Content and Metabolism of Indole-3-acetic Acid (IAA) in Healthy and Rust-Infected Wheat Leaf Segments

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The content of IAA in stem rust-infected susceptible wheat leaves shows a highly pronounced maximum 5−6 days after inoculation, shortly prior to the onset of sporulation. This auxin increase can not only be caused by a reduced degradation of IAA. Considerable amounts of IAA are also found in urediospores and germlings; the IAA is in part released by them into the germination medium.

IAA applied exogenously to wheat leaves is channelled into two different degradation pathways: (a) into the peroxidase-catalysed decarboxylation which leads to indole-3-methanol and subsequent products as well as into (b) a non-decarboxylative path which leads to a number of oxindolic compounds. Furthermore, IAA conjugates such as IAAglc and IAAsp are formed. The formation of the products is characteristically dependent upon the concentration of the IAA applied.

In rust fungus-infected wheat leaves, all IAA metabolites occur which are known in healthy leaves. The mode of their formation after "feeding" of radioactively-labelled IAA leads to the conclusion that the main part of the IAA in the infected leaves is present in a pool which does not permit a rapid exchange with the IAA taken up. The results lead to the hypothesis that IAA is present, to a major extent, in the structures of the fungus and is probably also produced by it.

Introduction

In many plant diseases endogenous regulators of the auxin type undergo concentration changes to a greater or lesser extent. Thus Shaw and Hawkins [1] reported a 20-fold increase in the IAA content during the sporulation phase in rust fungus-infected Little Club wheat. In a series of investigations it was attempted to correlate such effects with an alteration in the decarboxylation rate of IAA or an altered peroxidase activity. All in all however, these efforts have not led to very conclusive evidence so that the problem, at least in the case of the biotrophic fungal organisms, can still be considered to be unsolved. Neither the cause of concentration changes nor their significance in terms of the course of the disease are sufficiently known (see reviews [2−5]).

In this study we have tackled anew the auxin problem in rust-infected wheat plants. The intention was to provide answers to the following questions: how does the auxin content change during the whole course of the infection? − what causes the increase in the auxin level? − and are there indications that IAA is formed by the host cells or by the parasite and/or is localized there?

Materials and Methods

Biological materials

Wheat plants were cultivated under controlled conditions (light period 16 h, 30 Wm^{-2} Osram HQJ-E 400 W humidity 60%, temperature 21 °C/17 °C day/night).

Primary and secondary leaves of 10-day-old plants were inoculated with urediospores of *Puccinia graminis* f. sp. *tritici* Eriks. and Henn, race 32, by spraying them with a suspension of spores in Freon 112 (100 mg urediospores/50 ml Freon). Incubation took place in a plastic cage used as an humidity chamber in order to maintain high humidity conditions during the incubation period (16 h darkness fol-

lowed by 4 h light). The resulting degree of infection was about 90–110 pustules/cm² of leaf area.

The following wheat cultivars were used: *Triticum aestivum, cv.* Marquis and *Triticum compactum, cv.* Little Club: susceptible (infection type 4); *Triticum aestivum, cv.*'s Feldkrone and 417/65: resistant (infection type 0).

Determination of IAA

IAA was determined fluorescence spectroscopically as methyl indole-α-pyrone [6–8] as follows: Wheat leaves (10 g) were homogenized in a Waring blendor with 100 ml methanol and 10 mg BHT at 4 °C for 3×1 min and subsequently extracted for 10 min. Following filtration, washing with cold methanol until there was complete decolouration and addition of labelled IAA standard (5000 dpm [1-^{14}C]IAA), the methanolic extract was carefully concentrated and chromatographed on Sephadex LH-20 (System I, see below). Those fractions containing IAA were collected, and, after the addition of 3 mg BHT concentrated and chromatographed on a polyamide plate (System II). The IAA-containing zone was eluted with methanol in the presence of BHT, dried to completeness at 30 °C using a rotary evaporator, and dissolved in 400 µl methanol. This material was divided into 4 aliquots. One aliquot was used to determine the recovery by measuring the radioactivity of the added internal IAA standard; a further aliquot was used to determine the fluorescence background; the other two aliquots were used for the determination of IAA according to Iino *et al.* [8]. To achieve this, the samples were dried and, under exclusion of atmospheric moisture mixed with a precooled mixture of acetic acid anhydride/trifluoroacetic acid (1:1, 200 µl). After 15 min, the reaction was stopped by the addition of a mixture of acetic acid/water (28:2; 3 ml) and after a further 60 sec the fluorescence emission was measured (λ_{exc} = 440 nm; λ_{em} = 490 nm; Instrument: Jobin Yvon JY3D). A standard curve was plotted using IAA under identical conditions.

