Enzymology and regulation of the atropine metabolism in pseudomonas putida

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PhD THESIS

TO OBTAIN THE DEGREE OF DOCTOR IN MATHEMATICS
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ON THE AUTHORITY OF THE RECTOR MAGNIFICUS
DR L. KUKENHEIM Ezn, PROFESSOR IN THE FACULTY OF
LANGUAGE AND LITERATURE, IN THE PRESENCE OF A COMMITTEE OF THE
SENATE, TO DEFEND ON WEDNESDAY
18 JUNE 1969 EXACTLY ON THE STROKE OF 16.45

BY
WILLEM FRANS STEVENS
BORN ‘s HERTOGENBOSCH IN 1941

PROMOTOR: PROF. DR. A. RöRSCH

This thesis was written in the Dutch Language in 1969 and at that time deposited in the Repositorium of the Library of the Technical University, Delft, The Netherlands htpps://repository.tudelft.nl/view/tno; # 268336.

Only in 2019 the thesis was translated by the author in English as precise copy nearly page by page of the 1969 version (no update) and deposited at the same Library under nr htpps://repository.tudelft.nl/view/tno; #
The English translation is only meant to disseminate the scientific data, described in the Dutch version. It does not intend to add any new data. In case of differences in the interpretation of the intellectual property only the Dutch version counts.

Dedicated to

my parents

Yvonne

The thesis started off with the Homer-like poem in hexameter below. It is an explanation of the aim of this thesis. Atropos, as fate goddess on the Olympus, had the task to cut randomly the life-lines of the mortals with a pair of scissors. And as extract of Belladonna, she determined the fate of the new couple: by widening woman's eye: she was almost blind, he dazzled, so both made the wrong choice.
What will happen to You, now Hades' imperium has released a bacterium which contains an enzyme that will cut your life line?

Atropos in bronze,
by Peter Hoogerwerf

Atropos, gij waart godin van het noodlot en vrees'lijke rampspoed.
Gij waart bij machte ten gronde te richten, gij had een onzalige invloed
Door tot de Vader van Goden en Mensen het onheil te fluist'ren
Hiermee de sterv'ling voorgoed aan zijn blinde bestemming te kluist'ren
En als extract van bell'dona verwijdend het oog der beminde
Zodat gij aanstaande man alswel vrouw in hun keuze verblindde,

Welk lot brengt U een bacil die uit Hades' rijk vrij is gegeven
Die in haar handen de schaar heeft die knipt aan de draad van Uw leven ?

INTRODUCTION

The breakdown of a chemical compound in living organisms is accomplished usually by a sequence of enzyme catalyzed reactions. As a result, such a compound is broken down by a fixed pattern, the metabolic pathway. In most cases, the cells in an organism are able to control the synthesis and activity of the enzymes involved. In this way, the cells are able to fine tune the capacity of a metabolic pathway to the actual demand.

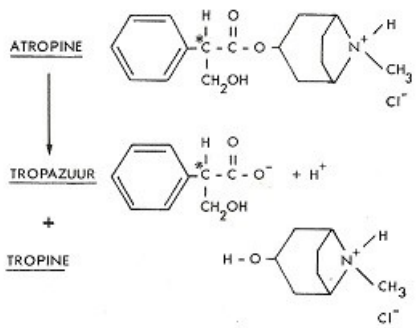
This PhD thesis research deals with the metabolism of the alkaloid atropine, in the bacterium Pseudomonas putida. It includes a study of the metabolic pathway of atropine in the bacterium and the enzymes involved. In addition, a special mechanism has been elucidated by which the regulation of this metabolic activity is accomplished.

Atropine is an alkaloid present in Nature a.o in the fruits of the plant Atropa belladonna L. and in other members of the Solanaceae family. The toxicity of extracts of atropa fruits is known within living memory (Hippocrates 400 BC); the active principle in these extracts was associated with the fate goddess Atropos who terminated life by cutting the thread of life.

The toxic action of atropine is based on the inhibition of transmission of nerve impulses in the parasympathetic nervous sytem. The compound has a number of important pharmacological applications that make use of this specific action. These comprise its action as antidotum in case of poisoning with organophosphorous compounds including many insecticides like parathion and chemical warfare agents.

The Medical Biological Laboratory (MBL) in the Dutch TNO Organization for Defense Research is carrying out a wide spectrum of projects to collect relevant data on the physiological activities of atropine. In this context, a study has been started on the interaction of atropine with its physiological receptor, this is the target structure for atropine. The binding of atropine to its receptor results in the disturbance of the impulse transmission in the central nervous system. So far, biochemical research of this receptor is not possible since the receptor can be recognized in vivo only. For this reason, a project has been started to find a more accessible biological structure, that shows a specific interaction with atropine and might be used as a kind of a model for the atropine sensitive receptor.

Literature data describe an enzyme present in rabbit serum, which can hydrolyze the ester atropine in the acid component tropic acid and the alcohol component tropine:



This enzyme is a rather aspecific esterase. Moreover, it is hardly to manage for enzymological research due to the low atropine esterase activity in Dutch rabbits.

A better study object became available when Rörsch and Berends isolated a bacterium from soil between the roots of Atropa belladonna (Berends et al 1969). This bacterium was able to grow in a synthetic medium with atropine as sole source of carbon. It was given name of PMBL-1 and has been identified by Bartlema and Wensinck as a Pseudomonas putida biotype A (Wensinck 1969). Rörsch and Berends were able to show the presence of an atropine-esterase in PMBL-1 grown with atropine as sole carbon source. In addition, they demonstrated the specificity of this bacterial enzyme for atropine and its suitability as an object of enzymological study.

