Enzymology and regulation of the atropine metabolism in pseudomonas putida

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CHAPTER 6

THE TROPIC ACID DEHYDROGENASE

6.1 INTRODUCTION

The identification of phenylacetic acid as metabolite of tropic acid (chapter 4) made it possible to search directly for the enzymes involved. The conversion of tropic acid in phenylacetic acid could be accomplished by oxidation and decarboxylation, leading in three steps to an elimination of the hydroxymethyl group in the aliphatic side chain of tropic acid (see annex 1). Therefore, the extract of Pseudomonas PMBL-1 cultivated in the presence of tropic acid was investigated for the presence of enzymes that could accomplish a dehydrogenation or decarboxylation of tropic acid. Initial experiments failed to find an enzymatic decarboxylation, but pointed in the direction of a tropic acid dehydrogenase. The extract was able to convert the hydrogen acceptor NAD^+ in NADH upon addition of tropic acid to the reaction mixture. The reduction of NAD^+ was not observed if the extract was previously heated or treated with a proteolytic enzyme (subtilisin or pronase). This indicated that a protein was the responsible component. The catalytic activity was absent in an extract prepared from PMBL-1 cultivated with phenylacetic acid, tropine or glucose as carbon source. This suggested the role of an inducible enzyme.

This chapter describes the investigation of the enzymatic dehydrogenation of tropic acid and the tropic acid dehydrogenase (TDH) involved. Once the conditions were established for a reproducible and quantitative assay of enzyme activity, a purification system was set up (6.3). The enzyme was partially purified (38 x). The stoichiometry of the dehydrogenation of tropic acid, the substrate specificity and several other properties of TDH were investigated with the purified enzyme (6.4).

A comparison was made between the pH dependence of a non-purified and a purified TDH sample that showed the same amount of enzyme activity at pH 9.5. At neutral pH the production of NADH by the purified enzyme was much less than that of the non-purified enzyme. Moreover, the kinetics in the case of the purified enzyme was abnormal (biphasic). The NADH production was fast at the start but after a short period it leveled off to slow. This phenomenon was studied in detail (6.5). The kinetics can be explained by the assumption that the NAD^+ dependent dehydrogenation of tropic acid is a reversible process and that, in the presence of TDH enzyme in the amount added in those experiments, the equilibrium was

reached quickly. The fast NADH production might correspond to the amount of NADH that is formed at the establishment of the equilibrium:

$$\text{tropic acid} + NAD^+ \overset{\text{TDH}}{\leftrightarrows} \text{2-phenylmalonic semi-aldehyde (pma)} + NADH + H^+$$

The following slow NADH production might be the result of the decarboxylation of pma accompanied by a shift in the equilibrium. This equilibrium theory has been confirmed by studying the effect of tropic acid, NAD^+, pma, NADH and H^+ on the establishment of the equilibrium in the presence of TDH and by demonstrating the enzymatic NADH-dependent hydrogenation of pma, the so- called back-reaction.

The experiments in which pma was added, did not agree quantitatively with the experiments that started with tropic acid and NAD^+. This led to an extensive study of the back-reaction (6.6).

The hydrogenation of pma also takes place according to biphasic kinetics. This kinetics appeared to be related to the fact that the pma added is predominantly in the enol form, whereas only the keto form of 2 phenylmalonic semi-aldehyde (keto-pma) can be hydrogenated by the TDH. So, at the start of the hydrogenation only that part of pma is converted, that is presented as keto-pma. Thereafter the rate of the hydrogenation is controlled by the rate of the tautomeric rearrangement from enol-pma into keto-pma.

To confirm the hypothesis that the rate of decarboxylation of pma is the rate-limiting step of the slow NADH production once the equilibrium has been reached, a calculation was made of the rate constant for a possible decarboxylation step (6.7). This value has been compared with the experimentally found rate constants for the spontaneously decomposition of enol-pma and keto-pma respectively. It was concluded that the decomposition of enol-pma proceeds too slowly to explain the slow production of NADH. In contrast, the decarboxylation of keto-pma occurs with a speed that corresponds with the slow NADH production.

The effect of keto-enol tautomerism on the establishment of the equilibrium will be discussed in 6.8. The equilibrium constant K_{eq} can be calculated:

$$K_{eq} = \frac{[\text{pma}]\,[\text{NADH}]\,[H^+]}{[\text{tropic acid}]\,[NAD^+]}$$

K_{eq} appears to be very small, i.c. $\pm\ 0.3 \times 10^{-12}$ M. In experiments dealing with the addition of enol-pma, a different value for K_{eq} has been found. This is attributed to the unsuitability of enol-pma to act as substrate for the enzymatic hydrogenation.

6.2 QUANTITATIVE ASSAY OF TROPIC ACID DEHYDROGENASE

A large number of dehydrogenases transfer the hydrogen that is withdrawn from the substrate to the cofactor nicotinamide-adenine-dinucleotide (NAD^+). This holds for tropic acid dehydrogenase TDH as well. By the action of this enzyme, oxidized tropic acid (2-phenylmalonic semi-aldehyde, pma), the reduced cofactor NADH and a proton are formed as products (fig 6.1):

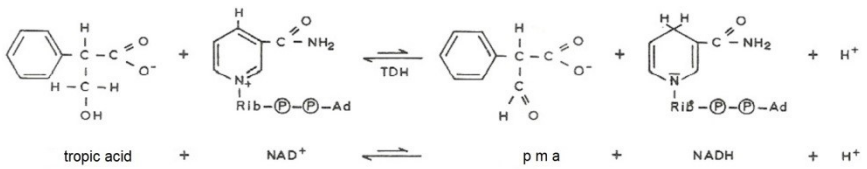


Fig 6.1

The ultraviolet spectrum of NADH differs from the spectrum of NAD^+: NADH has an absorption maximum at 340 nm, whereas NAD^+ does not absorb at this wavelength (Ciotti 1957). Since tropic acid, pma and phenylacetaldehyde do not absorb at this wavelength either, one can use the absorption increase at 340 nm as a measure for NADH production.
From the molar absorption coefficient of NADH at 340 nm, one can calculate, that an increase of absorption of 2.07 units corresponds with the formation of 1 µmol NADH under reaction conditions used (see 2.9.3). This spectrophotometric method has been used both in the investigation of the properties of the TDH as in the quantitative assay of the TDH activity.

The standardized assay of TDH activity was carried out with 10 mM tropic acid, 0.8 mM NAD^+ and 250 mM hydrazine in 33 mM K-carbonate buffer pH 9.5. Tropic acid and NAD^+ are present in excess. The enzyme does not show substrate inhibition at this tropic acid concentration. The choice of pH will be motivated in 6.4.

Hydrazine is added to remove the pma to prevent it to hinder the enzymatic dehydrogenation (see 6.5 and 7.2). In addition, the hydrazine acts with phenylacetaldehyde that can be formed by spontaneous decarboxylation of pma. In that way, it also prevents a possible disturbance of the TDH assay by removing the substrate for an NAD^+ dependent phenylacetaldehyde dehydrogenase that might be present in the extract as well.

Under standardized conditions, the enzyme activity is directly proportional with the amount of enzyme added. (fig 2.2)

6.3 PARTIAL PURIFICATION OF THE TDH

The TDH was purified partially according to the method as described in 2.11.2; compared with the starting material 38x with a yield of 18%. Table 6.2 gives a survey of the various purification steps.

In the enzymological investigations, TDH enzyme samples were used with a specific activity of ± 20. The enzymes 2-phenylmalonic semi-aldehyde decarboxylase and phenylacetaldehyde dehydrogenase could not be detected in these samples with the methods in 2.9.3. The samples were, however, contaminated with AtrE. This should be taken in account in the interpretation of the substrate specificity.

During the gel filtration over Sephadex G-100, the TDH had the behavior of a protein with a molecular weight of 46.000 (2.11.3). After iso-electric electrophoresis an isoelectric point of 5.2 was found in a pH gradient of LKB "Ampholytes".

Table 6.2

Partial purification of the TDH of PMBL-1

Sample	TA	yield	SA	Purification
Extract	4.6	100%	0.56	1x
After ammonium sulfate precipitation	2.2	48%	5.2	9x
After Sephadex G-100 gel filtration	1.58	34%	8.3	15x
After DEAE cellulose chromatography	0.82	18%	21.4	38x

The enzyme activity and the protein concentration were measured according to 2.9. TA in 10^3 U; SA in U/mg protein.