For the determination of IAA in urediospores and urediogermtubes, basically the same method was used. In each case 200 mg spores were dispersed in 200 ml water and incubated in the dark at 20 °C to induce germination. Following germination, the medium was separated from the spores by filtration, acidified to pH 2.5 and extracted with ether. For extraction, non-germinated spores or the germtubes were treated with cold methanol (1 h). An additional homogenization of the material was not necessary. In this case too BHT was used routinely to protect against oxidation and labelled IAA was added for standardization. Both in the ether phase, which contained the IAA from the germination medium, as well as in the spore extracts, thin layer chromatography sufficed for the pre-purification of the IAA; additional chromatography on LH-20 proved unnecessary.

Tracer experiments

Tracer experiments with radioactively-labelled IAA were performed either with the aid of the leaf injection technique or of the leaf segment technique. In the case of the injection technique (according to [9]; modified according to [10]), the aqueous IAA solution was injected by means of a special device into the intracellular space of primary leaves such that about an 8 cm long leaf section contained about 40 µl solution. In using this technique it was possible to avoid extensive injury to the leaf and the wound reaction remained restricted to the single site of injection.

The leaf segment technique offered, instead, the advantage of being more easy to carry out and a better reproducibility in quantitative determinations. In this case the leaves were cut into segments of 4 mm, washed with distilled water (2×10 min), and incubated in MES buffer (0.1 M; pH 5; 0.5 g leaf segments in 10 ml buffer or 2.5 g in 50 ml).

After addition of the radioactively-labelled substances, all samples were incubated with gently shaking in the dark at 20 °C. Following incubation, all leaf segments were washed with distilled water, dried on filter paper and pulverized in liquid N_2. To extract, the leaf powder was suspended directly in 80% methanol. The extracts were concentrated at a maximum of 30 °C. Aliquots were chromatographed as described below.

To investigate the IAA metabolism in germinated urediospores of the fungus, urediospores (200 mg) were washed with a 10^{-4} M Triton X-100 solution for 5 min in order to remove the germination inhibitor (according to W. K. Kim, personal communication). Finally they were left to germinate on water (20 ml) in the presence of the radioactively-labelled IAA (10^{-6} M, 16 h, 20 °C, dark). In such experiments the

spores took up about 50% of the activity. Following removal of the germination water with a pipette, the spores were carefully washed with distilled water (3 × 10 min, each 50 ml) and subsequently extracted, firstly with methanol (100 ml, 1 h) and then with chloroform (100 ml, 1 h).

The radioactively-labelled compounds were localized on the thin layer plate either from co-chromatographed inactive standards or by employing a β-camera system (Berthold BF 290 HR). To determine the incorporation of [1-^{14}C]IAA into the various products, the silica gel was scratched off the TLC plate and the radioactivity was measured after suspending the material in a scintillation cocktail containing thixotropic gelling powder. In all cases radioactivity was measured using a Berthold BF 8000 scintillation counter coupled to a Hewlett Packard 9815 A calculator programmed for automatic quench correction.

Systems used for chromatography

I: CC, Sephadex LH-20, solvent system chloroform/methanol/water (60:35:5). II: TLC, MN-polyamide UV$_{254}$, solvent system chloroform/ethyl acetate/acetic acid (70:25:5), addition of BHT (100 mg/l). III: TLC, silica gel with fluorescent indicator, solvent system ethyl acetate/2-butanone/ethanol/water/acetic acid (25:15:5:5:0.2). IV: TLC, same as III, but without acetic acid. V: HPLC, RP18, particle size 7 µm (250 × 8 mm), solvent system methanol/water (80:20), 3 ml/min. VI: same as V, but gradient elution methanol/water/acetic acid (75.5:19.5:1) →→ (39.5:59.5:1).