This atropine esterase appeared to be inhibited by various compounds of the organophosphorus type like dïisopropyl phosphorofluoridate (DFP). This gave support to the idea that the esterase belonged to a group of enzymes – the so-called serine-esterases - which are inhibited by organophosphates. It has been shown that this inhibition is caused by the reaction of the organophosphate with a highly active serine residue that is present in the catalytic center of the enzyme.

Serine-esterases have been subject of intensive study a.o. in the MBL during the last 20 years (J.A. Cohen, R.A. Oosterbaan, H.S. Jansz and F. Berends, 1959). The reaction of the organophosphate with the serine residue can be used to investigate the mechanism of action of these serine-esterases. These studies have made important contributions to our present understanding of enzymatic hydrolysis.

The atropine esterase in Pseudomonas PMBL-1 seemed to be interesting as a good model for the atropine-sensitive receptor and as a new representative of the serine esterases. This because of its specificity for atropine and its sensitivity for inhibiting organophosphorus compounds. The bacterial origin of the enzyme might offer the additional advantage to isolate mutants of the bacterium which produce an atropine esterase with small modifications in the protein molecule. The study of the effects of these modifications on the interaction of atropine with atropine-esterase and on the mechanism of action of this enzyme could provide important information on these processes.

The investigation of the metabolic pathway of atropine was started initially with the intention to isolate mutants with a modified atropine esterase. In this respect it was important to know the metabolic pathway of atropine in Pseudomonas in order to distinguish between mutants with a modification by mutation in the genetic information coding for the esterase versus mutants blocked further down in the pathway.

Soon, it became obvious that the search of the metabolism of atropine offered a quite different perspective. The synthesis of atropine esterase is - as discovered by Rörsch and Berends - subject to control: the atropine esterase cannot be detected in Pseudomonas grown with sole carbon sources like glucose or succinic acid. Apparently, the atropine esterase is an inducible enzyme, its synthesis dependent on the presence of a specific ingredient present in the cultivation medium.

During the investigation of the ability of the hydrolysis products tropic acid and tropine to act as sole carbon source, both compounds were metabolized by Pseudomonas. Growth on tropic acid appeared to result in the synthesis of atropine esterase whereas after growth on tropine the enzyme was absent. This is remarkable in view of the relation of the tropic acid to the enzyme. The induction of the esterase by its product tropic acid drew attention for the regulatory mechanism that controls the synthesis of the esterase. Therefore, it seemed of interest to elucidate the metabolism of tropic acid and to identify the enzymes involved. It might be that those enzymes could be induced together with the atropine esterase. A study of the induction of these enzymes could reveal the regulation of protein and enzyme synthesis in PMBL-1.

In addition, study of the further breakdown of tropic acid might contribute to our knowledge of microbial metabolism of aromatic acids with a branched side chain. Our knowledge of the metabolism of this class of compounds is still very poor.

These considerations have resulted in the investigation reported in this thesis on the metabolic pathway of atropine and the regulation of synthesis of the enzymes involved.

Chapter 1 of this thesis presents a summary of the literature available on the metabolism of atropine in higher animals, in plants and in micro-organisms. Chapter 2 describes materials, methods and techniques used in this study whereas chapter 3 deals with the synthesis, identification and the keto-enol tautomerism of 2-phenylmalonic semi-aldehyde, a compound that in this study appeared to be one of the intermediary metabolites in the breakdown of atropine.

In chapter 4 experiments are discussed that presented the first evidence for the metabolism of atropine and tropic acid in Pseudomonas PMBL-1. Studies were carried out on the adaptation of PMBL-1 during growth on tropic acid and various other aromatic carbonic acids. Pseudomonas cultivated in a medium with atropine or tropic acid appeared to be adapted for growth on phenylacetic acid. The role for phenylacetic acid in the metabolism of atropine and tropic acid was confirmed by the isolation of phenylacetic acid from the growth medium of a few mutants with a block somewhere in the breakdown of tropic acid.

These data were the basis for a working hypothesis: conversion of atropine in phenylacetic acid is accomplished by hydrolysis in tropic acid followed by two dehydrogenations and one decarboxylation (see annex 1). The chapters 5 through 8 describe the investigations to confirm this working hypothesis and the identification of the enzymes involved in PMBL-1 in the conversion of atropine in phenylacetic acid.

The breakdown of atropine proceeds through tropic acid, 2-phenylmalonic semi-aldehyde and phenylacetaldehyde catalyzed by the enzymes atropine esterase (AtrE), tropic acid dehydrogenase (TDH), 2-phenylmalonic semi-aldehyde decarboxylase (PDC) and phenylacetaldehyde dehydrogenase (PDH). Together these enzymes are called the “tropic acid enzymes”. The identification, the quantitative assay, the partial purification and some properties of each of these enzymes will be presented. These enzymes are proteins enzymologically well to handle with a large specificity for the corresponding substrate.

In case of atropine esterase (chapter5) a purification procedure has become available that allows an enrichment on protein basis of 600x compared with the enzyme in a crude extract. In this way an allmost pure enzyme preparation has become available for further investigation of the catalytic center of this enzyme.