6.4 PROPERTIES OF THE PURIFIED ENZYME

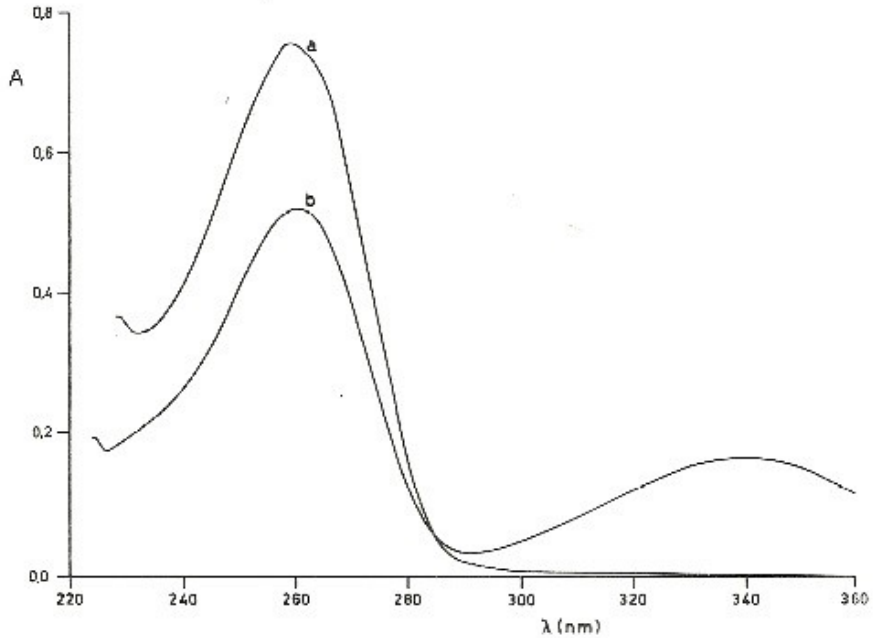
6.4.1 The analysis of the reaction products and the stoichiometry of the reaction

Pma, NADH and H^+ are expected as reaction products in the case of the NAD^+ dependent enzymatic dehydrogenation of tropic acid. The formation of NADH during the dehydrogenation is evident from the change in the spectrum during the incubation of tropic acid, NAD^+ and TDH (fig 6.3) [The effect of added NADH on the dehydrogenation under influence of TDH (6.5) and the ability of the reaction product to act as a H-donor for LDH (7.2) are additional arguments for the identity of the compound formed which absorbs at 340 nm].

The amount of NADH formed during dehydrogenation had a stoichiometric relationship to the amount tropic acid converted. This was shown by incubation

Fig 6.3

Spectral evidence for the formation of NADH during the enzymatic dehydrogenation of tropic acid.



The incubation mixture contained 1 mM tropic acid, 0.05 mM NAD^+ and TDH in 3 ml 33 mM K-carbonate buffer pH 9.5; the absorption was measured against the incubation mixture without NAD^+.
curve a : not incubated; curve b : after 10 min incubation at 25^0.

of a limited amount of tropic acid with 1 U TDH and an excess of NAD^+ (1.6 mM). The reaction was carried out at pH 10,0 in order to achieve a complete conversion. The absorption increased very fast at this pH and reached a final value within 1-2 min. The amount of NADH formed was calculated from the increase in absorption. This corresponded with the amount of tropic acid added. Starting from 0.06, 012 and 0.24 µmol tropic acid, 0.06, 0.11 and 0.23 µmol NADH were formed respectively. This shows the stoichiometric relationship. In the presence of hydrazine, which is added in the standardized activity measurement, a complete conversion can be realized at lower pH. Under these conditions, the stoichiometric relationship can be demonstrated as well. Using 1 U TDH, an excess of NAD^+ (1.6 mM) and 0.12M hydrazine in 33 mM K-phosphate buffer pH 8.5, tropic acid in amounts of 0.10, 0.20 and 0.30 µmol produced 0.10, 0.20 and 0.29 µmol NADH.

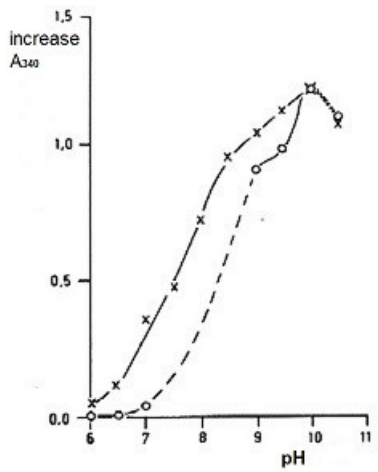
Due to its instability, the identification of pma, the conversion product of tropic acid, has been carried out in combination with the analysis of the product of the decarboxylase which will be discussed in the next chapter (7.2). In that experiment, the conversion of tropic acid in phenylacetaldehyde and carbon dioxide was demonstrated in the joint action of the TDH and the decarboxylase.

6.4.2 Effect of the pH on the enzymatic dehydrogenation.

The enzymatic conversion of tropic acid by purified TDH in neutral condition shows bi-phasic kinetics (6.5), which made it impossible to calculate the enzyme activity. Nevertheless, it was clear that the NADH production per unit of time was very small at pH 6-7 and maximal at pH 10 (see fig 6.4).

With a non-purified TDH sample, the bi-phasic kinetics was not observed in neutral condition. Moreover, the non-purified enzyme in an amount that had the same activity as a purified enzyme at pH 10, showed at pH 6-7 a much higher activity. The production of NADH was for both purified and non-purified THD samples maximal at pH10 (fig 6.4).

Fig 6.4
pH dependence of the enzymatic dehydrogenation



The reaction mixture contained 10 mM tropic acid, 0.8 mm NAD^+ in 33 mM K-phosphate buffer (pH 6.5 - 8.5) or in 33 mM K carbonate (pH 8.5 - 10.5). The enzyme activity was in either buffer nearly equal at pH 8.5.
Not-purified TDH sample x - x - x Purified TDH o - o - o
The ordinate shows the increase in absorption per min per ml enzyme.

It was not possible to assay the NADH production accurately at pH 10 or higher. At high pH-values, the phenylacetaldehyde formed by decarboxylation of pma gave rise to a considerable increase in aspecific absorption. Since TDH is also slowly inactivated at this high pH value, a pH of 9.5 is preferred for the standardized assay of TDH. The difference in reaction kinetics of the dehydrogenation for a purified and a non-purified enzyme sample will be discussed in detail in 6.5.

6.4.3 Specificity of the tropic acid dehydrogenase

Substrate specificity

A number of aromatic alcohols and hydroxyl carbonic acids (table 6.5.1, group 1) has been tested for dehydrogenation by the TDH under conditions that favor a fast-enzymatic dehydrogenation of tropic acid. However, except for tropic acid no other compound mentioned was dehydrogenated. It is therefore unlikely that the THD in the bacterium has a function in the breakdown of these compounds.

The enzyme has no function either in conversion of phenylacetaldehyde into phenylacetic acid, as is clear from the inability of phenylacetaldehyde to act as substrate for TDH (table 6.5.1 group 2). A number of aliphatic alcohols and hydroxycarboxylic acids (table 6.5.1 group 4) has been investigated, because THD might be involved in the conversion of any of these compounds. This could not be confirmed.

Apparently, the TDH is specific for tropic acid. The conclusion is justified that the enzyme in PMBL-1 is not an aspecific dehydrogenase. It is specifically involved in the breakdown of tropic acid.

Suitability of various compounds as hydrogen acceptor.

The suitability of the compounds nicotinamide adenine dinucleotide phosphate ($NADP^+$), potassium ferricyanide and dichlorophenol-indophenol sodium (DCIP) as hydrogen acceptor for TDH has been investigated.

$NADP^+$, which in its reduced form NADPH has the same spectral properties as reduced NADH, cannot be used as hydrogen acceptor to replace NAD^+. The $NADP^+$ produces only 1.1% of the absorption increase observed for NAD^+.

The compounds potassium ferricyanide and DCIP are not suitable either as hydrogen acceptor during incubation with a purified enzyme sample. Potassium ferricyanide and DCIP became reduced using a crude enzyme preparation and in the presence of tropic acid and NAD^+. This could be concluded from changes in absorption spectrum of the reaction mixture at 410 and 600 nm respectively. This reduction can be attributed fully to a “diaphorase system”, because in the absence of NAD^+ these spectral changes have not been observed.

Substrate specificity of TDH and PDH Table 6.5.1 Table 6.5.2

Substrate	TDH	PDH
Group 1: tropic acid (3-hydroxy-2-phenylpropanoic acid)	100	-
atrolactinic acid (2-hydroxy-2-phenylpropanoic acid)	0.1	0.1
3-phenyllactic acid (3-hydroxy-3-phenylpropanoic acid)	0.7	0.6
mandelic acid (2-hydroxyphenylacetic acid)	0.1	0.1
2-phenylpropanol-1 -	0.1	0.2
3-phenylpropenol -1	0.1	0.1
2-phenylethanol	0.1	0.1
benzyl alcohol	0.1	0.1
Group 2: phenylacetaldehyde	0.1	100
methyl ester pma	0.1	0.1
2-phenylpropanal	-	0.5
3-phenylpropenal	0.1	0.1
phenylglyoxyl hydrate	0.1	0.1
benzaldehyde	-	0.1
o-nitrobenzaldehyde	-	0.1
o-chlorobenzaldehyde	-	0.1
m-hydroxybenzaldehyde	-	0.1
p-nitrobenzaldehyde	-	0.1
p-dimethylaminobenzaldehyde	-	0.1
p-hydroxyl-m-methoxybenzaldehyde	-	0.1
Group 3 2-phenylacrylic acid	0.8	0.1
phenylpyruvic acid	0.2	0.2
phenylglyoxylic acid	0.1	0.1
Group 4 3-hydroxypropionic acid	0.1	0.1
lactic acid	0.1	0.2
maleic acid	0.1	0.1
succinic acid	0.1	0.1
citric acid	0.1	0.4
isocitric acid	0.1	0.1
n-decyl alcohol	0.1	0.1
n-heptyl alcohol	0.1	0.1
n-propanol	0.1	0.1
Group 5 acetaldehyde	0.1	0.1
formaldehyde	0.1	0.1
pyruvic acid-aldehyde	0.1	0.6

Table 6.5.1 Substrate specificity of the TDH
The substrates were added as a 10% solution in water or acetone to a final concentration of 3 mM. Optical active compounds were used as their racemic mixture. The TDH assay was carried out see 2.9.3 but in the absence of hydrazine. Enzyme activity with tropic acid as substrate was fixed on 100.

