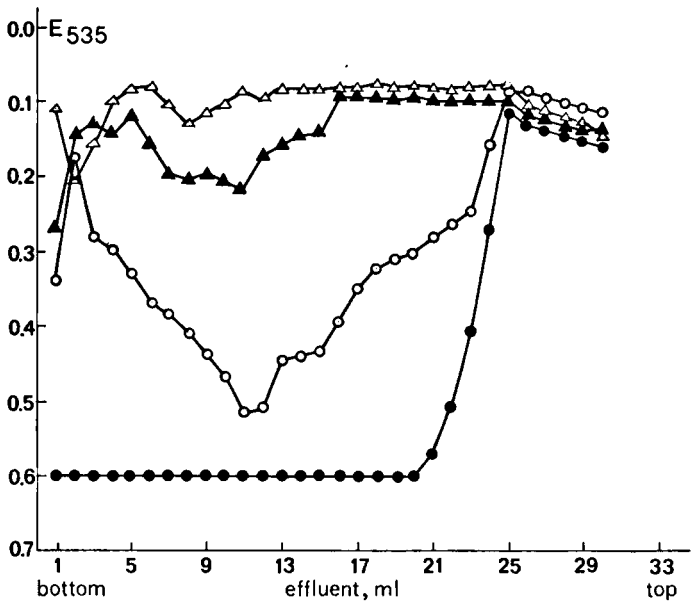

Fig. 5. Concentration dependent influence of acridine orange on the release of the enzymes from the membranes. Total release is effected at a concentration of 3.3×10^{-3}M in the 5 ml sample layered on the gradient. The gradients were made up in TCM buffers. The spontaneous release of the enzyme molecules is normal at the dye concentrations of 0.8×10^{-3}M and 1.6×10^{-9}M at pH 7.4. This release is partly inhibited at a dye concentration of 1.6×10^{-3}M.
●—● degradation of IAA by sample containing 3.3×10^{-3}M acridine orange.
○—○ degradation of IAA by sample containing 1.6×10^{-3}M acridine orange.
▲—▲ degradation of IAA by sample containing 0.8×10^{-3}M acridine orange.
△—△ degradation of IAA by sample containing 1.6×10^{-4}M acridine orange.

When the membranes were isolated from a TCMU or a TCMB preparation and mixed with a TCM supernatant the results were similar as those described above, i.e. free IAA degrading enzymes were coupled to these membranes. The results are shown in *figs. 7* and *8*.

When, however, the resuspended membranes from 10 ml of a TCMK preparation were treated with 100 mg PVP and then, after removal of this polymer, added to a TCM supernatant, no IAA degrading enzymes could be bound to these membranes as depicted in *fig. 6*. Treating a TCM supernatant with 200 mg PVP pro ml prior to mixing it with the TCMK membranes did not influence the binding of the protein complexes to these membranes, indicating that only compounds inherent to the membranes are involved in this coupling action.

The influence of acridine orange on the membranes is the same as exerted by PVP, i.e. no coupling of the IAA degrading enzymes to membranes isolated in TCMA buffer could be accomplished, as shown in *fig. 8*.