Chapter 6 describes tropic acid dehydrogenase (TDH). This enzyme catalyzes the dehydrogenation of tropic acid into 2-phenylmalonic semi-aldehyde (pma) by transfer of 2 hydrogens on the cofactor NAD^+. The study of the kinetics of the dehydrogenation has shown that this process is reversible in the presence of purified enzyme:

Establishment of the equilibrium was demonstrated both by dehydrogenation of tropic acid as well as by hydrogenation of pma. The slow production of NADH, that can be observed once the equilibrium has been reached, has been attributed to the shift of the equilibrium by spontaneous decomposition of pma.

Effect of keto-enol tautomerism of pma on the kinetics of the hydrogenation of pma and the position of the equilibrium has been studied in detail. The time course of the hydrogenation can be explained quantitatively on basis of the specificity of the tropic acid dehydrogenase for the keto form of pma and the rate of the tautomeric rearrangement enol-pma → keto-pma in aqueous condition. Although keto-pma not has been identified as the direct conversion product, the presented experimental evidence leaves no space for a conclusion other than keto pma being the direct metabolite of tropic acid.

PMBL-1 appears to possess a pma decarboxylase. This in spite of the Instability of keto-pma, what results in the spontaneous decarboxylation. Chapter 7 present the evidence for the presence of this enzyme, obtained by the study of a protein fraction that accelerates the enzymatic dehydrogenation at neutral pH many times. The identification as 2-phenylmalonic semi-aldehyde decarboxylase is based on the direct effect of this protein on the stability of pma and the formation of phenylacetaldehyde and CO_2.

Phenylacetaldehyde dehydrogenase (chapter 8) is the 2nd dehydrogenase contributing to the metabolism of atropine and tropic acid. Similar like tropic acid dehydrogenase it uses NAD^+ as cofactor. The enzyme is much less stabile compared with the other tropic acid enzymes, but can be stabilized by a buffer solution of special composition. Using this buffer, it is possible to purify the enzyme partially and investigate several of its properties.

Chapter 9 regards the functional sequence of the tropic acid enzymes as well as the presumable absence of an active system (permease) for the uptake of tropic acid. The metabolism of tropic acid in PMBL-1 is compared with that of mandelic acid in Pseudomonas ATCC 12633.

Chapter 10 deals with the regulation of synthesis of the tropic acid enzymes. The 4 enzymes in PMB-1 appeared to be induced in the presence of atropine or tropic acid in the growth medium. In addition, a few other compounds have been demonstrated to induce these enzymes. In a detailed study of the induction process in mutants of PMBL-1 evidence has been obtained that only phenylacetaldehyde and benzaldehyde induce the tropic acid enzymes that atropine and tropic acid have only the ability to do so if these compounds can be metabolized in phenylacetaldehyde. The apparent advantages of induction by

phenylacetaldehyde over the induction by atropine and tropic acid will be discussed.

The relevance of the elucidation of the tropic enzymes to the receptor model and to the mechanism of action of the atropine esterase will be discussed in the epilogue. Arguments will be presented for the hypothesis that the tropic acid enzymes are involved exclusively in the breakdown of atropine and probably not in a more central function in Pseudomonas. For this reason these enzymes might be very suitable for the further study of regulation of protein synthesis in general.

CHAPTER 1

OVERVIEW OF LITERATURE

The breakdown of atropine in mammalians, plants and bacteria has been studied quite intensively as far it concerns the hydrolysis in tropic acid and tropine. The atropine esterase has been shown to be involved in various organisms. In addition, in higher animals the conversion of atropine into some other metabolites has been demonstrated. Since these data have only limited relevance for the research presented in this thesis, only the most interesting data have been included in this overview.

1.1 THE BREAKDOWN OF ATROPINE IN MAMMALIANS

The first indications regarding the metabolism of atropine date from the year 1852 when Schroff, a physician in Vienna, noticed a relative insensitivity of rabbits, which fed themselves with the leaves of the belladonna plant. Rabbits elsewhere in Europe did not show this insensitivity. Nowadays these regional differences are not observed anymore. During the First World War various rabbit varieties in Europe have been mixed (Quinton 1966).

Fleischmannn (1910) and Metzner (1912) were the first scientists to relate the resistance against atropine and the ability of rabbit serum to inactivate atropine. Bernheim (1938, 1948), Glick (1940) and Ammon (1949) proved this inactivation to be caused by enzymatic hydrolysis of atropine in the pharmacological low active hydrolysis products tropic acid and tropine. The enzyme involved was named atropine esterase.

Resistance against atropine has a genetic basis and is transferred as a recessive feature (Sawin and Glick 1943). Recently, Werner (1967) has listed the mammalians that possess this enzyme. In addition to some rabbits, several guinea pigs are atropine resistant and possess the atropine esterase. The enzyme has not been observed in other mammalians or in humans.

Margolis and Feigelson (163, 1964) have made an extensive study of atropine esterase in rabbit serum. These investigators succeeded to purify the enzyme 70 times compared to the starting material and to prepare an antiserum against this atropine esterase for immune titrations using serum of atropine resistant and atropine sensitive rabbits. They concluded that the lack of atropine esterase activity was caused by the absence of the enzyme or an immunological

related protein and not by the presence of an activator of the enzyme.