Table 6.5.2 Substrate specificity of the PDH
This table will be discussed in chapter 8. The substrate concentration was 3 mM. The PDH activity was assayed according to 2.9.3; enzyme activity with phenylacetaldehyde as substrate was fixed on 100. Benzaldehyde, its derivatives and 2-phenylpropanal were assayed at pH 8.0 and compared with phenylacetaldehyde as substrate at that pH.

6.4.4. The stability of the TDH

The thermostability of the TDH has been investigated at various temperatures for a purified TDH preparation in HMP (0.18 mg protein/ml). The enzyme was incubated at various temperatures during 10 minutes. These experiments showed that the TDH was inactivated completely above 55^0. After incubation at 46^0, 20% of the activity remained, the enzyme was not inactivated at 40^0. Neither addition of 0.1 M KCl, 1mM substrate nor 1mM cofactor affected the inactivation. At room temperature, the enzyme is stable during several days.

The effect of pH on the stability of the TDH has been assessed with the same sample at 25^0C. The enzyme was stable at pH 7-9 for 20 min but was inactivated at pH 6.0 and at pH 10 in 20 min for 20% and 30% respectively. In 0.1 M K-carbonate pH 11 the enzyme lost its activity nearly immediately. However, this inactivation was much slower in 10 mM tropic acid and 0.8 mM NAD^+. In the presence of these compounds, a clear NADH production was observed at pH 11, which gradually decreased. Apparently, the enzyme is partially protected by the substrate and by NAD^+ against inactivation.

6.4.5 Some other properties of TDH

The enzymatic dehydrogenation in K-phosphate buffer pH 9.5 is inhibited by NADH. The nature of this inhibition was investigated in a number of experiments with NAD^+ concentration varying from 0.2- 1.1 mM and NADH concentrations fixed on 0, 0.1, 0.2 and 0.3 mM. From the initial reaction velocities measured, it was concluded using the method of Lineweaver and Burk (1934) that NADH inhibits competitively versus NAD^+, the inhibition constant being 6.1×10^{-5} M. The effect of NADH on the dehydrogenation at neutral pH will be described in detail in 6.5.

Phenylacetaldehyde and also phenylacetic acid, which are generated during the breakdown of tropic acid, appear not to have an effect of the TDH activity in a concentration of I mM. Compounds structurally related to tropic acid like 2-phenylpropanoic acid, 2- hydroxyphenylacetic acid and phenylglyoxylic acid did not show inhibition at the same concentration.

Tropic acid dehydrogenation is presumably not coupled to phosphorylation. Adenosine diphosphate, adenosine triphosphate (0.1mM) or inorganic phosphate (1mM) did not influence the enzyme activity in an otherwise phosphate free incubation mixture.

At a concentration of neither 1 mM Mg^{++}, Ca^{++} and Mn^{++} ions nor 1 mM EDTA did have an effect on the TDH activity. Preincubation during 5 min with reagents on the –SH compounds like p-chloromercuribenzoic acid, $Hg^{++}$and Cu^{++} ions at a concentration of 0.01 mM appeared to inhibit the activity for 80-100%. Na-arsenite and iodoacetic acid (1 mM) did not inhibit.

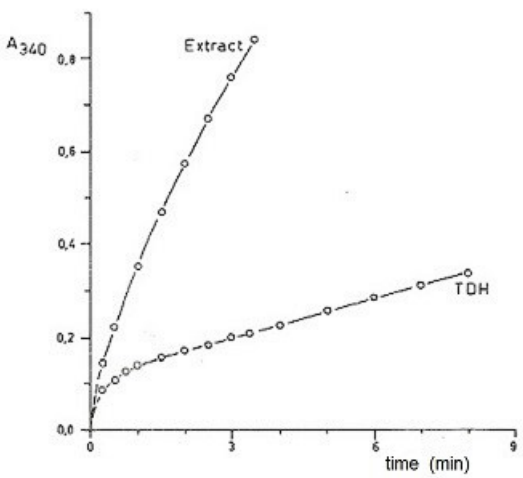
6.5 ENZYMATIC DEHYDROGENATION OF TROPIC ACID IN NEUTRAL ENVIRONMENT

In the study of the pH dependence of purified TDH samples, it came to light that during the dehydrogenation in neutral environment NADH was produced in relatively small amounts and in addition that the production of NADH had a different course than was observed with the non-purified enzyme.

The discrepancy in the dehydrogenation of tropic acid at pH 8.0 by a TDH in a crude extract versus a purified TDH sample is shown in fig. 6.6. The TDH samples used had the same activity (1.3 U/ml) when measured at pH 9.5. In the presence of the non-purified enzyme, the absorption was increasing continuously. In the dehydrogenation using the purified enzyme, a small amount of NADH was formed in an initially fast reaction. Thereafter, the NADH production suddenly decreased and changed in a slow mode. Since this phenomenon was not observed with the non-purified preparation, it seemed unlikely that the limited NADH production by the purified enzyme was due to a limiting factor in the incubation mixture.

However, it was strange that the enzyme preparation was not the limiting factor either; 10-fold more TDH appeared to have a minor effect only on the

Fig 6.6
Enzymatic dehydrogenation of tropic acid at pH 8.0



Enzymatic dehydrogenation of tropic acid in the presence of a cell free crude extract ("extract") and a purified TDH sample ("TDH"), both 1.3 U/ml. The incubation was carried out in 12 mM tropic acid, 2.0 mM NAD^+ and TDH in 2.5 ml mM K-phosphate pH 8.0.

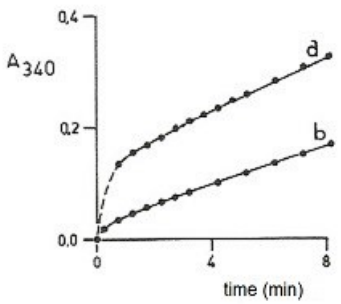
course of the NADH production: the initial absorption increase is faster in the presence of this large amount of enzyme, but the absorption at which the slow-down occurred and the slow NADH production thereafter were not affected.

The same phenomena were observed using NAD^+ sample from another supplier (Boehringer Mannheim). Addition of EDTA, metal ions, other cofactors or –SH protective agents did not change these kinetics either.

To find an explanation for the kinetics of the enzymatic dehydrogenation at the neutral pH, the effect of a number of parameters on this reaction was inquired more precisely. It appeared that addition of a small amount of NADH resulted in an obvious inhibition (fig.6.7). This indicated inhibition by one of the reaction products; however, the kinetics of the dehydrogenation could not be explained in detail.

Fig .6.7

Dehydrogenation of tropic acid by purified TDH. Effect of NADH.



curve a: 20mM tropic acid, 0.83 mM NAD^+ and TDH incubated in 3 ml 33 mM K-phosphate buffer pH 8.0

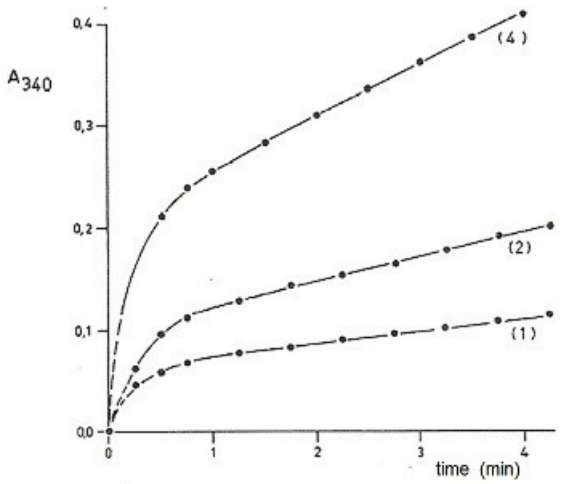
curve b: same, but now in the presence of 0.6 mM NADH; the curve is corrected for the absorption of the NADH added.

Indications for the mechanism of the enzymatic dehydrogenation in neutral environment were obtained in a study of the effect of tropic acid and NAD^+ concentrations on the kinetics of the reaction.

