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# Proposed Enzymes of Auxin Biosynthesis and Their Regulation II. Tryptophan Dehydrogenase Activity in Plants.

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**Abstract.** In pea, maize and tomato plants a hitherto undescribed L-tryptophan dehydrogenase activity (TDH) has been detected. This enzyme catalyzes the reversible formation of indolepyruvic acid (IPyA) from L-tryptophan (L-trp). TDH and L-glutamate dehydrogenase (GDH), related enzymes in their mode of action, could be separated by gel chromatography. Enzymatic activity of TDH was sustained by both pyridine coenzymes NAD/NADP. With pea TDH the coenzyme NAD displays, at optimum pH 8.5 and at room temperature, only about 40–70 % of the activity of NADP. The amination of IPyA is catalysed more actively than the deamination of L-trp. L-trp/IPyA, L-glu/ketoglutarate, L-ala/pyruvate reacted as dehydrogenase substrates; L-phe/ phenylpyruvate, D-trp and D-phe did not react with pea enzyme extracts. A considerable similarity between the active centres of TDH and GDH has been found using inhibitors: absence of heavy metals, presence of a carbonyl group, indispensibility of bivalent ions for the enzyme activity. Pea TDH and GDH were distinctly inhibited by sodium azide. For the activity of TDH the presence of SH groups is less important than for GDH. The TDH activity in the investigated plants was lower than the GDH activity. The possible role of TDH in the regulation of the IPyA pool is discussed.

The formation of indolepyruvic acid (IPyA) from L-trp may proceed in plants in several ways (*e.g.* Schneider and Wightman 1978, Kutáček 1985). Of these the system of L-trp-aminotransferase (TAT) is best investigated (Wightman and Cohen 1968, Truelsen 1972, Terziivanova-Dimova and Kutáček 1991). In the present study a further possible way of L-trp conversion to IPyA involving the reversible function of a pyridine nucleotide dependent dehydrogenase (TDH) is suggested.

It was assumed that only aliphatic acids may be substrates of pyridine nucleotide dependent dehydrogenases. In microorganisms, in plants and animals NAD dependent glutamate dehydrogenase (GDH) (EC 1.4.1.2.) and NADP dependent glutamate dehydrogenases (EC 1.4.1.3. and 1.4.1.4.) are well known enzymes. L-ala-dehydrogenase activity was demonstrated in microorganisms (EC 1.4.1.1.) (Krause *et al.* 1974).

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However, in our study an aromatic amino acid, *i.e.* L-trp, is reposted to serve as a substrate of a pyridine nucleotide dependent dehydrogenase in plants. Some preliminary investigations on pea TDH are described.

## MATERIAL AND METHODS

### Plant Material

Shoots of 7 to 9 d old etiolated pea plants (*Pisum sativum* L., *cv.* Jupiter), germinated under sterile conditions (Sahulka 1972), were used for preparation of enzyme extracts. Plants of maize (*Zea mays* L., *cv.* Ta 37/71 $o_2$), kohlrabi (*Brassica oleracea* var. *gongyloides* L., *cv.* Moravia) and tomato (*Lycopersicon esculentum* L., *cv.* Stupické field), cultivated for 7–9 d in the same manner, were investigated for the enzyme activity as well.

### Preparation of the Enzyme Extract

The enzyme was extracted by a procedure similar to that used for TAT (Truelsen 1972, Terziivanova-Dimova and Kutáček 1991). The enzyme was extracted with 50 mM Tris-HCl buffer of pH 8.5 containing 1 mmol $l^{-1}$ of mercaptoethanol (the only stabilizing agent used) either from fresh plant material, using a 1:2 ratio (m/v), or from the acetone powder. TDH activity was present in the fraction precipitated in the interval of 60 to 80 % ammonium sulphate. In some cases a decrease in the activity of the ammonium sulphate fraction was observed after dialysis. Better results were obtained by proceeding, after salting out, immediately to gel filtration. The enzyme fraction was purified on a Sephadex G 200 Fine column (138 × 2.5 cm) using 50 mM Tris-HCl buffer of pH 8.5 as eluent. The protein fraction with enzyme activity was collected and lyophilized.

