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# *N*-MALONYLTRYPTOPHAN METABOLISM BY SEEDLINGS OF CHINESE CABBAGE

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**Key Word Index**—*Brassica campestris* ssp *pekinensis*; Cruciferae, Chinese cabbage, IAA-biosynthesis, IAA-precursor, indole-3-acetaldoxime, *N*-malonyltryptophan.

**Abstract**—When [$^{14}$C]DL-*N*-malonyltryptophan (MTrp) was fed to hypocotyls and roots of five-day-old Chinese cabbage seedlings, three $CH_2Cl_2$-soluble metabolites were identified by HPLC as IAA, indole-3-acetaldoxime (IAOX), and indole-3-acetonitrile (IAN), respectively. The *in vitro* conversion of [$^{14}$C]MTrp by plasma membranes from Chinese cabbage seedlings enriched by aqueous two phase partitioning showed formation of IAOX as the only non-polar reaction product. The conversion of MTrp to IAOX showed a broad pH optimum between 5 and 8. The $K_m$ for MTrp at pH 8 was 6.25 $\mu$M, pH 5 6.6 $\mu$M. Simultaneous feeding of [$^{14}$C]MTrp and [$^{3}$H]tryptophan as well as inhibition experiments with [$^{14}$C]tryptophan and unlabelled MTrp showed that both substrates were converted by the same enzyme system. The formation of IAOX was reduced under anaerobic conditions, suggesting that the reaction was $O_2$-dependent; no other cofactors were needed. Other peptide-bound tryptophan derivatives were not accepted as substrates. It was shown that the enzyme system accepts only L-tryptophan as substrate. The role of MTrp as an auxin precursor is discussed.

## INTRODUCTION

*N*-Malonyltryptophan (MTrp) has been described as a natural compound in several plant species. It was first identified in wheat coleoptiles [1] and can be extracted from a number of fruits and vegetables [2]. Further identification of MTrp was achieved after incubation of spinach leaves with DL-tryptophan [3]. The isolated compound was characterized by TLC, thin layer electrophoresis and mass spectrometry. After incubation with [$^{14}$C]-labelled D-tryptophan it could be demonstrated that wheat roots are able to synthesize MTrp and that the compound was synthesized in the roots themselves and not transported therein from the shoots [3]. In pea [$^{14}$C] D-tryptophan was converted to MTrp, IAA, and IAAsp [4]. Thus, MTrp was postulated to be the storage form for the free amino acid. Other authors suggested a regulatory role for MTrp during IAA biosynthesis [5, 6]. It could be demonstrated that the MTrp content in drought-stressed leaves from several plant species was significantly enhanced, whereas no MTrp was detected in turgid leaves [6]. The MTrp synthesized during wilting was proposed to be a precursor for IAA biosynthesis after restoration of leaf turgescence. It was also observed that MTrp, as well as D-tryptophan, stimulated the growth of soybean and tomato tissue cultures when both substances were supplied instead of auxin [5]. Also MTrp promoted the root formation of excised young tomato leaves. [$^{14}$C] IAA and [$^{14}$C] D-tryptophan were found in soybean cells when they were incubated *in vivo* with [$^{14}$C] MTrp [5]. As all data cited above are from *in vivo* experiments, our aim was to investigate an *in vitro* system which is able to convert [$^{14}$C] MTrp to an IAA-precursor and to characterize the conversion of [$^{14}$C] MTrp to [$^{14}$C] indole-3-acetaldoxime (IAOX).

## RESULTS

### *Identification of* N-DL-*malonyltryptophan*

Identification of chemically synthesized MTrp was achieved by TLC analysis. On silica gel with BuOH–HOAc–$H_2O$ (12.3.5) as solvent, MTrp chromatographed at $R_f$ 0.7 [3]. Tryptophan ($R_f$ 0.57) showed a good separation from MTrp in this system. Mass spectra of MTrp showed the base peak of the quinolinium ion at *m/z* 130 and the malonyl group at *m/z* 87 (data not shown).