It appeared that both the amount of NADH produced in the initial phase as the subsequent slow NADH production became larger as the result of the increase of the tropic acid and the NAD^+ concentration. This effect is clearly demonstrated by the results of an experiment in which the tropic acid ánd the NAD^+ concentration were enhanced to the double and the fourfold value (fig 6.8). The absorption at the slow-down was about twice as high at the double concentration

of tropic acid and NAD^+. Similarly, at a fourfold amount of tropic acid and NAD^+ the slow-down occurred at a four-fold absorption value. The slow NADH production after slow- down was also in a ratio of 1 : 2 : 4.

Fig 6.8
Effect of tropic acid and NAD^+ concentration



Various amounts of tropic acid and NAD^+ incubated in 3 ml 33 mM K-phosphate pH 7.5.
(1) 10 mM tropic acid 0.83 mM NAD^+
(2) 20 mM tropic acid 1.67 mM NAD^+
(4) 40 mM tropic acid 3.33 mM NAD^+
Slow-down points (calculated by extrapolation of the initial phase and the slow phase) at 0.063, 0.106 and 0.219 respectively. Absorption increase per min after slow-down 0.012, 0.026 and 0.52 respectively.

This direct relationship between concentration of the reacting compounds and the kinetics of the dehydrogenation, the large effect of NADH (fig 6.7) and the small effect of the enzyme concentration have led to the hypothesis that the reaction underlying the enzymatic dehydrogenation of tropic acid is a reversible one and that TDH de facto catalyzes the establishment of the equilibrium:

tropic acid + NAD^+ $\leftrightarrows$ 2-phenylmalonic semi-aldehyde (pma) + NADH + H^+

The initial increase in absorption would then correspond with the amount of NADH produced at the establishment of the equilibrium. This increase would then be determined by the concentration of the reacting compounds and the equilibrium constant K_{eq}.

$$K_{eq} = \frac{[pma]\ [NADH]\ [H^+]}{[tropic\ acid]\ [NAD^+]}$$

From the reaction equation and from the formula of the equilibrium constant one can predict that a twofold or fourfold increase of both the tropic acid ánd the NAD^+ concentration will lead at to a twofold and fourfold increased NADH concentration at equilibrium. The slow NADH production, observed once the equilibrium has been established, could be attributed to a spontaneous first order decarboxylation of pma, resulting in a constant withdrawal of this compound from the equilibrium. This is in agreement with the observation of the water instability of pma (Struchov 1953; chapter 3.4).

If decomposition takes place as a first order reaction, the rate of the slow NADH production will then be directly proportional with the pma concentration formed at the establishment of the equilibrium. This will result in a ratio of 1 : 2 : 4 in the experiment in fig 6.8. This ratio has been confirmed experimentally.

The equilibrium hypothesis has been confirmed by a study of the effect of pma on the course of the enzymatic dehydrogenation of tropic acid and by demonstration the NADH dependent hydrogenation of pma in presence of TDH, the so-called back reaction.

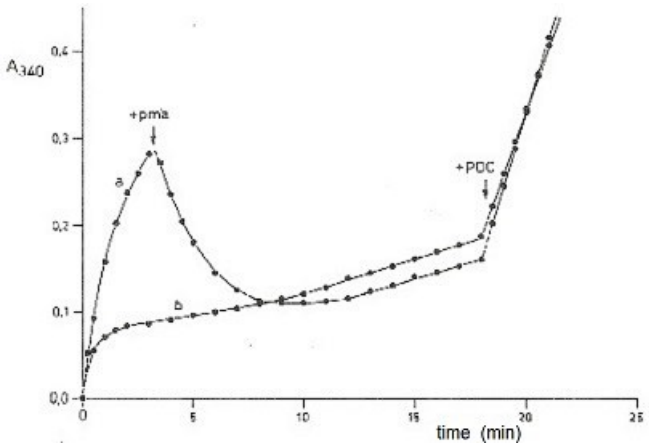
Addition of pma during the dehydrogenation of tropic acid (fig 6.9, curve a) resulted not only in the inhibition of the dehydrogenation, but even in a decrease of the absorption. After some time, a minimum value was reached after which the absorption slowly increased. Adding the same amount of pma at the beginning of the experiment resulted in a fast increase of absorption followed by a period of nearly constant absorption; after some time, absorption started to increase again.

The decrease of absorption observed in curve a of fig.6.9 demonstrates that the reaction is a reversible process. By addition of pma, the hydrogenation of NAD^+ is reversed in the dehydrogenation of NADH. This is in agreement with the supposed equilibrium reaction. As expected, addition of pma results in an adjustment of the concentration of all reactants at equilibrium: a part of the NADH and pma will be converted into NAD^+ and tropic acid. The increase in absorption observed after some time can be ascribed to a shift in the equilibrium due to decomposition of the pma.

In the second experiment in which the pma was added on time=0 (fig 6.9 curve b), a quick establishment of the equilibrium was observed. It is remarkable that in this case nearly the same NADH concentration is reached as after the establishment of the equilibrium after addition of pma in the previous experiment (curve a). The fact that in this system both by conversion of NAD^+ in NADH as well as by conversion of NADH in NAD^+ the same situation can be reached is a strong support for the equilibrium hypothesis.

Fig 6.9

Establishment of the equilibrium by dehydrogenation and hydrogenation



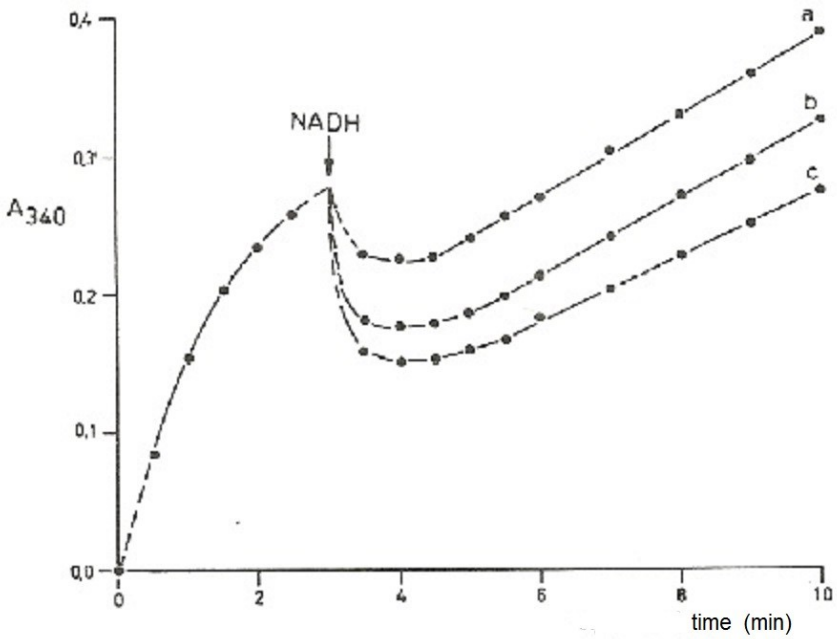
NAD^+ (3.33 mM), tropic acid (50 mM) and TDH (0.8 U) were added to 3 ml 33mM K-phosphate pH 7.5. The pma dissolved in diethyl ether was added in a final concentration of 1 mM on time = 3 min 15 sec (curve a) and on t=0 (curve b). The effect of PDC is discussed in chapter 7.

More proof was obtained in a similar experiment in which the NADH was added during the enzymatic dehydrogenation of tropic acid. The results are presented in fig. 6.10. The A_{340} is plotted against time. The A_{340} was corrected for the absorption of the NADH added. Here a decrease of absorption was noticed as well, the larger depending on addition of more NADH.

The increase of the absorption after the establishment of the equilibrium was larger if less NADH was used. This is also in agreement with the theory; the less NADH is added the larger the pma concentration at equilibrium. The NADH production after reaching the equilibrium is dependent on the amount of pma decomposing per unit of time. This will be all the larger as the concentration of pma is higher.

The back reaction was also demonstrated directly starting from NADH and pma. A decrease of absorption was noticed during incubation of a mixture of NADH and pma in the presence of TDH (fig 6.11.a). The NADH present in limiting amounts was almost completely converted in NAD^+. This conversion has only been observed with pma. The diethyl-acetal of pma, phenylglyoxylic acid, 2-phenyl propanal, phenylacetaldehyde and phenylpyruvic acid do not show this back reaction.

Fig 6.10
Effect of NADH on the establishment of the equilibrium



Tropic acid (50 mM), NAD^+ (33 mM) and TDH were incubated in K-phosphate pH 7.5 (33mM); after 3 min NADH was added: a) 0.25 µmol, b) 0.37 µmol, c) 0.50 µmol. The A_{340} was corrected for the absorption of the NADH added.

Formation of tropic acid from pma in the back reaction was demonstrated as follows: 5 mg pma was mixed with 4 U TDH enzyme and 18 mg NADH in 10 ml 0.1 M K -phosphate buffer pH 7.5. The mixture was incubated at 25^0 during 60 min. Thin layer chromatography was applied to investigate the reaction mixture. During incubation a compound was formed with the same R_f value as tropic acid using the elution fluids EMX and BEM (2.7).The demonstration of tropic acid as reaction product gives definite proof for the back reaction and the enzymatic dehydrogenation as equilibrium reaction.