### Determination of the L-trp-Dehydrogenase Activity

The TDH activity was determined on the basis of the oxidation or reduction of the coenzymes NADP or NADPH:

A. In the direction of the amination of the keto acid the assay mixture consisted of 6 mmol $l^{-1}$ of IPyA, 6 mmol $l^{-1}$ of $NH_4Cl$ and 0.16 mmol $l^{-1}$ of NADPH in 50 mM Tris buffer of pH 8.5, plus 0.5 ml of the enzyme extract. In the later experiments, $Ca^{2+}$ ions (0.8 mmol $l^{-1}$ $CaCl_2$) were added to the assay (Vacková *et al.* 1985). The final volume of the sample was 3 ml. The control did not contain the substrate (IPyA). The reaction took place at room temperature, absorbance (340 nm) was read at 2 min intervals for 10 to 15 min, starting from the time of addition of the coenzyme. TDH activity was estimated in the linear phase of the enzyme reaction, usually after 5 min.

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*Abbreviations used:* TDH – tryptophan dehydrogenase; GDH – glutamate dehydrogenase; IPyA – indole-3-pyruvic acid; NAD – nicotinamide adenine dinucleotide; NADP – nicotinamide adenine dinucleotide phosphate; L-trp – L-tryptophan; L-phe – L-phenylalanine; L-ala – L-alanine; EDTA – ethylenediaminotetraacetic acid; TAT – tryptophan aminotransferase; KG – α-ketoglutarate; IAA – indole-3-acetic acid.

B. In the direction of deamination of the amino acid the assay mixture consisted of 6 mmol $l^{-1}$ of L-trp, 0.8 mmol $l^{-1}$ $CaCl_2$ and 0.16 mmol $l^{-1}$ of NADP in 50 mM Tris-HCl buffer of pH 8.5 and 0.5 ml of the enzyme extract. The final volume of the sample was 3 ml. The control did not contain the substrate (L-trp). The incubation took place at room temperature and absorbance (340 nm) was measured at 2 min interval for 15 min. The TDH activity was determined in the linear phase of the enzyme reaction, usually after 5 min.

#### Determination of the L-glu and L-ala dehydrogenases

The activity was measured both in the direction of the oxidation and the reduction of the coenzymes NADP and NADPH. The procedure was the same as for TDH.

#### Identification of the Metabolites of L-trp Dehydrogenase Activity

##### A. Detection of the L-trp formed

At the end of a 20 min incubation the assay mixture was diluted with 20 ml of ethanol. After centrifugation, evaporation, reextraction with 70 % ethanol, evaporation and final solution in 70 % ethanol the sample was analysed by means of TLC, in 3 different solvent mixtures: n-butanol-acetic acid-water 4:1:2, 3 % aqueous acetic acid, water. The UV spectrum of chromatographically isolated L-trp was compared with the standard.

##### B. Detection of the IPyA formed

IPyA in the incubation solution was stabilized by conversion to 2,4-dinitrophenylhydrazone. After purification of the toluene extract (Wightman and Cohen 1968) the hydrazone was separated by TLC in three different solvent mixtures: isopropanol-ammonia-water 20:1:2, 3 % ammonia in 2-butanol, 3 % ammonia in n-butanol. In addition to this the identity of the IPyA formed was confirmed by spectrophotometry of the borate complex ($\lambda_{max}$ at 328 nm).

#### Inhibitors and Activators of L-glu and L-trp Dehydrogenases

A series of inhibitors (KCN, $CuSO_4$, $AgNO_3$, EDTA, p-chloromercuribenzoate, sodium azide) were tested for their effect on TDH and GDH. $Ca^{2+}$, $Mg^{2+}$, $Mn^{2+}$ ions were tested as TDH and GDH activators, at a 1 mmol $l^{-1}$ concentration. The activating effect of the above ions was determined in TDH inhibited with 6 mmol $l^{-1}$ EDTA. The ions were added after 5 min of the enzymatic reaction, which was than followed for another 10 min.

**Determination of Proteins**

The proteins in the enzyme extracts were determined with Coomassie Brilliant Blue G 250 (Bradford 1976).