Not only hydrolysis of tropine-esters, the hydrolysis of esters of choline and glycerol is accelerated as well. Therefore, this atropine esterase is a non-specific esterase. In contrast to the a-specificity is the remarkable preference of the enzyme for the (-) enantiomer of atropine and scopolamine[#]; (-) atropine is hydrolyzed 100 times faster compared with (+) atropine (Werner 1967). The enzyme has no preference for either of the optical isomers of homatropine.

Concerning atropine-like compounds one could add the following. For this atropine esterase only compounds with a 3' substituent of tropine and the N-methyl group in the trans position are suitable substrates. Therefore, Werner (1967) has proposed as the official name for this enzyme: trans (-) hyoscyamine-acyl hydrolase. The cis-isomer of atropine can be hydrolyzed by the serum of all investigated species including by that of human. The atropine esterase is inhibited by organophosphorus compounds di-ethyl-p-nitrophenyl phosphate (Margolis and Feigelson, 1964) and dïisopropyl phosphofluoridate (Berends, 1965 and Otorii, 1965).

The enzymatic hydrolysis of the ester atropine is not the only mechanism for detoxification of atropine in resistant animals. Also the metabolism and excretion of atropine in esterase deficient mammalians has been studied.

Kalser (1957), and Gabourel and Gosselin (1958) have applied radioactive atropine. Following intravenous application, the radioactivity was mainly recovered from urine, partially as atropine, partially conjugated to glucuronic acid.

In this way they confirmed earlier observations (Bernheim 1948) on the unaltered secretion by the kidney. A part of the radioactivity in the urine showed a positive reaction with reagents for the phenyl hydroxyl group: this points to a hydroxylation of the tropic acid residue in atropine. Only 0.3% of the radioactivity was excreted as tropic acid. Using paper chromatography, a number of non-identified products were found. Werner (1968) confirmed the hydroxylation of atropine and found evidence for an oxidative demethylation of the N-methyl group of atropine.

The conversion of atropine in rat liver has been studied by Matsuda (1966). This author compared the pharmacological action spectrum of intravenously applied atropine with that of intravenously applied atropanal (the tropine-ester

chemical formulas are presented in table 5.6 and in annex 1

of 2-phenylmalonic semi-aldehyde). Atropanal appeared to be 7x more toxic but was less active in parasympathetic activity. Atropine slowly applied in the portal vein gives the pharmacological action spectrum of intravenously applied atropanal. Matsuda concluded that atropine is oxidized to atropanal in rat liver.

1.2 THE BREAKDOWN OF ATROPINE IN PLANTS

The members of the plant family of *Solanaceae* are the natural producers of the tropa-alkaloids that include atropine, scopolamine, homatropine and cocaine. In these plans atropine is both synthesized and metabolized (Neumann and Tschöpe 1966). An atropine esterase for the breakdown of atropine has been demonstrated in Datura stramonium according to Kaczkowski (1964). This author also found evidence for the presence of an enzyme for the synthesis of atropine from tropic acid and tropine. Using column chromatography, it was possible to separate the esterase and the synthetase; therefore two different enzymes are involved in the synthesis and breakdown of atropine.

Vegetable atropine esterase has been studied in vitro (Jindra, Čihak, 1963) Enzyme activity is maximal at pH 5.3 and 30^0; it is inhibited by excess substrate and non-specific for one of the optical isomers of atropine.

Breakdown of tropic acid in plants is not known (Neumann and Tschöpe, 1966). However, samples of tropa-alkaloids, obtained by extraction of plant materials contain various more-basic unsaturated fatty acids, that might have been the result of breakdown of tropic acid (Flück, 1965).

[Phenylalanine and phenylpyruvic acid are direct precursors in the synthesis of tropic acid in vivo. According to Gibson and Youngken (1967) 1,3 ^{14}C phenyl pyruvic acid (C_6H_5-*CH_2-CO-*COOH) is converted in vivo in 1,2 ^{14}C tropic acid (C_6H_5-*$CH(CH_2OH)$-*COOH. This rearrangement is intriguing because it does not involve release of CO2. The rearrangement therefore occurs through an intramolecular group transfer.]

1.3 METABOLISM OF ATROPINE IN MIRO-ORGANISMS

A number of micro-organisms has been described to be able to metabolize atropine. Kedzia et al (1961) investigated the expiration date of belladonna eyedrops. He found micro-organisms in many droppers that could breakdown atropine. Such micro-organisms were found even in air samples from the hospital in Danzig and in soil samples in the surroundings. In total 53 strains were isolated, 38 of them appeared to be Pseudomonas.

The microbial decomposition of atropine has been studied by Kackowski (1959) in a bacterium isolated from soil underneath the Datura stramonium plant. The bacterium is probably an Athrobacter as mentioned by the author. In the extract of the bacterium, grown with atropine as carbon source, it was possible to demonstrate the presence of an atropine esterase: the products of hydrolysis tropic acid and tropine could be identified. Further data suggested the conversion of tropic acid in atropic acid (2-phenylacrylic acid) and the demethylation of tropine.

Niemer and Bucherer (1959, 1961) isolated a Corynebacterium named "belladonnae", able to utilize atropine as source of carbon. These authors demonstrated in this bacterium the presence of an atropine esterase and a dehydrogenase involved in the conversion of tropine into the corresponding ketone. A small amount of phenylacetic acid could be isolated from the growth medium that might have been formed by decarboxylation and dehydrogenation of tropic acid.