The experimental results presented here can be used to estimate the value of the equilibrium constant K_{eq}. The following calculation can be made based on data experiment 2 in fig 6.8. The NADH concentration “at equilibrium” was estimated from the turn-off point of the curve. This is 0.017 mM; the pma concentration is supposed to have the same value.

These values, the concentration of tropic acid and NAD^+ and the pH at the start of the dehydrogenation give for the equilibrium constant:

$$K_{eq} = \frac{[pma]\ [NADH]}{[Tropic\ acid]\ [NAD^+]} \times [H^+] = \frac{0.017 \times 0.017}{20 \times 1.67} \times 0.32 \times 10^{-7} = 0.28 \times 10^{-12}\ M$$

In this calculation, it is assumed that the tropic acid and NAD^+ concentration and the pH do not change during the reaction and that the amount of pma decomposed before the turn-off point is reached may be ignored. K_{eq} has been calculated similarly for the experiments (1) and (4) fig 6.8. Values are 0.38 and 0.30 x 10^{-12} M.

The very low value of the K_{eq} constant explains the strong effect of a relative small amount of NADH on the enzymatic dehydrogenation and the observation that in the back reaction a small amount NADH nearly completely is converted in NAD^+.

The equilibrium equation shows an important effect of the pH on the position of the equilibrium This agrees with the observation that the rise of the pH in the range of pH 7.5-9.5 had an effect on the course of the reaction like the rise in the concentration of tropic acid or NAD^+. The higher the pH, the higher the A_{340} at the point of turn-off and the higher the NADH production after establishment of the equilibrium.

The K_{eq} was also calculated from the experiment in which pma was added at the start of the incubation of tropic acid and NAD^+ with TDH (fig. 6.9 curve b); the calculated value was 2.5 x 10^{-12}, this is 8x as large as the equilibrium constant calculated from experiments on top of this page. Also in another aspect, this experiment deviated. After the equilibrium was reached, the NADH production was much slower than expected on basis of the pma concentration.

This discrepancy was the motive to make a detailed study of the back reaction. This will be discussed in 6.6.

The difference in kinetics observed with purified versus non-purified enzyme preparations can be explained as well with the equilibrium hypothesis if one assumes a component in the non-purified sample that accelerates the decarboxylation of pma. In that case, the equilibrium cannot be reached because the pma is continuously withdrawn from the reaction mixture leading to an expiring reaction. This component might be separated from TDH during purification. This would explain that the equilibrium can be observed using purified TDH. This will be discussed more in chapter 7.

6.6 THE EFFECT OF THE KETO-ENOL TAUTOMERISM ON THE ENZYMATIC HYDROGENATION OF PMA.

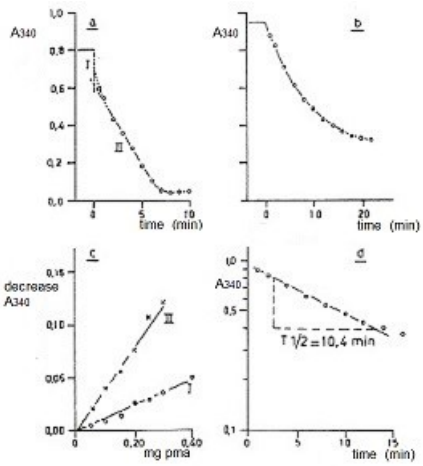
The decrease in absorption observed during incubation of pma , NADH and TDH is attributed to the enzymatic hydrogenation of pma, the so-called back reaction:

pma + NADH + H^+ → tropic acid + NAD^+

In a more precise investigation it was noticed that this reaction also showed biphasic kinetics (fig 6.11.a).

Fig 6.11

Effect of pma on the kinetics of the back reaction



All experiments were carried out at pH 7.5. In a total volume of 3 ml were mixed: 0.8 U TDH, 0.4-0.5 µmol NADH and a solution of pma in diethyl ether at 0^0. Buffer solution and amount of pma as specified :

a) 33 mM K-phosphate; 0.25 mg (1.5 µmol) pma;
b) 0.2 M tris-HCl; 0.06 mg (0.35 µmol) pma
c) 33 mM K-phosphate; various concentrations pma, noted down in mg/3ml,
I = values for phase I; II = values for phase II
d) as b) but plotted semi logarithmically

Start of the reaction : a) reaction started by addition of TDH ; b) c) by addition of pma

In the initial phase, a very fast decrease in absorption was observed (phase I) that thereafter changed in a much slower decrease (phase II). This biphasic kinetics was not influenced by addition of more enzyme or by rising the NADH concentration. It was influenced by changes in the amount of pma added.

Effect of the pma concentration and the effect of preincubation

Effect of the pma concentration was demonstrated in several experiments. Various amounts of pma were added to the NADH and TDH in phosphate buffer. Both the total decrease in absorption in phase I (calculated from the difference between the initial A_{340} and the value obtained by extrapolation of phase II to time t = 0 min) and the decrease in absorption per min in phase II appeared to be directly proportional to the pma concentration (fig 6.11.c).The amount of NADH oxidized in phase I was only a small part of the amount of pma added. After addition of 1.2 µmol pma (0.2 mg) only 0.012 µmol NADH was oxidized in phase I. It was remarkable that nevertheless a direct proportional relationship was found between the pma concentration and the absorption decrease in phase I. This arose the suspicion that either a conversion product of the pma or a contamination in the pma was responsible for absorption decrease in phase I.

Indication for a spontaneous conversion of pma in a compound that might be responsible for the initial decrease in absorption was obtained by preincubation of pma during 2 min in aqueous solution prior to the enzymatic hydrogenation. Thereafter, THD was added and the NADH decrease recorded. In a control experiment TDH and pma were added simultaneously (fig 6.13.a).

The amount of material which could be hydrogenated in phase I was increased during this preincubation. Apparently, this conversion of pma in “phase I material” occurs spontaneously under the conditions used. The suggestion seemed justified that pma in the form it was applied could not act as substrate for hydrogenation but had to be transformed in another compound. The decrease in absorption in phase I would correspond to the hydrogenation of the amount of that compound initially available, whereas the absorption decrease in phase II would be controlled by the rate of conversion of pma into this phase I material.

As reported in chapter 3, pma obtained by chemical synthesis was in the enol form. In solution in ether it is still in the enol form (2.13); in alcoholic solution, a rearrangement in the keto form was observed. (3.4).

In the experiments reported here, a solution of pma in diethyl ether was used. This compound was added in the enol form but might rearrange into the keto in aqueous solution. It was therefore obvious to assume that the effect of preincubation of pma on the kinetics of the hydrogenation was affected by the

rearrangement of enol-pma in keto-pma and that the keto-pma would be the actual substrate for the TDH. The keto-pma present at the start of the reaction could then be hydrogenated rapidly whereas the hydrogenation in phase II would be controlled by the rate of rearrangement of the enol-pma in the tautomeric keto-form.

In chapter 3 evidence has been presented on the conversion of the enol-pma in aqueous solution; the product of conversion was not identified. But it could be demonstrated that this conversion elapsed in 0.2 M tris-HCl pH 7.5 in a first order reaction with a rate constant of 0.073 min^{-1} (half-life time 9.5 min). If this conversion controls the rate of enzymatic hydrogenation in phase II, the hydrogenation of pma should proceed under these conditions according to a first order reaction with the same rate constant.

Conversion of a small amount of pma, conversion in ethanol at 0°

A limited amount pma (0.35µmol) was hydrogenated enzymatically in 0.2 M tris HCl pH 7.5. Phase I was not longer visible due to the small amount of pma (see fig. 6.11.b). The NADH consumption during phase II became gradually less. The hydrogenation proceeds as a first order reaction with a rate constant 0.067 min^{-1} (half-life time 10.4 min) as found by plotting the logarithm of the A_{340} against time (fig. 6.11.d). The nice agreement of this value with that for the spontaneous conversion of enol-pma under similar circumstances suggests that the hydrogenation of pma in phase II is controlled by the speed of conversion of the enol form in aqueous solution.

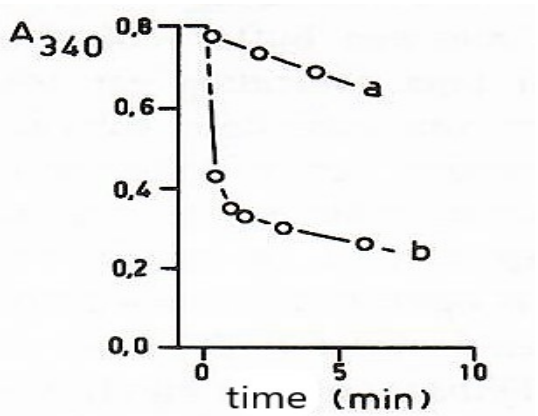
Since enol-pma in ethanol at 0^0 is converted slowly in the tautomeric keto-form, it has been possible to verify the assumption keto-pma being the actual substrate for the back reaction.