Chemicals

All chemicals were of highest purity available. OxIAA and 7-OH-OxIAA were gifts from Prof. R. S. Bandurski (Michigan State University, East Lansing, Michigan, USA) and IAAsp was a gift from Prof. W. Hilgenberg (Botanisches Institut, Universität Frankfurt, FRG). Dioxindole-3-acetic acid was prepared according to [11]. [1-^{14}C]IAA and [2-^{14}C]IAA were from Amersham/Buchler, Braunschweig, FRG.

Results

The IAA content in susceptible rust-infected wheat leaves showed noticeable changes during the course of the infection (Fig. 1). A slight increase in the IAA content was observed even at early stages of infection. A highly pronounced maximum arose on the 5th−6th day following inoculation; compared to the non-inoculated control plants, values of a 60−80-fold increase were found. Thereafter the values decreased; nonetheless, they were still clearly increased compared to healthy leaves even at the beginning of the sporulation phase (after the 8th day; Fig. 1, A + B).

In resistant wheat leaves however, no increase in the IAA content was established (Fig. 1C). The increased IAA content is apparently restricted to such regions of the leaves as are in fact affected by the infection (Fig. 2).

The appearance of the IAA peak described can be caused by a modified intensity of biosynthesis, conjugation or degradation of IAA in the host cell. It can, however, likewise be caused by an intensive IAA
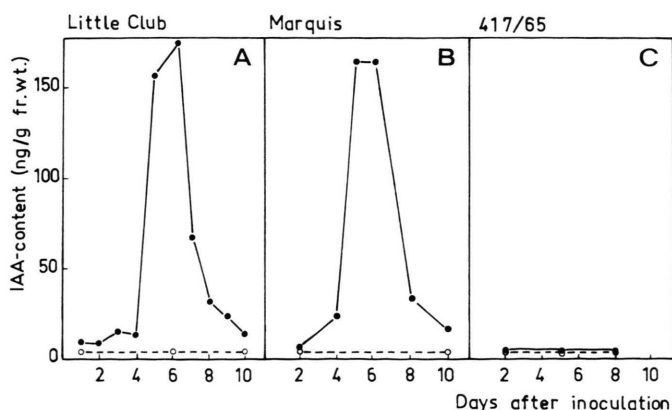

Fig. 1. IAA content of rust-infected wheat leaves: its dependence on the course of infection in susceptible leaves (A, B: infection type 4) and in resistant leaves (C: infection type 0). ●——●: inoculated; ○−−−○: control.
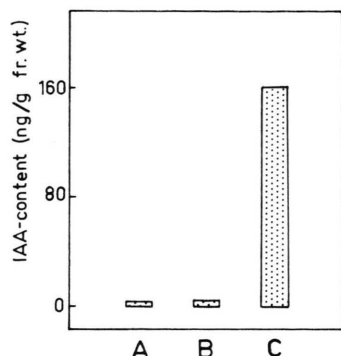
Fig. 2. IAA content in inoculated and uninoculated susceptible wheat leaf segments. A: uninoculated secondary leaves. B: lower uninoculated sections from secondary leaves which were inoculated in the upper part; 6 days after inoculation. C: corresponding to B, upper inoculated section.

biosynthesis by the fungus. The latter alternative is certainly to be born in mind since it was observed that urediospores also contain IAA and that the total amount of IAA detectable during the course of the spore germination increases, whereby the larger proportion of IAA is released into the germination medium (Fig. 3).

In studying the IAA metabolism in Gramineae we have similarly investigated the metabolism of IAA in rust fungus-infected wheat plants. Revealing information as regards the formation of the substances and the relationship prevailing in the infected leaf was gained from the investigation of the conjugates and the non-decarboxylated products following application of [1-^{14}C]IAA.
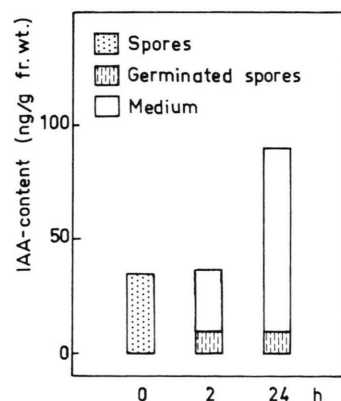

Fig. 3. IAA content of non-germinated and germinated spores of *Puccinia graminis f. sp. tritici*, race 32, as dependent on the duration of germination.