Also Jindra and Čihak (1963) communicated to have isolated from soil a Coryne bacterium "belladonnae" able to use atropine for its needs for carbon and nitrogen. Further details could not be found.

Published literature on the breakdown of atropine seems to be limited to the description of the hydrolysis into tropic acid and tropine; the atropine esterase involved has been demonstrated in a range of organisms. Literature data provide hardly any insight in the metabolism of tropic acid. No data are available regarding enzymes, involved in this metabolism.

CHAPTER 2

MATERIALS AND METHODS

2.1 NOMENCLATURE

Tropic acid has an optic active carbon atom. Therefore, atropine exists in two optical isomers. The name atropine is used for the racemic mixture; the optical isomers are (+) and (-) atropine, the latter is the isomer as it is found in Nature. The name hyoscyamine is also used for either of the stereo-isomers of atropine, but will not be used in this thesis.

The name tropic acid is used for the racemic mixture; the optical isomers are (+) and (-)tropic acid. The (-)tropic acid is the acid component of (-)atropine and is in the (S)-configuration.

As far as the systematic name of a compound is not being used, please see the chemical structures in the substrate-specificity table in chapter 5 and in the scheme in annex 1. Organic acids are named as the acid irrespective the degree of dissociation during the experimental conditions used.

The four enzymes atropine esterase (AtrE), tropic acid dehydrogenase (TDH), 2-phenylmalonic semi-aldehyde decarboxylase (PDC) and phenyl acetaldehyde dehydrogenase (PDH) as a group are indicated as the tropic acid enzymes.

Abbreviations of enzymes, compounds etc. trace back to their English names. Abbreviations like Atr for atropine and Tro for tropic acid are used exclusively to describe the phenotype of mutants.

2.2 MATERIALS

The (+) isomer of atropine, (+) and (-)tropic acid, the 3- chidinuclidinyl benzilate have been made available by Dr. H.L. Boter (Chem. Laboratory, National Defense Research Organization TNO); (-)atropine was obtained from the Nutritional Biochemicals Corporation U.S.A. The N-methyl iodide of (-) and (±)atropine was made available by Dr. F. Berends MBL; soman by Dr. P Christen, MBL. Technical tropic acid was bought from Mac-Farlan Smith Ltd (Schotland) and recrystallized twice from water.

The synthesis and identification of phenylmalonic semialdehyde is presented in chapter 3.

Phenylmalonic acid was isolated by alkaline hydrolysis of the diethyl ester and recrystallized from dichloroethane. Elementary analysis: found 59,83% C and 4.47% H, theoretical 60.00% C and 4.48% H.

The recipe for synthesis of the methyl ester of benzoic acid (Vogel 1959) was used as a guide to produce the methyl ester of tropic acid. Boiling point 149-152^0/12 mm, n_D^{20} 1.5218; literature boiling point 159-162^0/19mm (Beilstein E I 10 page 115. Phenylacetaldehyde provided by Aldrich Chem Co (Milwaukee U.S.A.) was used as such as carbon source; in case of enzymological research, it was distilled before use at reduced pressure under nitrogen gas. Boiling point 79-80^0/12 mm, n_D^{23} 1.5240; literature boiling point 85-90^0/18mm, $n_D^{19.6}$ 1.5255 (Beilstein E II 7, page 226).

Streptomycin, chloramphenicol, NAD^+, NADH were purchased at the Dutch Gist and Spiritus Factory (Delft); $NADP^+$ and LDH (from rabbit muscle) at Boehringer (Mannheim); hexokinase (type II from yeast) at Sigma (St.Louis U.S.A.); pyridoxal phosphate and 3-phenyllactic acid at Mann Research Lab Inc (New York); p-Cl-mercuri-benzoic acid at Bios Lab (New York); dithiotreitol at Calbiochem (California); (±)atropine at Brocades Stheeman (Amsterdam); scopolamine at Pharmacy Kipp (Delft); homatropine hydrobromide and atropine methylnitrate at the Amsterdamse Chinine Fabriek (Amsterdam); atropic acid and pseudo tropine at K&K Laboratories (California); benzaldehyde stabilisé at U.C.B. (Brussels); o-Cl benzaldehyde and 2-phenylpropionic acid at Schuchardt (München); vanillin and isovanillin at EGA –Chemie (W. Germany); phenylacetic acid, saponine (white) and o-nitrobenzaldehyde at The British Drughouse (London); silica gel G (according to Stahl) and epoxystyrol at E. Merck (Darmstadt); rhodamine at E. Gurr Ltd (London); all other less common chemicals were obtained from Aldrich Chem. Co (Milwaukee, U.S.A.) or from Fluka AG Buch SG (Switzerland).

Dialysis tube used was Visking tube 24/32"; DEAE-cellulose was provided by Serva (Heidelberg); Sephadex G100 by Pharmacia (Uppsala); Muncktell's cellulose powder by Grycksbo-Pappersbruk AB (Sweden).

2.3 ISOLATION OF PSEUDOMONAS PMBL-1

The Pseudomonas bacterium, able to grow with atropine as sole source of carbon was isolated by Rörsch and Berends (MBL) from a soil sample taken around the roots of Atropa belladonna L in the botanical garden of the Technical University in Delft, The Netherlands.