A solution of enol-pma in ethanol contains after 4 hours at 0^0 a considerable amount of material, which was hydrogenated by TDH according to phase I (fig. 6.12). The pma 0.3 µmol in this experiment was dissolved in ethanol at 0^0 and assayed after dissolution immediately (curve a): the hydrogenation of the same amount of this solution after 4 hours at 0^0 is presented in curve b. The formation of the material, which is converted at 0^0 in ethanol in phase I corresponds with the formation of keto-pma in ethanol at 0^0, as has been demonstrated by infrared spectroscopy. This leads to the conclusion that TDH is specific for the keto-pma and that the amount of the keto-pma present at the start of the dehydrogenation reaction is responsible for decrease of absorption in phase I.

On basis of the similarity of the kinetics of the hydrogenation in this experiment with the kinetics after preincubation van pma in aqueous solution, one may deduce that enol-pma is converted in keto-pma in aqueous solution. The rate at

which enol-pma is converted in aqueous solution, as measured with the ferrichloride method, corresponds with the rate of hydrogenation in phase II. One may conclude that the rate of pma hydrogenation in phase II is controlled by the rate of the tautomeric rearrangement.

Fig 6.12 Shift of the keto-enol equilibrium in ethanol at 0^0



The pma (0.3 µmol) was added to 0.4 µmol NADH and 1.6 U TDH in 33 mM K-phosphate pH7.5. a) pma dissolved in ethanol at 0^0 and measured immediately. b) pma dissolved in ethanol at 0^0 and measured after 4 hours.

To complete the argumentation for the explanation of the absorption changes in phase I and phase II, the effect of preincubation at pH 5.5 and the effect of the buffer concentration was studied.

The effect of preincubation was investigated by incubation of pma at pH 5.5 during 20 seconds. Thereafter, the kinetics of hydrogenation was studied by addition of NADH and TDH. The effect of preincubation at pH 7.5 during 20 sec was measured for comparison. This experiment displayed in fig 6.13.b shows that the amount of keto-pma increases as result of the preincubation at pH 5.5. Catalysis by H^+ is generally found for tautomeric rearrangements.

Higher concentration of buffer resulted both in more decrease of absorption in phase I as well as a higher rate of decrease of absorption in phase II. In 1 M K-phosphate buffer pH 7.5 the hydrogenation proceeded even exclusively as phase I (fig 6.13.c). Apparently, the tautomeric rearrangement of enol-pma is so fast in I M buffer that this reaction is no longer rate limiting for the enzymatic dehydrogenation and that this hydrogenation takes place exclusively in phase I.
This experiment also shows that a stoichiometric amount of NADH is oxidized during the hydrogenation in 1 M buffer (added 0.30 µmol pma; decrease of

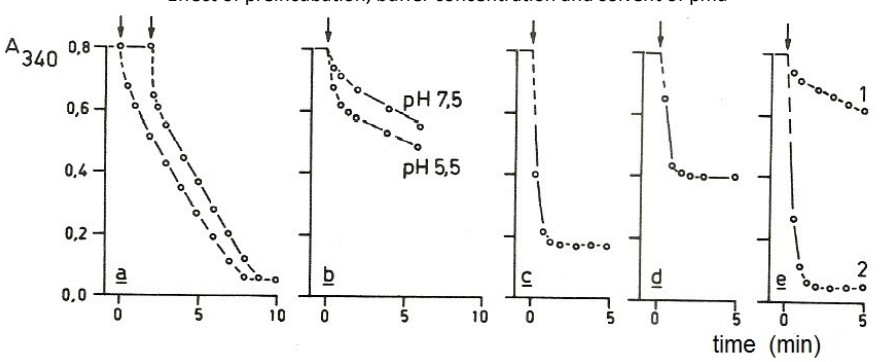
absorption A_{340} 0.62 = 0.30 µmol NADH). Due to the stoichiometry of this conversion, a quantitative assay for pma became available for further research.

Effect of the solvent used for pma

A large effect of solvent on the tautomerism of pma was already noticed from the difference between pma dissolved in diethyl ether and pma dissolved in ethanol. (3.4.2). The effect of the solvent was also obvious in a solution of pma in 50 µL ethanol filled up to 1 ml with water. This solution was tested in the NADH system with a buffer concentration of 33 mM. The solution of pma in diethyl ether had been shown a biphasic hydrogenation (fig 6.11.a). In case of the solution in ethanol-water the hydrogenation appeared to proceed exclusively by phase I (fig 6.13.d). Apparently, a complete conversion of enol-pma in keto-pma has taken place in this solvent, probably catalyzed by the low pH of the mixture (pma has a carboxylic group).

The stability of the keto-pma in the ethanol-water solvent at room temperature was striking (half life time 40 min.). The hydrogenation of pma dissolved in ethanol-water 1-20 did not consume the stoichiometric amount of NADH. Apparently, under these conditions of the enzymatic hydrogenation a part of the keto-pma decomposes at pH 7.5 in the time required for its conversion.

Fig 6.13 a – e
Effect of preincubation, buffer concentration and solvent of pma



TDH 1.6 U and 0.4 µmol NADH was used in 3 ml K-phosphate buffer pH 7.5 in experiments a c d e.
-a preincubation at pH 7.5: 1.5 µmol pma + NADH in 33mM buffer; TDH added on time t=0 min and t=2 min
-b preincubation at pH 5.5: 0.5 µmol pma in 0.5 ml 5 mM buffer pH 5.5 or pH 7.5; after 15 sec (time t=0) were added 0.4 U TDH, 0.4 µmol NADH and 2.5 ml 33mM K-phosphate buffer pH 7.5
-c effect 1 M buffer: hydrogenation of 0.3 µmol pma in I M buffer
-d pma in ethanol-water (1-20): 0.3 µmol pma added to NADH and TDH in 33 mM buffer
-e1 pma in carbon tetrachloride: added to TDH and NADH in 33 mM buffer
-e2 pma in tetra-water (1-20): added to TDH and NADH in 33 mM buffer

The rapid formation of keto-pma has been observed in a similar experiment with carbon tetrachloride as solvent instead of ethanol. Fig 6.13.e shows the enzymatic hydrogenation of pma, added to the incubation mixture as a solution in carbon tetrachloride (curve 1) or as a solution in carbon tetrachloride - water 1-20 (curve 2). Because pma in carbon tetrachloride is in the enol-form nearly completely (3.4.2) this experiment suggests with regard to phase 1 in curve 2 that the hydrogenation may not attributed solely to the presence of keto-pma already in the pma at the start but also may be caused by a fast-tautomeric rearrangement during the mixing of the a-polar pma solution and the incubation mixture, after all, for a short period of time resulting in an acidic aqueous environment.

Quantitative explanation of phase I

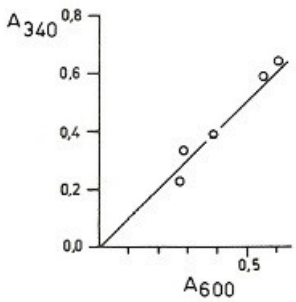
A quantitative explanation for phase I was obtained by taking samples during the rearrangement in ethanol at various moments at 0^{o}. Approximately, the following relationship was found:

total pma[1] minus keto-pma[2] = enol-pma[3]

[1] assay decrease A_{340} pma +TDH +NADH in 1 M buffer
[2] assay decrease A_{340} pma + TDH +NADH in 33 mM buffer phase I
[3] assay A_{600} pma using ferrichloride method

This connection is shown graphically in fig. 6.14. It follows that the amount of pma hydrogenated in phase I is equal to the amount of pma added minus the amount of pma present as enol-pma at the start of the reaction. This is a further confirmation of the explanation of the biphasic course of the enzymatic hydrogenation of pma, as given before.

Fig. 6.14 Relationship between the enol-content of pma and the hydrogenation in phase I



A solution of pma in ethanol (2.5 mg/ml) was incubated at 0^{0}. At different times the total decrease in absorption was assayed with 20 µL of this solution in 1 M K-phosphate buffer at pH 7.5 and the decrease in phase I in 33 mM buffer. The difference between these values was plotted against the absorption at 600 nm of 100 µL of the pma solution, assayed according the ferrichloride method 2.13.

The stoichiometry of the conversion in 1 M buffer, the identification of tropic acid as product of the reaction (6.5), the effect of the pH on the tautomerism and the relationship pma (total) – pma (faseI) = pma (enol) confirm the explanation of the kinetics of the enzymatic hydrogenation of pma presented here. In addition, the hydrogenation in 1M buffer can be used for a quantitative assay of pma and ethanol water 1-20 as solvent of pma has provided a rather stable solution of keto-pma. This allowed a further study of the decomposition of the keto-pma (6.7) and the effect of keto-pma on the establishment of the equilibrium catalyzed by TDH (6.8).