### In vivo *metabolism of* [$^{14}$C]DL-*malonyltryptophan by seedlings of Chinese cabbage*

The metabolism of [$^{14}$C]MTrp when fed to hypocotyl and root tissue of five-day-old dark grown Chinese cabbage seedlings after an incubation time of 1 hr revealed three major dichloromethane-soluble metabolites (Fig. 1). indole-3-acetaldoxime (IAOX); indole-3-acetonitrile (IAN) and IAA. Tentative identification of the reaction products was achieved by HPLC using internal non-labelled standards. IAOX was only found as the *E*-isomer. 0.25 nmol [$^{14}$C] MTrp, (50% of the total amount of [$^{14}$C]MTrp fed) was taken up by the seedlings and 0.05 nmol (20% of [$^{14}$C]MTrp uptake) was found in $CH_2Cl_2$-soluble products.

### In vitro *conversion of* [$^{14}$C]DL-*malonyltryptophan by plasma membranes of Chinese cabbage seedlings*

The enzymatic conversion of [$^{14}$C] MTrp by phase partitioned membranes from Chinese cabbage seedlings revealed IAOX as the only reaction product in the neutral organic phase. The identification of IAOX was achieved

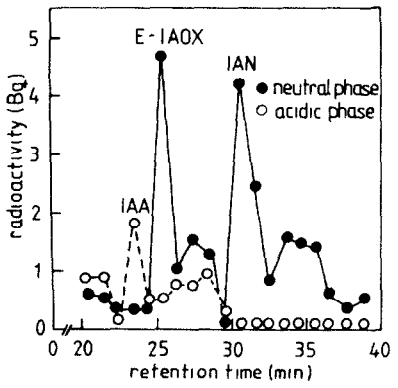


Fig 1 Metabolism of MTrp to $CH_2Cl_2$-soluble products in 5-day-old Chinese cabbage seedlings *in vivo*, incubation time was 1 hr Distribution of radioactivity after HPLC-separation (reverse phase), solvent 38% MeOH for neutral reaction products, MeOH–Na citrate, pH 3 5 (19 31) for IAA, flow rate 0 8 ml/min

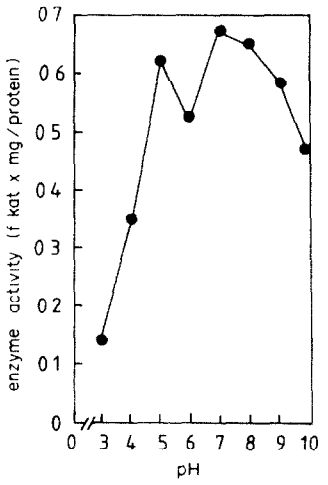


Fig 2 pH-Dependence of the conversion of MTrp to IAOX by phase partitioned membranes of Chinese cabbage seedlings after background subtraction of chemical conversion

by TLC and HPLC as described elsewhere [7] The conversion of MTrp showed a linear IAOX formation up to 2 hr and maximum formation of IAOX after 4 hr. After subtraction of the chemical IAOX formation at low pH the pH-dependence curve showed a broad pH-optimum between 5 and 8 (Fig 2) Compared to the conversion of [$^{14}$C] L-tryptophan to IAOX the conversion of [$^{14}$C] DL-MTrp is much higher The $K_m$ value obtained for MTrp from the Lineweaver–Burk plot was 6 25 $\mu$M at pH 8 (Fig. 3), and 6 6 $\mu$M at pH 5 Reduction equivalents as well as metal ions did not increase IAOX formation Incubation with [$^{14}$C]DL-MTrp under aerobic and anaerobic conditions showed that the enzyme activity under anaerobic conditions is reduced by *ca* 35% after 1 hr incubation compared to aerobic conditions