In this case the formation of at least 9 different substances was observed (for illustration see Fig. 6). They could, in part, be tentatively characterized [13]: The identity of substances 1 and 2 is unclear. Substance 3 is an amino acid conjugate of IAA. It was possible to identify it as IAAsp by co-chromatography with marker substances. Substance 7 was identified as IAAglc with the aid of chromatographic and mass spectrometric analysis as well as from its behaviour under enzymatic and alkaline hydrolysis. Substance 8 is also, probably, a glucose ester of IAA which, during the preparation, can form non-enzymatically by acyl migration from the 1-0-glucoside (see [14]). In this connection it should be mentioned that IAA-myoinositol was not detected. Substances 4, 5, 6 and 9 are apparently closely related to one another biochemically. Substance 9 was tentatively identified as OxIAA by co-chromatographing with reference compounds using TLC and HPLC (Systems III, IV, V, VI). Substance 6 is most probably to be viewed as DiOxIAA. Substances 4 and 5 are β-D-glucosylated forms of substances 6 and 9, respectively. 7-OH-OxIAA or its glucose ester were not detected.

Since experiments with leaf segments as well as those in which IAA was injected into intact leaves produced effectively the same results, artefacts due to injury could be largely excluded.

The pattern of the formation of the different products was noticeably dependent on the concentration of the substrate applied: Pulse-chase application of 10^{-4} M [1-^{14}C]IAA (spec. act. 1.85 MBq/100 μmol) to healthy wheat leaf segments led to the result that the major part of the IAA taken up is first glucosidized but is then increasingly incorporated into the substances 1−6 (Fig. 4A). This indicates that the formation of the IAAglc is not a rate-limiting step, and apparently this reaction enables large amounts of IAA to be rapidly conjugated with glucose. By lowering the IAA concentration applied to 10^{-6} M (spec. act. 1.85 MBq/μmol) it became clear that the IAAsp too, is subject of a rapid turnover (Fig. 4B). Altogether these data show that the concentration of the free IAA in the leaf is highly effectively regulated to a low level. The formation of the glucoside already commences, apparently without a lag phase, during the initial phase of the IAA pulse whereas the formation of the aspartate as well as the pair of substances 1 and 2 is retarded. Furthermore it became clear that substances 4, 5, 6 (the related substance 9 was not
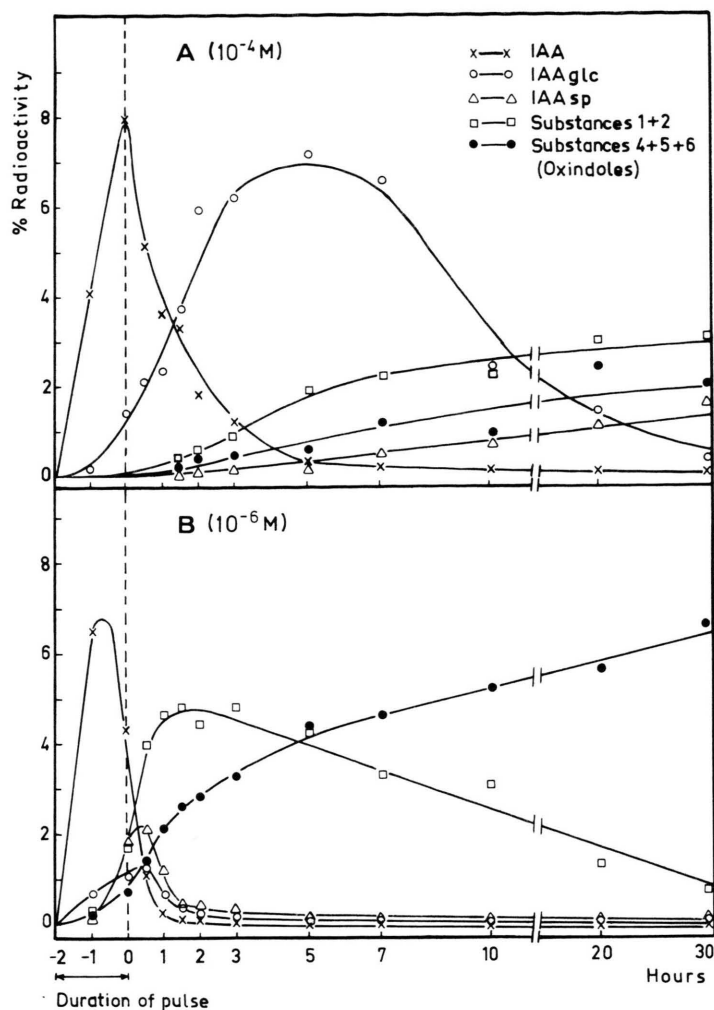
Fig. 4. Time course of incorporation of radioactivity into the products after pulse-chase feeding of 10^{-4} M and 10^{-6} M [1-^{14}C]IAA on uninoculated wheat leaf segments.