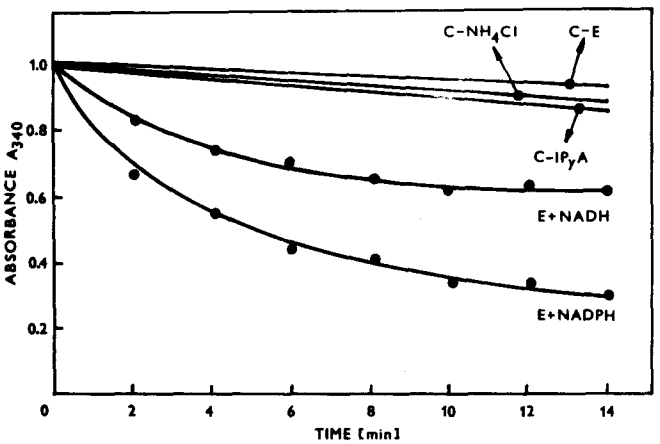


Fig. 1. Activity of L-tryptophan dehydrogenase from pea. Amination with coenzymes NAD(P)H. Assays and control with omitted substrates.

## RESULTS

**Characteristics of the Enzymatic Activity**

The reaction of TDH took place in both directions: in the presence of oxidized coenzyme NADP deamination of L-trp was observed, while in the presence of reduced coenzyme NADPH amination of IPyA took place (Fig. 1, 2). When denatured by boiling, the enzyme extract was no longer active. With increasing enzyme concentration TDH catalytic activity increased.

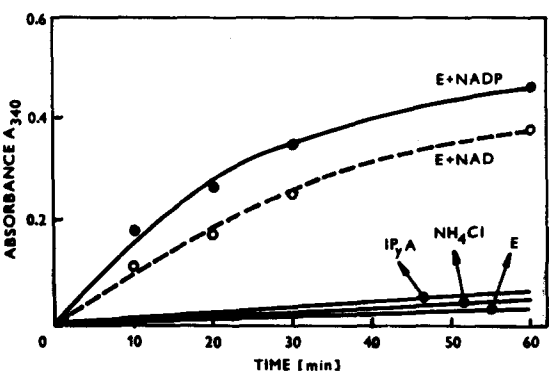


Fig. 2. Activity of L-tryptophan dehydrogenase from pea. Deamination with coenzymes NAD(P). Assays and controls with omitted substrates.

The TDH activity is NADP dependent (Fig. 1, 2). Coenzymes NADP and NADPH catalyzed the reversible reaction more actively; after 5 min NAD and NADH achieved only 40 % and 70 % of the conversion catalyzed by NADP and NADPH. The deamination of the amino acid took place less intensively than the amination of the keto acid, on average only 21 % of the amination activity after 5 min. Still, the asymptotic course in the keto acid amination was reached within 12–14 min, while the asymptotic course of the amino acid deamination was attained after 60 min. The effect of coenzyme concentration of the activity of the reaction is evident from Fig. 3.

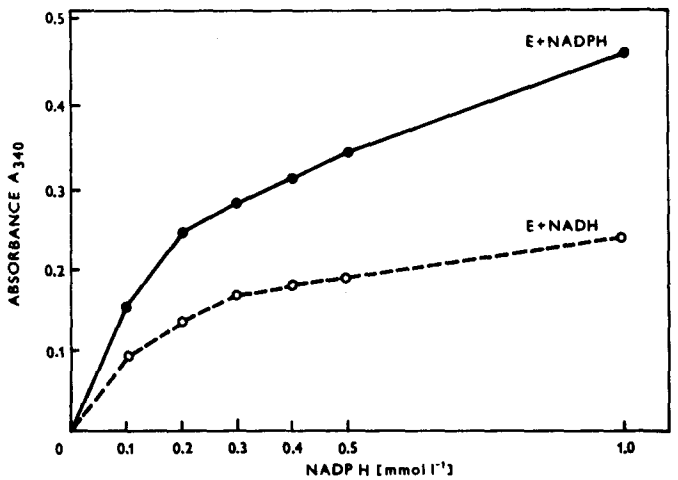


Fig. 3. Effect of the concentration of the coenzymes NAD(P)H on the activity of L-tryptophan dehydrogenase from pea.