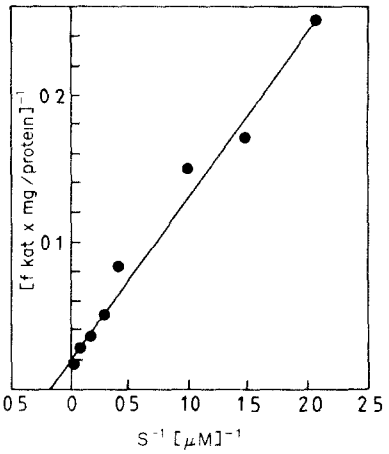


Fig 3 Lineweaver–Burk plot of substrate kinetics for MTrp at pH 8

*Inhibition experiments*

Inhibition experiments with unlabelled D- and L-MTrp showed that only L-MTrp is converted to IAOX, because only this isomer is able to inhibit IAOX formation significantly As shown (Table 1) IAOX formation under the influence of 1 mM L-MTrp is reduced by *ca* 25%, whereas D-MTrp at the same concentration showed no inhibitory effect Other peptide-bound tryptophan derivatives were apparently not accepted as substrates, as feeding of non-labelled L-tryptophyl-tryptophan and L-leucyl-tryptophan simultaneously with [$^{14}$C] DL-MTrp in concentrations 10-fold higher than the labelled substrate showed no inhibition of IAOX formation compared with the control

Table 1 IAOX formation from [$^{14}$C]DL-MTrp (in the presence of) 180 $\mu$M unlabelled L-MTrp and D-MTrp

| Condition | IAOX formation sp act (fkat/mg protein) | % of control |
|---|---|---|
| Control | 12 5 | 100 |
| +D-MTrp | 12 3 | 98 4 |
| +L-MTRP | 9 3 | 74 4 |

*Conversion of tryptophan and MTrp by the same enzyme system?*

Feeding of [$^{14}$C] tryptophan and unlabelled L-MTrp simultaneously to the plasma membranes led to a competitive type of inhibition (Fig 4) The $K_i$ value was determined as 4 5 mM which is not identical with the $K_m$ value for DL-MTrp of 6 6 $\mu$M Double label experiments with [$^3$H]tryptophan and [$^{14}$C] MTrp suggested that the formation of IAOX must take place at the same active site of the enzyme Fifty-six per cent of the total radioactive IAOX was found after an incubation time of 1 hr in the $^{14}$C-fraction, and 44% in the $^3$H-fraction compared to the IAOX formation when both substrates were fed at the same concentrations separately (Table 2)

*Conversion of D-tryptophan by microsomal membranes of Chinese cabbage seedlings*

Feeding [$^{14}$C] DL-tryptophan to phase partitioned membranes of Chinese cabbage leads to a reduced IAOX

Table 2. Double label experiment with [$^3$H]tryptophan (sp act 46 mCi/mmol) and [$^{14}$C]MTrp (sp act 50 $\mu$Ci/mmol)

| Condition | % Radioactivity of IAOX found in | |
|---|---|---|
| | $^{14}$C-fraction | $^3$H-fraction |
| [3H]Tryptophan | 0 | 100 |
| [$^{14}$C]MTrp | 100 | 0 |
| [$^3$H]Tryptophan + [$^{14}$C]MTrp | 56 | 44 |

Phase partitioned membranes from Chinese cabbage seedlings were incubated for 1 hr with 25 kBq [$^3$H]tryptophan and 0 25 kBq [$^{14}$C]MTrp.

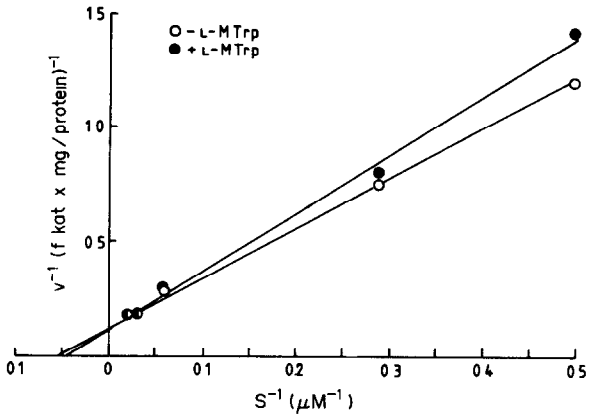


Fig 4 Lineweaver–Burk plot of substrate kinetics for [$^{14}$C] L-tryptophan with simultaneously feeding of L-MTrp at 1 mM substrate concentration The correlation coefficient for both curves after linear regression was 0 99

formation of *ca* 40% compared to L-tryptophan alone. Obviously only the L-isomer of tryptophan is converted to IAOX in Chinese cabbage