measured owing to the very small amounts appearing in the leaf) in the present system are to be considered end products without recognizable turnover.

Additionally it was established that under these experimental conditions about 75% of the IAA taken up flows into the non-decarboxylative metabolism described above whereas ca. 25% are channelled into the decarboxylative IM pathway. Moreover it was found that as regards the appearance of the non-decarboxylated products considerable deviations between the different Gramineae species arise [13] (results not shown here).

Pulse-chase experiments with infected wheat leaf segments were performed on the 6th day after inoculation since at this time in compatible systems possible modifications in the IAA metabolism were to be

expected owing to the high endogenous IAA content. Furthermore, at this time in the compatible systems, the differences in the IAA decarboxylation rate between inoculated and non-inoculated plants were minimal (see Discussion).

In incompatible interactions no noticeable changes with respect to the non-decarboxylative IAA metabolism were established to have been effected by the infection [13] (results not shown here). In contrast, the situation prevailing in the compatible system was completely different. In this case the uptake of IAA by the infected leaf segments was significantly increased (14.1% cf. 9.5% in healthy segments). Nonetheless the radioactivity measured in IAA was reduced in the infected leaf material (Fig. 5). This meant that IAA in these leaves (in

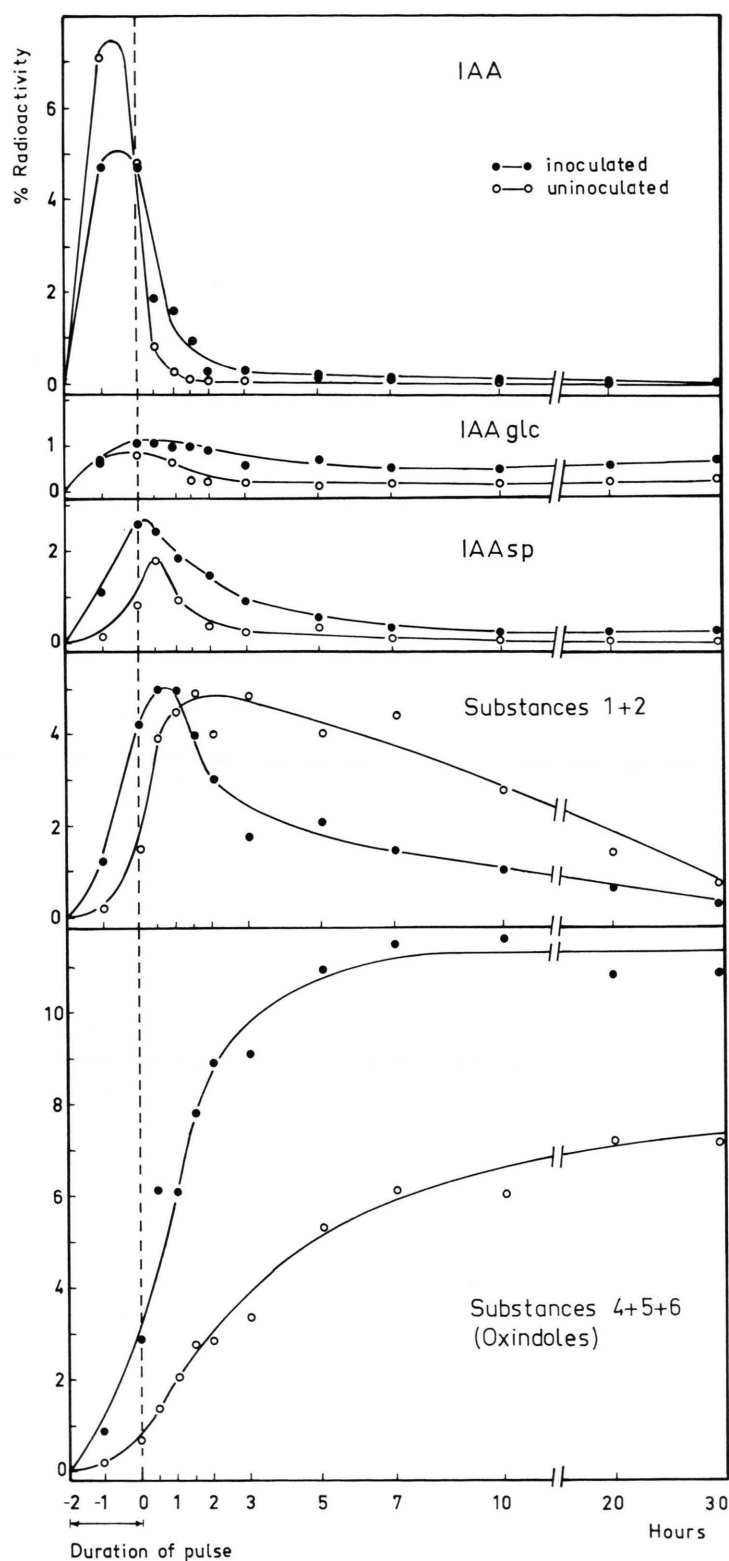Fig. 5. Time course of incorporation of radioactivity into the products after pulse-chase feeding of 10^{-6} M $[1\text{-}^{14}C]IAA$ on inoculated or uninoculated wheat leaf segments (cultivar Marquis; susceptible); 6 days after inoculation (see also Fig. 6).