The soil was mixed with a synthetic medium (2.4) with atropine as sole carbon source and held for several days at 30^o and 37^O. After 3 days, growth was observed at 30^o. From the film at the surface, a bacterium was isolated able to grow on atropine as sole carbon source. The bacterium was identified as Pseudomonas by Dr. H.C. Bartlema (MBL). It is a Gram negative rod, it is motile thanks to one or more polar flagellae and it produces a green fluorescent pigment during growth in glycerin bouillon. The bacterium grows optimal at 28-29^o, is not

chemo-autotrophic and exhibits only oxidative degradation of carbohydrates added. In an elaborated determination according to the method of Stanier (1966), this strain has been classified by Wensinck (1969) as a Pseudomonas biotype A. The strain has been deposited in the strain collection of the MBL under the code number PMBL-1.

Eight other Pseudomonas species able to breakdown atropine have been isolated by Rörsch at a later stage of this project. These were isolated from garden soil of the MBL and from soil taken at the Botanical Garden of the University of Leyden. The properties of these strains – as far as known and related to the atropine metabolism - will be discussed in chapter 9. In this context, it is of interest to mention that none of the Pseudomonas species – already present in the bacterial collections of the MBL or in that of the Technical University Delft – is able to metabolize atropine.

2.4 CULTIVATION OF THE BACTERIA

The synthetic medium used for the cultivation of Pseudomonas contained per liter distilled water: 1 g NH_4Cl, 6 g Na_2HPO_4, 5 g KH_2PO_4, 0.5 g NaCl, 0.2 g $MgSO_4.7aq$, 11 mg $ZnSO_4.4aq$, 1.5 mg $MnSO_4$, 5 mg $FeSO_4.7aq$, 0.4mg $CuSO_4.5aq$,0.25 mg $Co(NO_3)_2.6aq$, 0.2 mg $Na_2B_4O_7.10aq$, 0.2 mg $(NH_4)_6Mo_7O_{24}.4aq$ and 2.5 mg EDTA (Cohen, Bazire et al. 1957).

The carbon source of choice was added immediately before use. The final pH was 7.0 - 7.1. This synthetic medium has been also used with 1.5% agar as solid medium. The compounds to be tested as carbon source or inducer were dissolved in water if possible, neutralized to pH 7.0 and sterilized by Seitz filtration. Compounds which dissolved very poorly in water were added as such to the medium.

Small quantities of bacteria were cultivated in fluid synthetic medium in flasks kept at 29^0. The flask was filled to a maximum of 15% with medium and shaken thoroughly (± 80 strokes per minute). Sterile air (100-200 ml/min) was blown on the culture when it was expected that the absorption at 700 nm (A_{700}) would exceed 0.8 during cultivation. The A_{700} was routinely used to estimate the concentration of the bacteria in the culture. This was measured using a Zeiss spectrophotometer or a Vitatron Colorimeter (703 nm filter). For very accurate measurements, a counting chamber was used. In case the medium became turbid caused by the insolubility of a compound, counting plates were used. At the end of the cultivation, the culture was cooled down to 4^0; the bacteria were collected by centrifugation during 10 min at 7000 x g and extracted as described in 2.10.

Larger amounts of Pseudomonas PMBL-1 were cultivated in a 25L reactor in synthetic medium with 0.2% tropic acid as the carbon source. In this way

80 – 100 g Pseudomonas could be produced in one run. Bacteria were collected by centrifugation and extracted (2.10) or stored at -20^0.

2.5 THE ISOLATION OF PSEUDOMONAS MUTANTS

In order to treat PMBL-1 with a mutagenic agent, it was inoculated into a Fluid synthetic medium with 0.1% glucose. After overnight cultivation, the bacteria were transferred 1:10 to the same medium. At A_{700} = 0.9 the culture (5 ml) was mixed with 1 ml of a N-methyl-N'-nitro-N-nitrosoguanidine solution (1 mg/ml in HMP) and 4 ml synthetic medium. The mixture was shaken calmly at 30^0 during 45 min. The fraction surviving bacteria after this treatment was about 10^{-3}. The bacteria were collected by centrifugation, suspended in 0.1% glucose in synthetic medium and divided in 40-80 subcultures for growth overnight with efficient aeration. In the next step, the cultures were plated on a solid medium with glucose as carbon source in a sufficient dilution to obtain a so-called mother plate with about 100 colonies after cultivation. Next, each mother plate was used to make a number of prints on a daughter plate using the replica-plating method (Lederberg and Lederberg 1952). By the right choice of the carbon source in these daughter plates, it was possible with this technique to investigate large numbers of bacteria on possible disturbances in their pattern of growth. Colonies with a modified growth pattern were purified by plating and subsequently several times tested for their special behavior. If the observed abnormality could be confirmed, the mutant was added to the mutant collection of the MBL. It was given an isolation number preceded by the code PMBL. From each subculture, only one mutant with a certain phenotype was kept; one can almost exclude that mutants with different isolation number have acquired an identical change. All mutants used in this project originate from PMBL-1.

The mutants disturbed in the breakdown of phenylacetic acid have been listed in table 3.1. Out of these mutants, PBML-107, PMBL-112 and PMBL-114 with the phenotype $Tro^-Pac^-Php^+$ have been used for experiments shown in fig. 4.4. and 4.7. The other mutants will be mentioned in table 10.7.