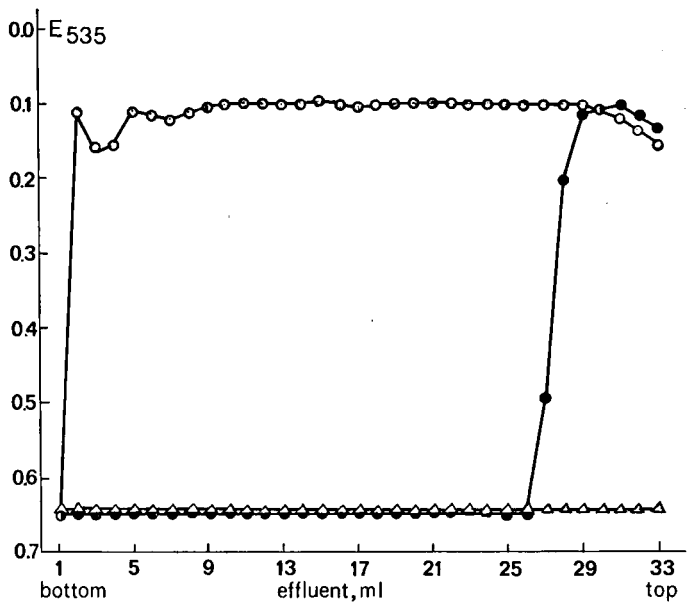

Fig. 6. The influence of KCl and PVP on the sedimentation velocity of IAA degrading enzymes. Treatment of the membranes with 50 mM KCl removes all IAA degrading activity from this fraction but this compound does not inhibit rebonding of the free IAA degrading complexes from a TCM supernatant to these structures. After treatment of the TCMK membranes with PVP the bonding sites are removed from the lipoproteins and recoupling of enzymes to the membranes can not be accomplished. In this case the free IAA degrading enzymes from the TCM supernatant give rise to the activity peak at the top of the tube.
The pH of the gradients and the samples was 7.4. TCMK membranes from 10 ml pea root homogenate were resuspended in 2 ml TCM buffer and this was added to 10 ml of a TCM homogenate from which the membranes had been removed. Samples of 3 ml were layered on the gradients. When PVP was added to the membranes an amount of 100 mg was used.
△—△ IAA degradation by TCMK membranes resuspended in TCM buffer.
○—○ IAA degradation by enzymes from a TCM supernatant coupled to TCMK membranes.
●—● IAA degradation by enzymes from a TCM supernatant after addition of PVP-treated TCMK membranes.

Treatment of TCM membranes with 10^{-3} M H_2O_2 prior to mixing them with a TCM supernatant also abolished the coupling action, probably by oxidizing the intermediate compound necessary for this coupling (see discussion).

Heat denaturation of TCM membranes also resulted in loss of this coupling faculty, probably indicating that the intermediate compound is at one side attached to the proteins of these lipoproteineous structures.

When heat denatured membranes were mixed with TCMK membranes treated with PVP and the mixture was added to a TCM supernatant, no coupling of enzymes to the membranes could be observed, indicating that the coupling

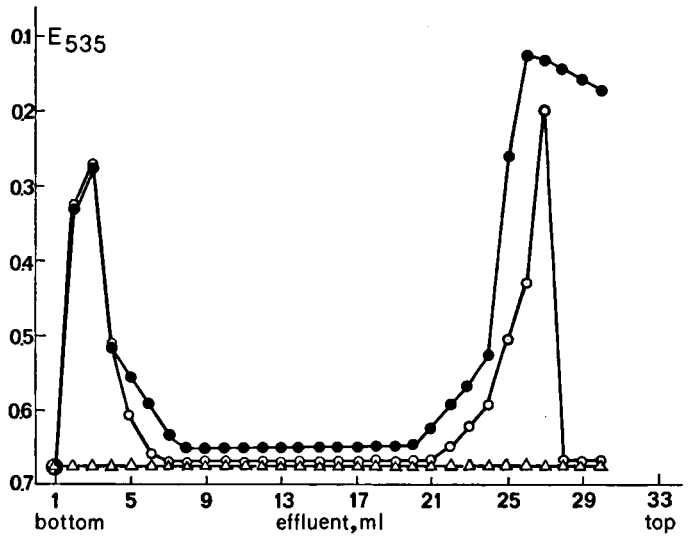

Fig. 7. The influence of urea on the sedimentation characteristics of IAA degrading enzymes. Membranes treated with 6M urea are devoid of these enzymes but rebonding takes place when these structures are added to the free enzyme complexes from a TCM supernatant. The influence of caffeic acid added to the original sample on the degradation of IAA by the high molecular weight fraction is nil. The influence of this compound is discernible at the top of the gradient. Directly under this layer degradation of IAA is apparent. Caffeic acid was added to the 3 ml sample in a concentration of 100 ppm. The gradient was made up in Mc Ilvaine's buffer.

△—△ IAA degradation of TCMU membranes resuspended in TCM buffer.

●—● IAA degradation by enzymes from a TCM supernatant bonded to TCMU membranes.