The optimum pH in the amination of IPyA in the presence of both coenzymes NAD(P)H was 8.5.

#### Separation of L-trp-Dehydrogenase Activity by Gel Chromatography

In gel chromatography on a Sephadex G 200 Fine column TDH was eluted with the high molecular mass protein peak, closely followed by the activity maximum of GSH. The TAT activity was eluted later (Fig. 4).

#### Substrate Specificity

It was observed that the enzyme extract from pea catalyzed the reversible dehydrogenation of the following pairs of amino/keto acids: L-trp/IPyA, L-glu/ketoglutarate (KG). The enzyme extract did not react with L-phe/phenylpyruvate, D-trp and D-phe. The L-ala-dehydrogenase activity was found in the pea enzyme extract as well. With pyruvate as the L-ala-dehydrogenase

substrate the enzymatic activity was relatively high (106 % of the TDH activity with IPyA as substrate), while in the direction from L-ala as substrate it was lower (only 43 % of the TDH activity with L-trp as substrate).

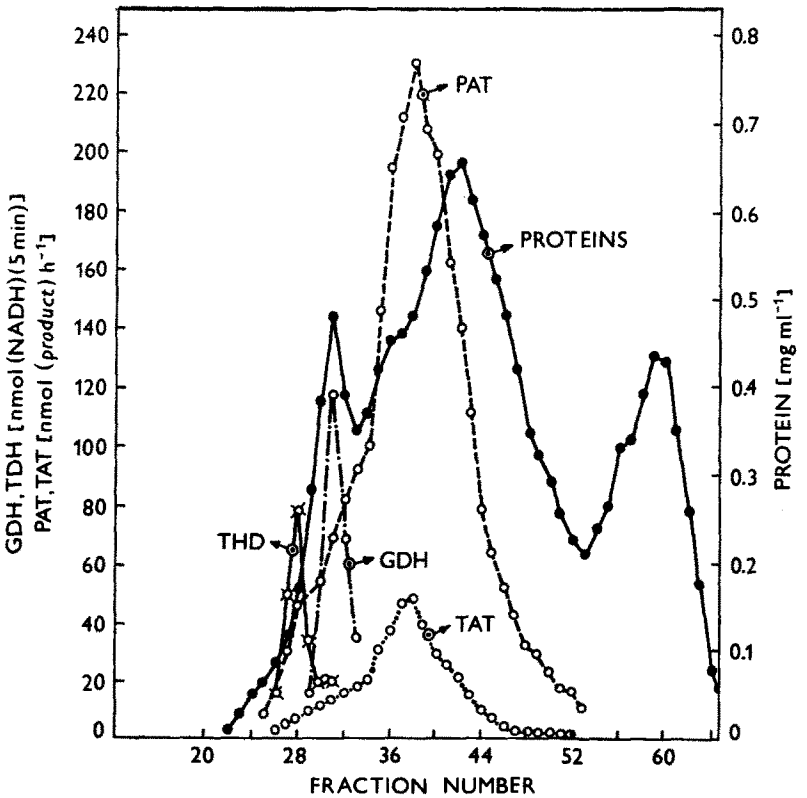


Fig. 4. Separation of L-tryptophan (TDH) and L-glutamate (GDH) dehydrogenases and L-tryptophan aminotransferase (TAT) from pea by means of gel chromatography on a Sephadex G 200 Fine column (138 × 2.5 cm).

**Identification of the Metabolites of L-trp Dehydrogenase from Pea**

The formation of L-trp as a metabolite was confirmed by comparison with a standard, using TLC in three solvent systems, and also by the UV spectrum of a chromatographically purified product.

On the chromatogram of the products of deamination of L-trp in experiments with both coenzymes NAD and NADP the spot of IPyA hydrazone was evident (Fig. 5). The formation of IPyA was also confirmed by the UV spectrum of its borate complex.

**The Effect of Inhibitors; Comparison of L-trp and L-glu Dehydrogenases**

The effect of inhibitors was investigated in the following concentration ranges: $10^{-3}-10^{-7}$ M for TDH and $10^{-5}-10^{-7}$ M for GDH (Table 1). The effect of the

selected inhibitors on the TDH and GDH activity was comparable. Sodium azide caused a strong inhibition of both enzymes. TDH inhibited by sodium azide could be reactivated up to 80 % by dialysis.