In a proper mutagenic treatment, one should start from a culture that came from one pure colony. At the start of this research, this precaution was not taken. A culture was used with a history of several months at 4^0 on solid medium. After mutagenic treatment, an unusual number of mutants with the phenotype $Atr^-Tro^-Tpn^-$ was observed (more than 5%). It was found that many of these mutants were already present in the culture prior to the mutagenic treatment. These mutants seem to have been formed spontaneously from the wild type. Starting the mutagenic treatment from one well identified colony resulted in 0.1% Atr^- mutants.

The spontaneous mutation to the Atr^- Tro^- Tpn^- phenotype might be linked to an extra-chromosomal location of these characteristics; however, experimental data that support this suggestion have not been found. The Atr phenotype seemed not to be sensitive to ethidium bromide or acridine orange treatment, the Atr^- mutation is not coupled with a resistance for antibiotics and highspeed equilibrium centrifugation* did not result in the demonstration of satellite DNA.

2.6 ASSAY OF THE OXYGEN CONSUMPTION AND THE CO_2 PRODUCTION.

The amount of oxygen consumed by Pseudomonas cultures was quantified using the manometric technique according to Warburg (Umbreit et al. 1951). The bacteria were cultivated in synthetic medium with 0.2% carbon source. The cultures were harvested in the logarithmic growth phase and incubated for an additional 4 hours in a synthetic medium in the absence of a carbon source. As a result, endogenous reserves had been consumed, the spontaneous oxygen consumption was reduced.

The main compartment of a Warburg mini container was loaded with 2.7 ml suspension containing bacteria (2-3 mg dry weight), the side compartment with 0.2 ml 2.5% carbon source and the middle compartment with a small piece of filter paper containing 0.1 ml 10 N potassium hydroxide. Pure oxygen was passed through during 15 minutes. The Warburg mini containers were closed and shaken in a thermostat bath of 30^0 with a frequency of 120 strokes per minute. The change in the internal pressure, caused by the spontaneous oxygen uptake, was followed during 20 min. Next the bacteria and the carbon source were mixed. The change in the pressure was observed as a function of time.

The amounts of oxygen consumed were calculated from the change in pressure measured and the manometer constant for oxygen; the data were corrected for the oxygen uptake in the absence of carbon source. In separate experiments, it was confirmed that the amount of carbon source was in excess related to the amount of micro-organisms used. The oxygen consumption of every bacterial culture was measured in duplicate. The average values of these duplicate observations that usually showed only minor discrepancies have been plotted in the graphics in chapter 4.

The same method was used for the assay of the CO_2 production during dehydrogenation and decarboxylation of tropic acid by the enzymes TDH and PDC. The incubation mixture was deposited in the main compartment; the side compartment contained 0.2 ml 4N H_2SO_4. After perfusion using nitrogen gas during 5 min, the mini containers were closed and shaken during 60 min at 30^0. This incubation was carried out at pH 8.5; in this condition, the CO_2 produced remained as bicarbonate in the solution.

*this experiment has been carried out by C. Knijnenburg MBL

By the addition of the sulfuric acid, the carbon dioxide was liberated from the incubation mixture. The resulting change in pressure and the equipment constant of the manometer for CO_2 were used to calculate the amount of CO_2.

2.7 THIN LAYER CHROMATOGRAPHY

This technique was used to identify atropine metabolites extracted from a cultivation medium or obtained by enzymatic conversion in vitro.

To start extraction, the medium was acidified to pH 2 using concentrated HCl and subsequently extracted three times with a suitable amount of diethyl ether. Etheric extract was dried using anhydrous Na_2SO_4 and concentrated in vacuo.

Silica gel G was used as stationary phase in thin layer chromatography (Stahl, 1957); 30g silica gel was mixed thoroughly with distilled water using a Waring Blendor and subsequently spread on a glass pate of 20 x 20 cm with a spreading device in a layer with a thickness of 0.25 mm. Prior to use the plates were heated during 60 minutes at 110^0.

Samples were deposited preferentially in a solvent with more elutive properties compared with the liquid phase, in order to reduce the amount of material remaining on the starting spot to a minimum. Liquid phases used:

EMX:	Ether : Xylene : Formic acid : Water	=	50 : 30 : 10 : 3	(v/v/v/v)
BEM:	Benzene : Ethyl formate : Formic acid	=	75 : 24 : 1	(v/v/v/)

Prior to the chromatography, the plates were brought into contact with the vapour phase in the chromatography tank; thereafter the process was started by addition of more liquid phase.

The liquid phases EMX and BEM have a large resolution power for the aromatic acids. The R_f values of some particular compounds are in EMX and BEM respectively: 3.4-dihydroxyphenylacetic acid 0.47 and 0.10; tropic acid 0.50 and 0.21; phenylglyoxylic acid 0.64 and 0.33 resp.; phenylacetic acid 0.70 and 0.55 resp.; phenylacetaldehyde 0.83 and 0.85 resp.

The thin layer plates to be used for chromatography of phenylacetaldehyde were run first with one of the two liquid phases and dried at 110^0. This pretreatment was required because freshly distilled phenyl acetaldehyde shows 3-4 spots with either of the liquid phases. This compound is apparently sensitive for a contamination in the silica gel.