○—○ IAA degradation by enzymes from a TCM supernatant bonded to TCMU membranes in the presence of caffeic acid.

intermediate is not interchangeable. The addition of tryptophan, tyrosine, catechol, p-coumaric acid and caffeic acid, all compounds known to adsorb to PVP (Dougherty & Schepartz 1969, Andersen & Sowers 1968), to PVP treated membranes did not reinstitute bonding.

Caffeic acid, an inhibitor of IAA degradation, also does not show binding to the enzymes themselves, as depicted in *fig. 7*. In this experiment TCMU membranes were brought together with a TCM supernatant to which 100 ppm caffeic acid had been added. After density gradient centrifuging the incubation time with IAA and p-coumaric acid of the fractions was so chosen that lack of the substrate could not influence the results. If caffeic acid had adhered to the enzymes a larger amount of cofactor would have been needed to obtain a reaction comparable to the untreated enzymes (KONINGS 1964). *Fig. 7* shows, however, that the amount of IAA degraded by membrane-bound enzymes is the same at an equal concentration of the cofactor. When the experiment was repeated with 100 ppm p-coumaric acid instead of caffeic acid and the resulting

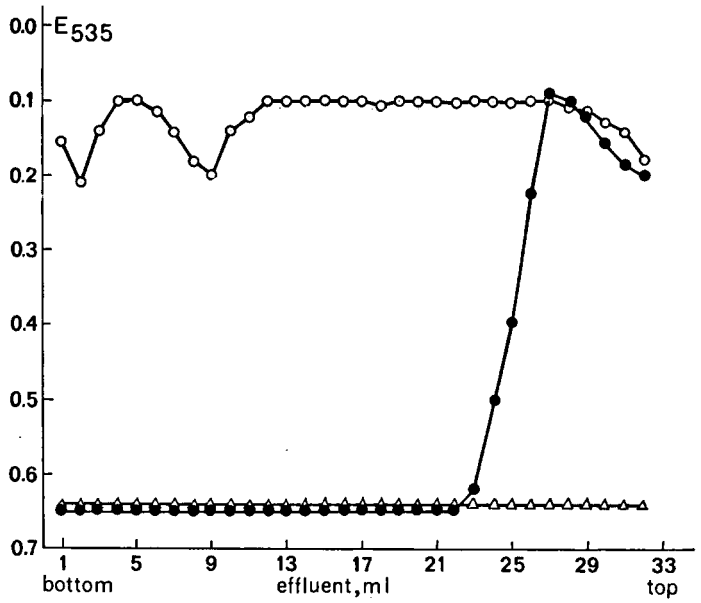

Fig. 8. The influence of cationic and anionic dyes on the sedimentation of IAA degrading protein complexes. Both biebrich scarlet and acridine orange uncouple the enzymes from the membranes. Biebrich scarlet treated membranes retain the ability to bind free enzyme complexes. This property is abolished by acridine orange. Samples of 3 ml, containing either 1.6×10^{-3}M biebrich scarlet or 3.3×10^{-3}M acridine orange were layered on gradients in TCM buffer.

△—△ IAA degradation by TCMA or TCMB membranes resuspended in TCM buffer.

○—○ IAA degradation by enzymes from a TCM supernatant bonded to TCMB membranes.

●—● IAA degradation by enzymes from a TCM supernatant after addition of acridine orange-treated membranes.

fractions were incubated without this cofactor, no degradation of IAA could be observed by membrane-bound enzymes, indicating that the cofactor also does not show binding to these enzymes.

4. DISCUSSION

The results mentioned in *fig. 1* show the presence of IAA degrading enzymes in fractions containing the larger structures. Treatment with deoxycholate, lauryl sulphate or butanol removed these structures indicating their membraneous nature. As the IAA degrading enzymes do not bind to the ribosomes the presence of these enzymes in the fractions containing these organelles is probably due to smaller membrane fragments which function as their carrier. This was confirmed by the action of the detergents which also removed the IAA degrading activity to the fractions containing the free enzyme complexes.

Fig. 2 shows that ultracentrifugation yields essentially the same results as gel chromatography. It can also be seen from this figure that the spontaneous release of enzyme molecules is dependent upon the pH of the density gradient. At pH 5 a negligible amount of enzyme molecules is released in the middle of the gradient as indicated by the IAA conversion.