6.7 THE RATE OF THE SPONTANEOUS DECOMPOSITION OF PMA

The explanation of the tropic acid dehydrogenation by purified TDH has been formulated in the supposition that the slow NADH production following the establishment of the equilibrium is controlled by the rate of decomposition of pma under the actual circumstances (6.5). The rate of decomposition of both the enol- and the keto-form has been assessed using the quantitative assay of pma, described in the previous paragraph. These data have been compared with the rates calculated from the NADH production mentioned. In this way, confirmation has been obtained for the supposition above.

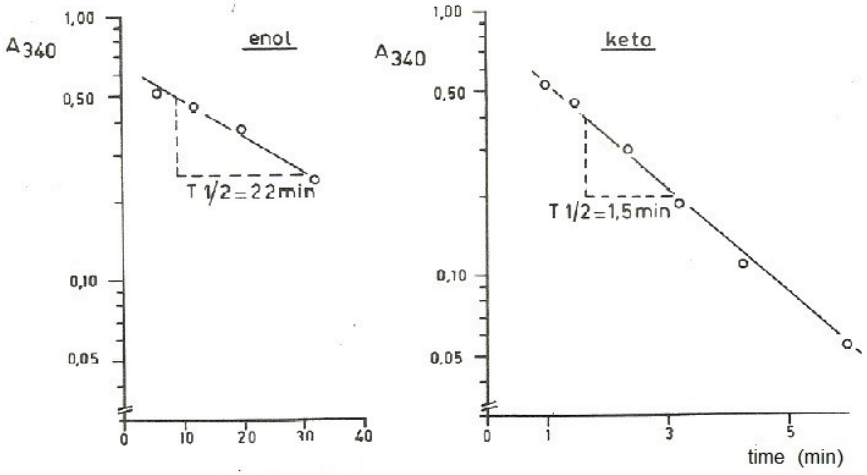
In order to measure the speed of decomposition of keto-pma, 2.5 mg pma was dissolved in 50 µL ethanol, water was added up to 1 ml. Samples of 40 µL of this keto-pma solution were preincubated with 0.4 ml 33 mM K-phosphate pH 7.5 during various periods of time. Thereafter, NADH and TDH were added in 33 mM K-phosphate buffer. Absorption was measured after 5 min. The difference between absorption after 5 min and absorption of a reference sample (minus pma) is a measure for the keto pma concentration after preincubation. This difference is plotted semi-logarithmically against the preincubation time. The keto compound appeared to decompose according to a first order reaction with a rate constant of 0.46 min^{-1}, this a half-life time of 90 sec.

The stability of enol-pma was investigated in a similar way. Starting point were samples of 20 µL of a solution of enol-pma in diethyl ether (2.5 mg/ml). To measure the amount of pma still present, incubation was carried out in NADH and TDH in 1M K-phosphate buffer pH 7.5. The results reported in fig. 6.15 show that in this solvent pma disappeared in first order kinetics with a rate constant of 0.032 min^{-1}. (half-life time 22 minutes). In 1 M phosphate enol + keto are measured. The keto pma has only a very limited stability under these conditions; therefore, the data obtained can be ascribed to the enol- pma. The rate constant will relate to decomposition of the enol-form (probably through the keto-form).

In this way, a large difference in stability has been demonstrated between the keto- and enol-pma.

Fig 6.15

Spontaneous decomposition of enol- and keto-pma



The enzymatic assay of pma was carried out with NADH (0.13 mM) and TDH 1.6 U in K-phosphate buffer pH 7.5. Buffer concentration in the assay for enol-pma 1M; in assay for keto-pma 33mM.

The rate of the spontaneous decomposition can also be calculated from the NADH production after the establishment of the equilibrium. It is not simple to calculate the half life time of pma from the NADH production; this NADH production does not proceed according to first order kinetics, since pma is continuously supplied as the result of the shift of the equilibrium.

Because the adjustment of the equilibrium, given the amount of TDH used, is a rapid reaction compared with the spontaneous decomposition of pma and because the changes in the concentrations of tropic acid, NAD^+ and H^+ may be ignored when only a small part of the tropic acid is dehydrogenated, one can formulate the following equations for the reactions:

$$\text{A (tropic acid)} + \text{B } (NAD^+) \underset{}{\overset{K_{eq}}{\rightleftharpoons}} \text{C (pma)} + \text{D (NADH)} + H^+$$

$$\text{C (pma)} \xrightarrow{k} \text{E (phenylaceetaldehyde)} + CO_2$$

$$\frac{d[E]}{dt} = k\,[C] \qquad (1)$$

$$\frac{d[C]}{dt} = -\frac{d[E]}{dt} + \frac{d[D]}{dt} \qquad (2)$$

$$\text{suppose } [C] \times [D] = Keq \cdot \frac{[A]\,[B]}{[H^+]} = K^1 = \text{constant} \qquad (3)$$

differentiation of (3) gives:

$$\frac{d[C]}{dt} = -\frac{K^1}{[D]^2} \cdot \frac{d[D]}{dt} \qquad (4)$$

Substitution of (1), (2) and (3) in (4) leads after integration to (5). D_0 and D are the concentrations of NADH on time t=0 and t=t respectively; t=0 is any time after establishment of the equilibrium.

$$\ln \frac{[D]}{[D_0]} + \frac{[D]^2 - [D_0]^2}{2\,K^1} = k.t \qquad (5)$$

The value of k has been calculated following equation (5) for the curves 2 and 4 in fig 6.8 from the NADH production after the establishment of the equilibrium. In curve 2, the A_{340} of the NADH formed is 0.124 after 45 sec (t=0). At 3 min 45 (t=3) the A_{340} is 0.203. The NADH concentrations D_0 and D are calculated being 0.0200 and 0.0326 mM respectively.

If the amount of pma decomposed during the establishment of the equilibrium is ignored, then C = D and $K^1 = (0.020)^2 = 0.0004\ mM^2$. Substitution of these values in (5) gives k= 0.44 min^{-1} (half life time 95 sec). Since the value taken for C is too high, the real k value will be a little bit higher (estimated 5-10%). From fig 6.8 curve 4 a value of k being 0.40 min^{-1} can be calculated in the same way (half life time 104 sec).

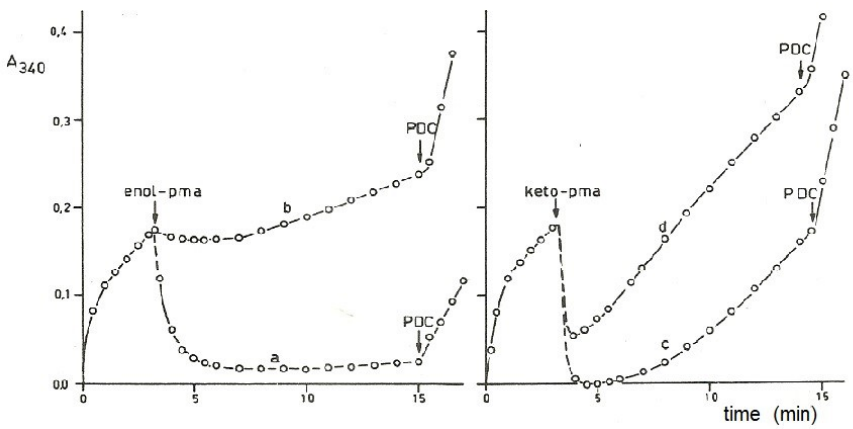
These values are in good agreement with the decomposition constant of keto-pma, as calculated from the experiments earlier in this chapter. One may conclude that the NADH production after establishment of the equilibrium is controlled by the decomposition rate of the keto-pma. It cannot be explained by the instability of enol-pma under reaction conditions.

6.8 THE EFFECT OF KETO-PMA ON THE ESTABLISHMENT OF THE EQUILIBRIUM

If the keto-form of pma is the product of enzymatic dehydrogenation of tropic acid, as can be deduced from the rates of decomposition of the reaction product, keto-pma will have a much larger effect on the establishment of the equilibrium compared with that of enol-pma. Therefore, a comparison was made of the effect of enol-pma and keto-pma on the establishment of the equilibrium (see fig 6.16). Tropic acid, NAD^+ and TDH were incubated during 3 min and 15 sec; next was added 3 µmol enol-pma (curve a), 0.3 µmol enol-pma (curve b), 3 µmol keto-pma (curve c) and 0.3 µmol keto-pma (curve d) ; the absorption at 340 nm was followed during some time.

The left part fig. 6.16 shows a clear effect of 3 µmol enol-pma on the position of the equilibrium. This experiment looks like the experiment shown in fig 6.9 curve a: a similar regression of the NADH production is noticed after the establishment of the equilibrium. A small effect was observed with 0.3 µmol pma. The right part of fig 6.16 shows the effect of keto-pma. It is obvious that similar amounts of keto-pma have a larger and faster effect on the establishment of the equilibrium. This effect however is only of short duration.

Fig 6.16
The effect of enol-pma and keto-pma on the establishment of the equilibrium



Tropic acid 50 mM, NAD^+ 3.3 mM and TDH 4 U were mixed with 3 ml 33mM K-phosphate pH 7.0. After 3 min 15 sec were added: 3 µmol and 0.3 µmol enol-pma (dissolved in diethyl ether) curve a and b respectively; 3 µmol and 0.3 µmol keto-pma (dissolved in ethanol-water 1-20) curve c and d respectively.
Data corrected for the absorption of the incubation mixture in the absence of enzyme.
The effect of PDC will be discussed in chapter 7.