## DISCUSSION

L-Tryptophan is always believed to be the primary precursor of IAA in the most important pathways of IAA biosynthesis [8], whereas MTrp is only thought to be a storage or regulatory product of the free amino acid [4], but there were hints that MTrp has auxin activity and that *in vivo* IAA was formed when MTrp was fed to soybean cells [5]. The *in vivo* metabolism of [$^{14}$C]DL-MTrp in Chinese cabbage seedlings revealed three $CH_2Cl_2$-soluble products· IAOX, IAN, and IAA. These three reaction products were also found after incubation of 30-day-old root segments of *Brassica napus* cv. swede [9]and of five-day-old hypocotyls of Chinese cabbage seedlings [10] with [$^{14}$C]tryptophan, so that the pathways from DL-MTrp and L-tryptophan to IAA seem to be similar Further *in vitro* studies have recently shown that a plasma membrane bound enzyme system, which is described elsewhere [7], is able to convert DL-MTrp to IAOX. In order to test which isomer of MTrp is preferred, unlabelled D-and L-MTrp were fed simultaneously with [$^{14}$C]DL-MTrp. Until now always D-MTrp is assumed to be the only biological relevant isomer of MTrp [5, 6] The experiment showed that labelled IAOX is only reduced when L-MTrp was given additionally. This leads to the conclusion that only the L-form of MTrp is converted to IAOX and not the D-isomer. Other peptide substrates such as tryptophyl-tryptophan and leucyl-tryptophan were not accepted as substrates. No significant inhibition of IAOX formation from DL-MTrp was observed with these two compounds. So this indicates high substrate specificity for DL-MTrp.

A significant enhancement of the reaction by cofactors was not reproducible, but a slight enhancement with hydrogen peroxide in the incubation medium was observed (Ludwig-Müller, J., unpublished results). The reaction mechanism of IAOX formation from DL-MTrp is not yet clear As it was shown, no specific cofactors are needed for IAOX formation from MTrp, whereas $H_2O_2$, $Mn^{2+}$ and 2,4-dichlorophenol enhanced IAOX formation from tryptophan. Preliminary results showed a partial $O_2$-dependence for the conversion of MTrp to IAOX. Under anaerobic conditions ($N_2$) IAOX formation was reduced by *ca* 35%. It is suggested that oxidation of MTrp is catalysed by $O_2$ and/or membrane lipid peroxides presumably formed during membrane preparation [11]. Inhibition studies with [$^{14}$C]L-tryptophan and unlabelled L-MTrp showed a competitive type. Thus, the recently described enzyme system converting tryptophan to IAOX [7] and the system described in this paper could be the same (Fig. 4) As the $K_i$ value was 4.5 mM (compared to a $K_m$ for DL-MTrp of 6.6 $\mu$M) it is not so clear whether it is really the same enzyme system. Additional double label experiments with [$^3$H]L-tryptophan and [$^{14}$C]DL-MTrp support the statement for an identical active site of the enzyme. The values show no additional IAOX-formation in the total radioactive fraction, but a 14:11 ratio between [$^{14}$C]IAOX and [$^3$H]IAOX after an incubation time of 1 hr. Probably these results can also be explained as a competition for $O_2$ and peroxides which are obviously necessary for both reactions (Table 3). Different cofactors and also different pH-optima and kinetic behaviour (Table 3) suggest that the conversion of L-tryptophan and DL-MTrp to IAOX is catalysed by two different enzymes which are both located on the plasma membrane The broad pH-optimum can be rationalized considering the processes presumably taking place during IAOX formation which are (i) oxidation, (ii) splitting of the peptide bond and, possibly, (iii) trans-membrane transport The $K_m$ values for DL-MTrp at pH 5 and 8 do not differ very much, but the difference from the $K_m$ value for L-tryptophan is significant (Table 3).