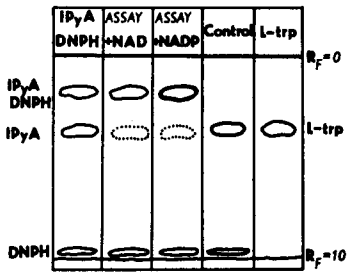


Fig. 5. Determination of indolylpyruvate (IPyA) stabilized as 2,4-dinitrophenylhydrazone, the product of L-tryptophan deamination by L-tryptophan dehydrogenase from pea plants by TLC on cellulose layer. Solvent: 2 % $NH_4OH$ in 2-butanol.

TDH activity was inhibited by p-chloromercuribenzoate only slightly, while GDH activity more intensively. 8-hydroxyquinoline was inactive. Both enzyme activities were inhibited by KCN, maleic acid hydrazide and isonicotinic acid hydrazide (Table 1).

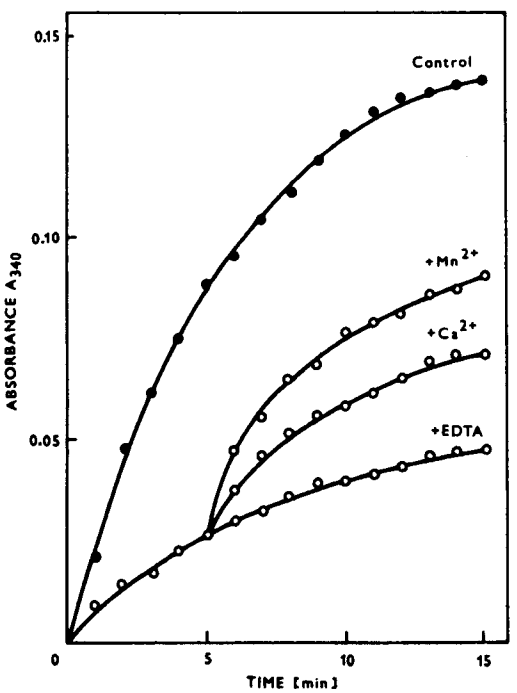


Fig. 6. Effect of EDTA (6 mm) on the activity of L-tryptophan dehydrogenase and the reactivation of the enzyme with $Mn^{2+}$ and $Ca^{2+}$ cations (1 mM); coenzyme NADH; the ions were added after 5 min of the enzyme action.

TABLE 1.

Effect of inhibitors (I) in various concentrations (c) on the L-tryptophan dehydrogenase (TDH) and on the L-glutamate dehydrogenase (GDH) activities in % of the control

| | I | KCN | | 8-hydroxy-chinoline | | EDTA | | $CuSO_4$ | | $AgNO_3$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | c | NADH | NADPH | NADH | NADPH | NADH | NADPH | NADH | NADPH | NADH | NADPH |
| TDH | $10^{-3}$ M | − 52.14 | − 50.03 | − 20.83 | − 12.40 | − 89.33 | − 89.50 | − 58.33 | − 62.50 | − 80.40 | − 87.00 |
| | $10^{-4}$ M | − 20.83 | − 21.87 | − 8.33 | − 3.12 | − 70.00 | − 74.00 | − 25.00 | − 20.75 | − 72.18 | − 65.47 |
| | $10^{-5}$ M | − 9.17 | − 12.50 | 0 | 0 | − 52.82 | − 46.15 | − 12.50 | − 9.07 | − 33.33 | − 45.00 |
| | $10^{-6}$ M | − 3.05 | − 7.50 | 0 | 0 | − 16.15 | − 11.80 | − 7.35 | − 4.20 | − 8.33 | − 6.25 |
| | $10^{-7}$ M | + 1.77 | + 0.98 | 0 | 0 | − 5.96 | − 2.57 | − 2.00 | 0 | + 4.47 | + 3.00 |
| GDH | $10^{-5}$ M | − 18.40 | − 23.77 | − 11.54 | − 7.20 | − 55.00 | − 50.11 | − 35.00 | − 28.20 | − 29.62 | − 20.00 |
| | $10^{-6}$ M | − 6.52 | − 9.03 | 0 | 0 | − 37.50 | − 32.40 | − 11.67 | − 9.11 | − 12.50 | − 8.23 |
| | $10^{-7}$ M | − 2.12 | − 4.10 | 0 | 0 | − 2.08 | − 1.29 | − 4.17 | − 1.27 | − 4.67 | − 2.14 |

TABLE 1.