Using data on keto-enol tautomerism these observations can be explained as follows: keto-pma has a direct and large effect on the establishment of the equilibrium, because keto-pma is the product formed as the result of the dehydrogenation. The duration of the effect is short due to the instability of the keto form (half life time 90 sec); as a consequence, the NADH production is after several minutes again on a level comparable with the NADH production before the addition of the keto-pma. The enol-pma has a much smaller effect, however its duration is much longer. This can be explained by assuming that enol-pma itself has no effect but is exclusively effective because it can be converted into the keto form. The long duration of the effect is due to the slow tautomeric rearrangement, resulting in the production of small amounts of keto-pma during a longer period of time. In spite of the spontaneous decomposition of the keto-pma, so much keto pma is formed from enol-pma during 10 min that the equilibrium only shifts marginally and hardly any NADH is produced.

Also differences can be explained in values of the equilibrium constant found in experiments with and without pma. Since the pma added was mainly in the less-active enol form, the calculation of the equilibrium constant was based on a too high pma value, resulting in a constant of too high value.

In accordance with this explanation, a value for the equilibrium constant can be calculated from the data with keto-pma in experiment 6.16. The equilibrium constant has been calculated from the absorption minimum of curve d. It is estimated that 25% of the pma added after 4 min and 70% of the pma formed (during dehydrogenation) was decomposed. This taken in account, the calculation leads to the value of $\pm$ 0.4 x 10^{-12}. This value corresponds with data from earlier experiments in which no pma was added (see table 6.17).

6.9 DISCUSSION

The dehydrogenase in Pseudomonas PMBL-1 involved in dehydrogenation of tropic acid is specific for this substrate. The enzyme transfers the hydrogen to the cofactor NAD^+, therefore its official name of the enzyme is proposed as

tropic acid : NAD^+ oxidoreductase.

Tropic acid dehydrogenase appears to catalyze both the NAD^+-dependent dehydrogenation of tropic acid and the NADH-dependent hydrogenation of keto-pma; the enzyme accelerates the establishment of an equilibrium in the presence of the substrate and the cofactor. A detailed study of the enzyme kinetics has shown that the fast initial absorption increase at the dehydrogenation of tropic acid in neutral environment corresponds with the establishment of the equilibrium

and that subsequent slow NADH production is controlled by the rate constant k of the spontaneous decarboxylation, causing the pma formed being withdrawn from the equilibrium. The equilibrium constant was determined at various concentrations of substrate and cofactor and at various pH values (table 6.17). This constant has a value of ± 0.30 x 10^{-12} M, as calculated from experiments in which the equilibrium was reached by dehydrogenation of tropic acid.

The constant calculated from experiments in which enol-pma was present, seemed to be 5-8 times larger, but this is caused because not the enol-pma but keto-pma is the real substrate in the hydrogenation by TDH. Accordingly, from the experiment with keto-pma K_{eq} = ± 0.4 x 10^{-12} was calculated.

Various NAD^+ dependent dehydrogenases catalyze reversible reactions. Well known examples are ethanol dehydrogenase and lactic acid dehydrogenase. These enzymes catalyze the dehydrogenation of ethanol and lactic acid and the hydrogenation of acetaldehyde and pyruvic acid respectively. The equilibrium constants are 8.0 x 10^{-12} (Bäcklin 1958) and 2.4 x 10^{-12} (Kubowitz 1943). Comparing the equilibrium constant for the by equilibrium catalyzed by TDH with these values, it is apparent that in case of the TDH the hydrogenation reaction is even more favoured.

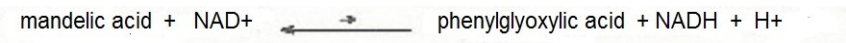
Table 6.17
Equilibrium constants for the NAD^+ dependent dehydrogenation of tropic acid

Fig	tropic acid mM	NAD^+ mM	pma enol mM	pma keto mM	NADH mM	H^+ M	K_{eq} M
6.6	12	2.0	–	–	0.020	10^{-8}	0.17 x 10^{-12}
6.7	20	0.83	–	–	0.022	10^{-8}	0.29 x 10^{-12}
6.8	10	0.83	–	–	0.010	0.32 x 10^{-7}	0.39 x 10^{-12}
6.8	20	1.67	–	–	0.017	0.32 x 10^{-7}	0.28 x 10^{-12}
6.8	40	3.33	–	–	0.035	0.32 x 10^{-7}	0.30 x 10^{-12}
6.9	50	3.33	1.0	–	0.013	0.32 x 10^{-7}	2.5 x 10^{-12}
6.16	50	3.33	1.0	–	0.003	10^{-7}	1.9 x 10^{-12}
6.16	50	3.33	0.1	–	0.027	10^{-7}	1.6 x 10^{-12}
6.16	50	3.33	–	0.083	0.0081	10^{-7}	<0.40x 10^{-12}

The direct assay of the rate of the spontaneous decarboxylation showed that the rate found for the decomposition of the keto-pma (k=0.46 min^{-1}) is in nice agreement with the decomposition constants that have been calculated k = 0.40 and 0.44 min^{-1} (data in 6.7). So there is no doubt that the NADH production after establishment of the equilibrium is caused by decomposition of the keto-pma.

The effect of the pH on the rate of the decomposition of keto-pma could not be investigated accurately due to experimental problems. But the impression was obtained that this rate is constant in the pH range of 5-9. The enol-pma in aqueous solution appeared to have a stability optimum around pH 7.0. The enol-pma is probably decomposed via conversion in the less stable keto-form. The pH optimum for stability might indicate that the tautomeric rearrangement proceeds more quickly at both high and low pH.

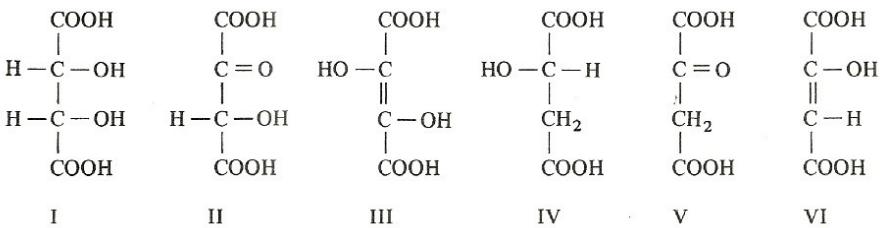
In the Pseudomonas ATCC 12633, mandelic acid (2-hydroxyphenylacetic acid) is oxidized to phenylglyoxylic acid by means of a mandelic dehydrogenase. This enzyme seems to be unable to use NAD^+ as hydrogen acceptor (Kennedy 1968). One could imagine that in the hypothetical reaction :



the position of the equilibrium is located so far to the left that experimentally the production of NADH cannot be observed. However, no indications could be found to support this assumption. Neither by spectrophotometry nor by thin layer chromatography, it appeared possible to demonstrate the hydrogenation of phenylglyoxylic acid in the presence of NADH and an extract containing the mandelic acid dehydrogenase.

In the literature two investigations have been reported on the effect of keto-enol tautomerism on the enzymatic (de)hydrogenation. Kohn and Jacoby (1968) have investigated the NAD^+ dependent dehydrogenation of mesotartaric acid (I) catalyzed by the malic acid dehydrogenase (fig. 6.18). They isolated as reaction product not the oxaloglycolic acid (II) but the tautomeric form dihydroxyfumaric acid (III). The authors propose the reaction to proceed through (II) as intermediate, but have only limited evidence.

Fig.6.18



Loewus (1955) investigated with the same enzyme the effect of the keto-enol tautomerism of oxalacetic acid (V), formed after the enzymatic dehydrogenation of malic acid (IV). It was shown that in the back reaction the keto-form (V) in phosphate buffer was much faster hydrogenated compared with the tautomeric enol-form hydroxymaleinic acid (VI). This suggested that the enzyme could not convert (VI) as such, but that VI first had to be rearranged to V in an aqueous environment. In the presence of Mg^{++} ions both V and VI were hydrogenated with the same speed, indicating that under these conditions the tautomeric rearrangement of VI not was rate limiting any more. Qualitative confirmation was obtained by chemical investigation of the keto-enol tautomerism of V and VI. Mg^{++} indeed accelerated the tautomeric rearrangement. A quantitative relationship between the various effects of Mg^{++} was not found.

The study of the hydrogenation of pma by TDH has led to the elucidation of the effect of the keto-enol tautomerism on the kinetics of dehydrogenation at pH 7.5. By a combination of enzymological research and infrared spectroscopy (3.4) and the quantitative assay for enol, it could be proven that the TDH is specific for the keto form, that the initial fast NADH consumption in phase I of the back reaction corresponds with the hydrogenation of the keto form and that the subsequent slow phase is controlled by the tautomeric rearrangement of enol-pma into keto-pma.