MTrp is probably better converted to IAOX because of its higher stability at lower pH. Chemical oxidation of

Table 3 Comparison of the conversion of MTrp and tryptophan to IAOX by phase partitioned membranes of Chinese cabbage seedlings

| Feature | Tryptophan | MTrp |
|---|---|---|
| pH-Optimum | 8–9 | 5–8 |
| $K_m$-value pH 5.0 | — | 6 6 $\mu$M |
| pH 8.0 | 20 $\mu$M | 6 25 $\mu$M |
| Cofactors | $H_2O_2$, $MnCl_2$, 2,4-dichlorophenol | $H_2O_2$ (?) |
| Time course max | > 5 hr | 4 hr |
| $O_2$-Dependence | yes | yes |

tryptophan is only possible in the non-protonated form of the amino nitrogen. The $pK_a$ value of tryptophan is 10, so that only above pH 8 the amino acid is in the non-protonated form. That was shown with oxidation kinetics of tryptophan by sodium hypochlorite [12] These results agree with the pH optimum of 8–9 for the conversion of tryptophan to IAOX (Table 3) by microsomal membranes The properties of the peptide bond (MTrp) can be described as (i) the C–N bond is a single bond with no overlap between the nitrogen's lone pair and the carbonyl carbon and (ii) a double bond between the amino nitrogen and the carbonyl carbon, where the nitrogen atom bears a charge of + 1 ($3 < pH < 4.5$) [13], so that chemical oxidation of MTrp is possible above pH 3. If the enzyme system is located on the outer plasma membrane [7], these results are probably relevant for the *in vivo* situation (low pH in the cell wall compartment)

It is not yet clear when the malonyl group is released, but it was shown that the investigated membranes contain peptidase activity After incubation of 500 $\mu$l membrane suspension with 1 mM tryptophyl-tryptophan, a liberation of tryptophan after 1 hr of 23% was observed. When D- and L-MTrp were fed in separate experiments to the membranes, both substrates led to formation of tryptophan. Feeding of L-MTrp led to tryptophan formation of 29%, whereas tryptophan release from D-MTrp was only 8%, which means higher affinity of the peptidase for the L-isomer (Ludwig-Muller, J , unpublished results) Hydrolysis of DL-MTrp to tryptophan by a membrane bound peptidase activity before oxidation of MTrp to IAOX does not seem to be involved in IAOX formation from 5000 Bq L-tryptophan *ca* 33 3 Bq IAOX can be formed during 1 hr incubation time This corresponds to 0.6% of the total radioactivity fed to the membranes In the same incubation time *ca* 17 Bq IAOX were formed from 500 Bq DL-MTrp Maximum tryptophan release by the peptidase was determined as *ca* 30%, 30% of 500 Bq MTrp is *ca* 166 Bq tryptophan from which only 0 6% are converted to IAOX, which is in our example 1 Bq That corresponds to the radioactive background determined in each experiment Thus, we are measuring the IAOX formed from MTrp and not that from tryptophan release.

It was proposed that D-tryptophan could be an IAA precursor [4] and that regulation of IAA biosynthesis should be via conversion of L-tryptophan to D-tryptophan by a racemase, while D-MTrp should be the storage form A malonyl transferase which converts 1-aminocyclopropane-carboxylic acid (ACC) to malonyl-ACC as storage form [14] has been recently described Malonyl-ACC is converted back to ACC for the synthesis of ethylene when needed [15] There was evidence for the conversion of L-tryptophan to D-tryptophan in cabbage, but it was not believed to be relevant for IAA biosynthesis [16] After feeding a mixture of D- and L-tryptophan to microsomal membranes of Chinese cabbage, we found only half of the IAOX formation in comparison to only L-tryptophan as substrate In our system D-tryptophan is not metabolized so that we can only assume a role as an intermediate in the conversion of L-tryptophan to MTrp and reverse, but not as an IAA precursor itself Law [4] found a conversion of [$^{14}$C]D-tryptophan to MTrp, IAA and IAAsp in pea. Phase partitioned plasma membranes from pea showed a conversion of DL-MTrp and L-tryptophan to IAOX, but not of D-tryptophan (Ludwig-Muller, J , unpublished results).

Probably MTrp has a more general role as IAA precursor (not only as a storage form), because we found a conversion of MTrp to IAOX not only in Chinese cabbage but also in pea. The conversion of tryptophan to IAOX has also been found in other plant species, e.g. maize, sunflower, tobacco, and pea [7] All membranes were prepared by aqueous two phase partitioning and are rightside out. Thus, it is possible that the system converting DL-MTrp to IAOX is widely distributed in the plant kingdom.