(continued)

| | I | p-chloromercuri benzoate | | malein-hydrazide | | isonicotin-hydrazide | | $NaN_3$ | |
|---|---|---|---|---|---|---|---|---|---|
| | c | NADH | NADPH | NADH | NADPH | NADH | NADPH | NADH | NADPH |
| | $10^{-3}$ M | –26.67 | –20.50 | –75.53 | –80.10 | –55.00 | –61.20 | – | – |
| | $10^{-4}$ M | –16.67 | –11.25 | –60.34 | –72.31 | –42.90 | –76.56 | – | – |
| TDH | $10^{-5}$ M | –10.15 | –7.03 | –46.15 | –46.15 | –38.46 | –35.90 | –87.50 | –91.30 |
| | $10^{-6}$ M | –5.48 | –2.50 | –11.54 | –13.46 | –15.39 | –20.51 | –70.00 | –84.32 |
| | $10^{-7}$ M | 0 | 0 | –1.75 | –3.25 | –3.85 | –2.56 | –40.00 | –58.12 |
| | $10^{-5}$ M | –58.40 | –50.90 | –45.84 | –31.02 | –39.56 | –30.00 | –79.17 | –61.11 |
| GDH | $10^{-6}$ M | –45.31 | –37.03 | –28.00 | –22.10 | –19.17 | –17.00 | –64.59 | –50.01 |
| | $10^{-7}$ M | –20.55 | –17.20 | 0 | 0 | –4.17 | 0 | –55.00 | –42.00 |

TABLE 2.

Comparison of L-tryptophan dehydrogenase (TDH) and L-glutamate dehydrogenase (GDH) specific activities [μmol $l^{-1}$ (substrate) $mg^{-1}$ (protein) $min^{-1}$] in different plants.

| PLANT MATERIAL | TDH | | GDH | | GDH/TDH | |
|---|---|---|---|---|---|---|
| | NADH | NADPH | NADH | NADPH | NADH | NADPH |
| *Pisum sativum (Fabaceae)* | 52 | 70 | 155 | 120 | 2.98 | 1.71 |
| *Zea mays (Poaceae)* | 45 | 64 | 182 | 164 | 4.04 | 2.56 |
| *Solanum lycopersicum (Solanaceae)* | 28 | 39 | 140 | 122 | 5.00 | 3.13 |
| *Brassica oleracea* var. *gongylodes (Brassicaceae)* | – | – | 135 | 100 | – | – |

**Inhibition of L-trp Dehydrogenase with EDTA, Reactivation with Bivalent Ions**

EDTA inhibited both the TDH and the GDH activity. The addition of $Ca^{2+}$ and $Mn^{2+}$ ions (1 mmol $l^{-1}$) decreased the inhibition of TDH with EDTA (6 mmol $l^{-1}$). The $Mg^{2+}$ ions were not active under the conditions of the experiment (Fig. 6).

**Activities of L-trp and L-glu Dehydrogenase in Individual Plants**

The specific activities of TDH and GDH were compared in four plants belonging to various families (Table 2).

The following results have been achieved: a) in the case of TDH from all of the plants investigated the coenzymes NADP and NADPH were more active. The opposite is true of GDH, where the coenzymes NAD and NADH were preferred, b) in all investigated plants GDH was more active than TDH, c) the most active TDH was found in pea and the most active GDH in maize, d) in plants of the *Brassicaceae* family no TDH activity could be detected, only GDH was active.

## DISCUSSION

The possibility of an alternative or parallel enzyme system, complementary to L-trp aminotransferase, has remained open over the whole period of the investigations of auxin biosynthesis.