spite of the high endogenous IAA content) must have been much more rapidly degraded or conjugated. Contradictory to this, at first sight, is the observation that the radioactivity measured in IAA after the end of the pulse decreased with a slight delay compared to the healthy leaves and during the entire course of the experiment was found at a slightly increased level. The increased rate of IAA metabolism in the infected susceptible leaf found expression in the increased incorporation rate into the different products of IAA during the pulse. As in the case of IAA, a somewhat delayed reduction in the radioactivity in IAAglc and IAAsp was, moreover, recognizable. In contrast, the radioactivity in substances 1 and 2 decreased rapidly in comparison to that in the healthy control leaves, whereas the radioactivity in the substances 4, 5 and 6 increased much more quickly. Since the radioactivity was determined, for technical reasons, in groups (*i.e.* in 1 + 2 and 4 + 5 + 6) it is not possible to deduce whether all those substances belonging to one group were uniformly affected by the changes. The β-photographs obtained from the TLC plates were assessed visually: an unambiguous shift from substance 2 to 4 in the infected leaves is responsible for the effect (Fig. 6). The high incorporation of radioactivity into substance 4 finally resulting reflects the increased IAA uptake of the infected leaves.

The results described above agreed completely with those obtained with inoculated or uninoculated leaves from wheat cultivars tested additionally *i.e.* Feldkrone (resistant) and Little Club (susceptible). The typical changes effected by infection were similarly obtained in random sample experiments when the IAA solution was injected into the leaves in order to circumvent the unavoidable wound effect in the segment technique [13] (results not presented here).

To examine the IAA metabolism in the rust fungus, urediospores (mycelium was not available for such investigations) were germinated in the presence of the radioactively-labelled IAA (10^{-6} M). In such experiments, the spores took up about 50% of the radioactivity. Following extraction of the germlings none of the decarboxylated and non-decarboxylated products formed in the leaves could be detected. Instead, both after application of [1-^{14}C]IAA and [2-^{14}C]IAA practically all the radioactivity taken up was found in two highly non-polar substances which could be extracted with chloroform. Both substances were not characterized further since they did not occur in infected wheat leaves.