## EXPERIMENTAL

*Chemicals* L-[methylene-$^{14}$C]Tryptophan, sp act 1 98 GBq/mmol and L-[side chain-2,3-$^{3}$H]tryptophan, sp act 1 70 TBq/mol were purchased from Amersham DL-[$^{14}$C]MTrp was prepared from DL-[methylene-$^{14}$C]tryptophan, sp act 2 18 GBq/mmol (ratio D L 1 1, Amersham, personal communication) as described in the following DL-[$^{14}$C]Tryptophan (1 85 MBq) were suspended in 200 $\mu$l m KOH and mixed with equal parts of ethylchlorformylacetate (Merck) The reaction was carried out for 10 min at 100° The pH was lowered to 2 5 and MTrp was extracted twice with $Et_2O$ The combined phases were dried with $Na_2SO_4$ and directly submitted to TLC for purification Silica gel plates (Merck) with BuOH–HOAc–$H_2O$ (12 3 5) as solvent were used MTrp was localized by fluorescence quenching at 254 nm and the $R_f$ values corresponded to those given elsewhere [3] The [$^{14}$C] MTrp was eluted twice with $Et_2O$, evapd to dryness and resuspended in 1 ml $H_2O$ containing 2% EtOH and stored at −20° Further identification of MTrp was achieved by MS with a non-labelled standard prepared by the procedure described above Unlabelled IAOX was synthesized from IAAld-$NaHSO_3$ (Sigma) according to ref [17]

*Plant material* Seeds of Chinese cabbage (*Brassica campestris* subsp *pekinensis* cv Granat) were cultivated under sterile conditions in the dark at 23° and 90% humidity Seedlings were harvested for all expts after 5 days

*Feeding of* [$^{14}$C]*malonyltryptophan* The appropriate amount of [$^{14}$C]MTrp was added to 2 ml of 100 mM MES–KOH-buffer, pH 6 Hypocotyls and roots of 5-day-old seedling were cut into 2 mm segments and washed in substrate free buffer After drying the plant material on filter paper, 1 g of material was added to the feeding soln, incubation time was 1hr at 25° Further extn and identification of the reaction products were carried out according to ref [18] Radioactivity of the HPLC separated samples was determined by liquid scintillation counting

*Enzyme preparation* Phase partitioned membranes from 5-day-old seedlings were prepared as described in ref [7] For the pH-dependence study membranes were centrifuged for 45 min at 50 000 *g* and washed twice with the appropriate buffer to adjust

the pH to between 3 and 10 After the last centrifugation step the membranes were resuspended in a small vol. for the enzyme assay and the pH was controlled. For pH 3–6 20 mM triethanolamine, for pH 7–8 50 mM Tricine, and for pH 9–10 10 mM borate buffer was used.

*Enzyme assay for IAOX formation* The standard enzyme assay contained 500 $\mu$l enzyme ext and 1 $\mu$M DL-[$^{14}$C]MTrp. For substrate kinetics the substrate concn ranged from 0.4 to 20 $\mu$M. Unlabelled D- and L-MTrp were given at a concn of 180 $\mu$M. The reaction mixt was incubated for 1 hr at 30°. The reaction was stopped and the reaction mixt ext with 500 $\mu$l $CH_2Cl_2$. The $CH_2Cl_2$ phase was directly used for TLC analysis. The determination of radioactivity and the identification of IAOX were carried out as described in ref [7]. For the double label expt 1 $\mu$M DL-[$^{14}$C]MTrp (sp act 1 85 MBq/mmol) and 100 $\mu$M L-[$^{3}$H]tryptophan (sp act 1.702 Gbq/mmol) were added to the membrane prepn DL-[$^{14}$C]tryptophan and L-[$^{14}$C]tryptophan were added at 3 $\mu$M each. L-Tryptophyl-tryptophan and L-leucyl-tryptophan were used at 10 $\mu$M final concn in the inhibitory test Anaerobic conditions were obtained under a continuous flow of $N_2$ for 1 hr.

*Protein determination* was according to ref. [19], or alternatively with the BCA-protein-assay reagent (Pierce Chemical Company).

*Statistical treatment of data* All expts were repeated 3–5 times using independent enzyme prepns. Means of different independent expts are given. The $K_m$ value was calculated by linear regression analysis of the data after Lineweaver–Burk transformation The $K_i$ value was determined according to the equation for competitive inhibition according to ref [20]

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