When centrifugation was carried out at the same pH but the substrate and the cofactor were added to the gradient the conversion of IAA in this middle region of the gradient was complete when 2 ml pea root supernatant was used. A comparison between *fig. 2* and *3* shows that only a small amount of the IAA degradation is due to released enzyme molecules and that the larger part is converted by enzyme complexes still attached to the membranes. This indicates that the binding sites do not interfere with the catalytic function of these proteins.

As shown by *fig. 4* total release is effected close to pH 9. As this is near the I.E.P. of the protein complex it seems likely that the positive charges of these complexes are involved in binding these enzymes to the membranes.
KCL, $CaCl_2$, urea and biebrich scarlet all seem to exert the same influence as they uncouple the IAA degrading complexes from the membranes but leave the site or sites of attachment intact so that recoupling can be accomplished as shown by the *figs. 6*, *7* and *8*.

Spectroscopic studies with histone and biebrich scarlet suggest that the dye molecules lie on the outside of the polymers, probably in a radial orientation (WINKELMAN & BRADLEY 1966). Possibly the action of biebrich scarlet is also exerted on the surface of the enzyme molecules under study here, which is in accord with the position of the positive charges of the enzymes.

Acridine orange has remarkable penetrative powers as it binds to approximately 90% of the RNA-phosphates in intact ribosomes of Escherichia coli within 30 seconds (FURANO *et al.* 1966). They also found that the dye could be displaced by stoichiometric amounts of polylysine.

As shown by the figures 6 and 8 the action of acridine orange and of PVP on the membranes is irrevocable.

It is possible that bonding of the enzyme complexes to these membranes is achieved through an intermediate compound or compounds. The removal of this compound by PVP could mean that a polyphenolic substance is involved, which is destroyed by 10^{-3} M H_2O_2, while the influence of acridine orange indicates that electrostatic forces are operative in binding this compound to the membranes. It seems likely that on the membraneous side of attachment, outside the electrostatic forces, also steric effects are operative, as alterations of the configuration of the membraneous proteins by heat also abolished the bonding of the IAA degrading enzymes to the membranes. Exchange of the intermediate substance coud not be accomplished. This compound is not present in supernatants but is inherent to the membranes.

A possible model of a bound IAA degrading complex is given in *fig. 9*.

It could be argued that acridine orange only binds to the negative groups at the non-membraneous side of the intermediate compound and thus makes im-

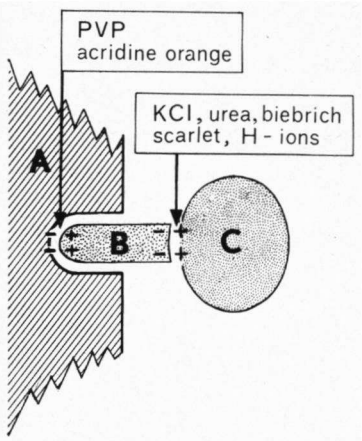

Fig. 9. Model of the bound state consisting of (A) a fragment of a lipoproteineous structure, (B) an intermediate compound and (C) an IAA degrading protein complex. The charges probably indicate hydrogen bonds. At the membraneous side of the intermediate compound possibly steric effects are also operative. The place of attack of various compounds is indicated.

possible the rebonding of free enzyme molecules. In that case, however, low molecular weight substances with a higher affinity for this dye, present in the TCM supernatant to which these membranes were added, would have displaced the bound dye molecules and recoupling of enzyme complexes would have been possible. This was not found.

The intermediate compound could not be substituted by tryptophan, tyrosine, catechol, p-coumaric acid or caffeic acid. The latter compound does not adhere to the enzymatic surface, as is shown in *fig. 7*, although it remains possible that attached caffeic acid was lost during the passage of the proteins through the gradient solution. However, it can also be seen from this figure that at the top of the centrifugation tube high IAA degrading activity is apparent just outside the layer containing this inhibitor. Thus its inhibiting function is probably not exerted by an alteration of the enzyme resulting in a blockage of the catalytic site. This phenomenon was also found with p-coumaric acid. As shown by *fig. 4* this cofactor and the substrate stabilize the bond between the IAA degrading enzymes and the intermediate compound at high pH. The stabilisation could not be achieved with the cofactor alone.

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