Discussion

In compatible wheat-rust systems we have observed the appearance of a pronounced IAA maximum with 60−80-fold increased concentrations on the 5th and 6th day after inoculation, shortly before the onset of sporulation. Thus it was possible to specify more exactly previous studies in which only the late stages of infection were examined and in which, correspondingly, the maximum was not recognized.

One possible cause of the auxin increase in rust-infected wheat leaves suggested earlier is an altered rate in the decarboxylation of IAA [1]. In compatible reactions, the latter alters in three phases: whereas it is increased during the early stages of infection, it sinks on the 4th and 6th day of infection to roughly the values of healthy control plants, and during the sporulation phase clearly reduced decarboxylation rates are measured [15]. A new investigation of IAA decarboxylation with those wheat-rust combinations used in the present study confirmed the results quoted above. Further it was established that also
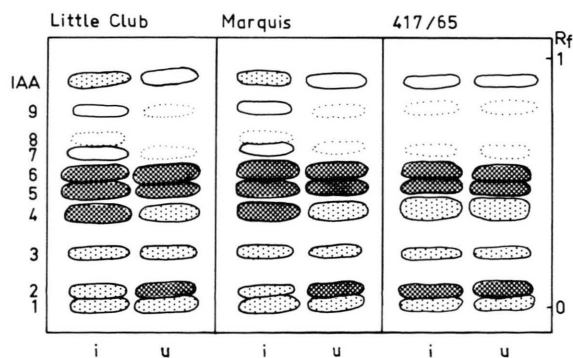

Fig. 6. TLC chromatogram of 80% MeOH extracts of inoculated (i) and uninoculated (u) secondary wheat leaf segments (cv. Little Club, susceptible; cv. Marquis, susceptible; cv. 417/65, resistant) after feeding of 10^{-6} M [1-^{14}C]IAA; 6 days after inoculation. Time of incubation 5 h; TLC system III; the hatching corresponds to the radioactivity according to visual assessment (drawing made from β-photographs). In compatible interactions the infection effects a characteristic change in the appearance of the IAA metabolites (decrease in substance 2 in favour of substance 4; see also Fig. 5). See text concerning the identity of substances 1−9.

the appearance of IM-glucoside and ICA-glucoside as a result of the decarboxylation reaction reflects, as is to be expected, the changes observed in the decarboxylation rate (Langenbeck-Schwich and Wiese, unpublished results). If these results are compared with the IAA content of the rust-infected leaves, it becomes evident that the altered decarboxylation rate cannot be counted responsible either for the early auxin increase or for the auxin maximum itself. Only at later stages in the infection is it conceivable that the decarboxylation rate influences the IAA content. In any case other factors must lead to the increase in the auxin level in infected wheat leaves.

Exogenously-applied IAA is channelled, in wheat leaves, both into the IM pathway leading to the above-mentioned decarboxylation products [12] as well as (to a larger extent) into a non-decarboxylating path. Changes in the non-decarboxylative degradation of IAA are similarly unlikely to be the cause of the increased auxin level since it was shown that IAA applied exogenously is more rapidly converted to non-decarboxylated metabolites in infected leaf segments than in healthy ones (this study).

The non-decarboxylated products are represented in the current study by substances 4, 5, 6 and 9; they have been tentatively indentified as oxindole-3-acetic acid and its derivatives. Compounds of this type have been intensively studies in the past, in particular in maize seedlings [see 14]. It appears to be plausible to assume that both metabolic pathways serve for the degradation of the active IAA molecule. The pulse labelling experiments further showed clearly that in addition to the IAA-degrading paths, other systems exist which are capable of regulating the IAA concentration in the tissue rapidly and effectively: namely the conjugation of IAA with other reaction partners, in the present study with glucose and aspartate.

In leaf segments of resistant plants neither the IAA uptake from the incubation medium nor the formation of the different non-decarboxylated IAA products was modified as a result of the infection, as was to be expected. In leaf segments of susceptible plants, the infection effected, in contrast, both an increased uptake of IAA from the incubation medium and alterations in the appearence of the non-decarboxylated products. The results of the pulse-labelling experiments which were performed on the 6th day following inoculation when the IAA content was at maximum indicate that the endogenous IAA in the host-parasite complex is, at least partially,
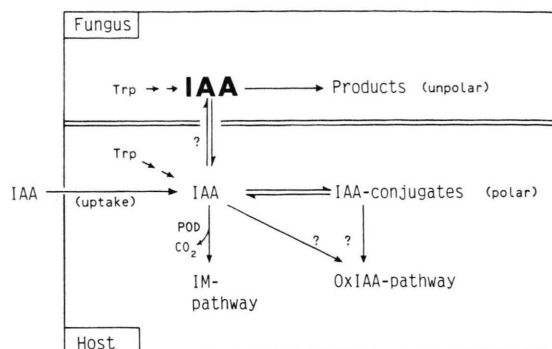