Thus, for example, on the basis of indirect observations Muir and Lantican (1968) envisaged a possible deamination of L-trp by a flavin-enzyme of the amino acid oxidase type, occuring in bacteria and animals; the occurrence of which in plants is improbable (Davies *et al.* 1964).

Our experimental data indicate the existence of a pyridine nucleotide dependent dehydrogenase activity (TDH) which reversibly converts L-trp to

IPyA the presence of which was confirmed in several plants species of different families. From the studies with various inhibitors following conclusions might be drawn about the structure of TDH active centre: a) it does not contain a heavy metal (inactivity of 8-hydroxyquinoline); b) the SH groups are not of

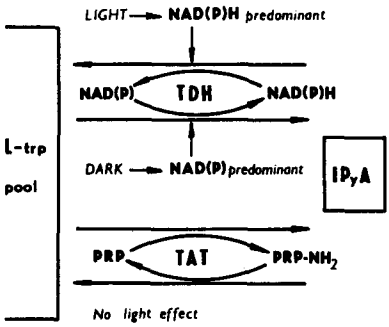


Fig. 7. Scheme of the coupled effect of L-tryptophan aminotransferase (TAT) and L-tryptophan dehydrogenase (TDH) in etiolated (dark) and photosynthesizing plants (light).

great importance (*p*-chloromencuribenzoate was only slightly inhibitory); c) carbonyl group is of importance (inhibition by KCN, hydrazides of maleic and isonicotinic acids); some ions, especially bivalent interacting with EDTA might be of importance for TDH function. These conclusions together with kinetic data of TDH are similar to those of GDH. A characteristic trait of GDH activity is its cooperation with bivalent cations. This is true also for TDH: the pea enzyme was inhibited by EDTA and reactivated by $Ca^{2+}$ and $Mn^{2+}$, for spinach TDH activation by $Ca^{2+}$ was demonstrated in both directions of catalysis (Vacková *et al.* 1985). However, differences have been observed which show that TDH might be a separate enzyme. TDH is activated preferentially by the coenzyme NADP, less so with NAD, while for GDH the opposite is true. TDH shows a lower dependence on SH groups than GDH. The separation on a Sephadex G 200 column showed that TDH has a relative molecular mass higher than $M_r$ = 200 000, differing only little from that of GDH, but considerably from that of TAT.

The occurence of TDH activity was confirmed in a series of model plants: pea, maize, tomato as well as in *Prosopis juliflora* (Ebeid *et al.* 1985). The plants of the *Brassicaceae* family are exceptions, here TDH activity could not be detected, unlike the activity of GDH. In plants of this family the activity of L-trp aminotransferase could not be detected either (Terziivanova-Dimova and Kutáček 1991). This indicates that the biosynthetic pathway for IAA has to be different in these plants (Kutáček and Kefeli 1968, Kutáček and Králová 1972, Mahadevan and Stowe 1972).

As expected, TDH can coact in the first step of auxin biosynthesis, in close connection with TAT. The basic problem in the regulation of IAA synthesis seems to exist in the maintainance of the concentration gradient between the

metabolic pool of amino acid L-trp and the hormonal pool of IAA (Terziivanova-Dimova and Kutáček 1991).

We suggest that the role of TDH is in the regulation of the IPyA level in plants (Fig. 7). We consider as relevant the higher activity of the enzyme in amination of IPyA than in deamination of L-trp. This preference of the keto acid as substrate is also known for GDH. As further possibility in the regulation of TDH activity could be the ratio of oxidized to reduced pyridine nucleotide coenzymes. This ratio depends *in vivo* on different factors, *i.e.* light, which has a known effect on the IAA level in plants (*e.g.* Suzuki *et al.* 1981).

The investigation of TAT and TDH cellular compartmentation has shown that the maximal activity of both enzymes is connected with the organelle fraction, namely that of chloroplasts (unpubl. results). The TDH activity of the cytosol enzymes was weak. In contrast TAT activity in chloroplasts was much less distinct (Vacková *et al.* 1985). Thus the TDH activity may contribute to IAA synthesis in plastids which represent an important site of IAA synthesis (Heilmann *et al.* 1981a,b, Sandberg *et al.* 1982).

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