Fig. 7. The results described in the present paper support the hypothesis that in the rust-wheat complex the major part of the endogenous IAA is present in structures of the fungus. Exogenously-applied IAA is metabolized in the host cells with formation of all the substances known in healthy leaves. A rapid mixing of both IAA pools *i.e.* of the exogenously-applied IAA with the fungal IAA, can be excluded.

compartmentalized in such a way that the radioactively-labelled IAA taken up is not mixed with the total endogenous IAA pool. The simplest interpretation assumes that the major part of the IAA in compatible host-parasite interactions is present in structures of the rust fungus (see Fig. 7). The following arguments support such a hypothesis or are in agreement with it, *viz.:*

1. Urediospores are probably capable of synthesizing auxin during germination; in addition they release IAA into the surroundings (this study). Earlier studies with the isolated mycelium of another rust fungus, namely *Melampsora lini,* have shown that this fungus synthesizes IAA from exogenously-applied tryptophan [16]. Altogether these observations suggest that rust fungus mycelium also contains auxin in the host-parasite complex and also that it synthesizes it itself.

2. The uptake of IAA from the incubation medium is increased in the infected leaves despite the increased level of free endogenous IAA. This result was surprising since it was rather more expected that leaves with an increased endogenous IAA content would take up auxin less well than those with a low IAA level. This speaks against the total free IAA being localized in the host cells.

3. The metabolism of exogenously-applied IAA takes place in infected wheat leaves apparently only in the host cells. All IAA products appear that are known in healthy leaves. Urediospores (and accord-

ingly probably also the mycelium) form exclusively non-polar products from exogenously-applied IAA. These products never appear, however, in experiments with infected leaves.

4. The conjugation and the non-decarboxylative metabolism of exogenously-applied IAA are accelerated in the infected susceptible leaf segments at a stage when the endogenous IAA content had reached its maximum. This indicates that the exogenously-applied IAA is not mixed with the total pool of free IAA in the host-parasite complex. Then were the latter to be the case, more than a 100-fold increase in the amounts of IAA would have to be converted in the infected leaves during the same length of time (considering the 60–80-fold increase in endogenous IAA and also the finally highly increased formation of substance 4). This is improbable since healthy wheat leaf segments by no means metabolize excess amounts of IAA (taken up) faster than lower; in such a case they form (during the course of the experiment) predominantly IAAglc first of all. It is just this however which is not synthesized more in the infected susceptible leaves compared to the other products.

The accelerated metabolism of IAA is reflected in the increased turnover of substance 2 and the highly increased formation of substance 4. This shift from substance 2 to substance 4 is an overt effect of the rust-infection on the IAA metabolism. The relevance of this effect for the course of the infection can at present not be judged, especially since the identity and the physiological significance are unknown, in particular of substance 2.

5. Noticeably, the turnover of the substances present in the leaves following the pulse (IAA, IAAglc, IAAsp) appears to be slightly reduced. On a speculative basis, this effect can be understood if it is assumed that the radioactively-labelled fraction of the substances at the site of metabolism is diluted by the slow release of inactive IAA from the fungus and its subsequent conversion into the compounds under consideration. Alternatively, it may also be assumed, that very small amounts of the radiolabelled IAA is taken up by the fungus and then slowly released again into the host cell.

The hypothesis put forward, which states that the major part of the IAA in the host-parasite complex is present in structures of the fungus, is supported by the above-mentioned arguments. To confirm this hypothesis it is necessary in the future to aim at a direct demonstration of the localisation of auxin in the fungus and a release of auxin into the host cell. Up till now this has not been experimentally feasible. In particular it will be necessary to find an answer to the question as yet unanswered: What is indeed the function of IAA in the host-parasite interaction?

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