The liquid phase CD and BAW were used for the chromatography of atropine and tropine

CD:	Chloroform : Diethylamine	=	90 : 10	(v/v)
BAW:	n Butanol : Acetic acid : Water	=	4 :1 :5	(v/v/v)

Detection was carried out by spraying with one of the following reagents:

1. Bromocresol green, dissolved in weak alkaline ethanol: acids show up as yellow spots in a blue background.
2. 2.4-Dinitrophenylhydrazine, dissolved in 10% H_2SO_4 in ethanol and then diluted 1:3 with distilled water: aldehydes and ketones show up in an orange-yellow color.
3. Fluoresceïn or rhodamine dissolved in ethanol. The plates were observed under an UV lamp (254 nm); many compounds appear as dark spots against a shining background.
4. Equal parts concentrated H_2SO_4 and HNO_3, thereafter heated 20-30 min at 170^0. This variant of the well-known H_2SO_4 destruction has been developed to detect tropic acid. In the standard destruction, this compound only turns black once the whole plate turns into dark brown. This method has been used many times for the detection of atropine, tropic acid and phenylacetic acid. Benzoic acid, benzaldehyde and phenylacetaldehyde cannot be detected in this way due to their volatility. These aldehydes can be detected with this reagent if the plate is first sprayed with 2.4-dinitrophenylhydrazine.

Preparative thin layer chromatography was carried out by applying the material in a broad band. After chromatography only a small vertical strip was used for detection. The relevant areas were scratched from the plate and extracted.

2.8 ISOLATION OF ^{3}H TROPIC ACID; UPTAKE ^{3}H TROPIC ACID IN PSEUDOMONAS

^{3}H-Atropine 0.1 mg (a-specifically labeled; 172 mCi/mMol; 0.6 mCi/mg; Radiochemical Centre Amersham England) was completely hydrolyzed using AtrE. Tropic acid and tropine 10 mg each were added as carriers. The products of hydrolysis were separated using Dowex-50 column (2 ml) equilibrated with 0.1 M ammonia formate (pH7.0). Tropic acid was eluted with the ammonia formate, while tropine remained bound to the ionexchange material. The radioactivity of the eluate corresponded for more than 99% with tropic acid. This was demonstrated by thin layer chromatography (2.7), followed by the counting of the radioactivity (see below) in the silica gel zones. The eluate was used as such for the experiments with ^{3}H-tropic acid.

To estimate the ^{3}H uptake, a bacterial culture was used in the logarithmic growth phase. This culture was washed with and suspended in synthetic medium and diluted to A_{700} = 1.5. This suspension (1 ml) was mixed with 0.2 µmol ^{3}H tropic acid (± 1.6 x 10^5 disintegrations per minute, dpm) and incubated during 60 min at 30^0. The incubation was terminated by cooling to 4^0. Thereafter, 10 µmol non-labelled tropic acid and a 10 fold amount of carrier bacteria were added.

In the next steps, the bacteria were washed in synthetic medium, centrifuged and lysed. Lysis was accomplished by incubation with 0.02 ml lysozyme (2mg/ml) during 15 min at 37^0, followed by addition of 0.07 ml SDS 20%. The lysate was filled up with water up to ± 0.4 ml and transferred to counting bottles containing 11 ml scintillation fluid (2.1 ml Triton X-100, 8.9 ml toluene with 0.5% 2,5-diphenyloxazole and 0.005% 1,4-bis-2-(5-phenyloxazolyl)benzene. The radioactivity was counted using a Mark-I scintillation counter (Nuclear Chicago)

2.9 ENZYME ACTIVITY ASSAYS

2.9.1 General

The unit of enzyme activity, according advice of the Commission of Enzymes (1961), is the amount of enzyme able to catalyze the conversion of 1 µmol substrate per minute at 25^0 under defined conditions. These conditions are defined for the AtrE in 2.9.2, for the enzymes TDH, PDC and PDH in 2.9.3.

The enzyme activity of a sample is expressed as the number of units (U) per ml. The total enzyme activity (TA) is the total of units present in the sample. The specific activity (SA) is the number of units per mg protein. The total enzyme activity before and after a purification procedure is the basis for the calculation of the yield. The specific activity is an indication of the purity of the sample.

The protein content in extracts of bacteria was measured using the biuret method (Layne 1957), in case of low protein concentration according to Lowry (1951). The method of Lowry was used for purified enzyme samples as well (2.11). For a rough estimation of the protein concentration in separate fractions after chromatography or electrophoresis, the relation A_{280} = protein concentration in mg/ml was used.

2.9.2 Quantitative assay of the AtrE-activity

The assay of the activity of the AtrE was done by acidimetric titration of the liberated tropic acid at constant pH. In this assay, pH stat equipment (Radiometer Autotitrator TTT-1b and Titrigraph SBR-2c) was used that can add and register automatically the amount alkaline per unit of time required to keep pH constant.

The normal activity assay was carried out at pH7.0 and 25^0 in 10-25 ml 0.4 mM (-)atropine, 0.1 M KCl and 0.02% saponin. After addition of 50-100 µl of an enzyme sample, the acid production was followed during 5-10 min. The amount of enzyme able to hydrolyze per min under these conditions 1 µmol (-)atropine was defined as the unit of activity.

The activity of AtrE in this routine assay has been shown over a large range

to be directly proportional with the amount of enzyme added (see fig 2.2).

The spontaneous hydrolysis of atropine in this solution is very small and can be usually ignored. In assays in enzymological research at high pH, with other substrates or at very low enzymatic activity a correction was applied for this spontaneous consumption of alkali. In addition, nitrogen gas was lead over the incubation mixture to prevent disturbance of the titration by carbon dioxide.

2.9.3 Quantitative assay of the TDH-, PDC- and PDH- activity

The determination of the activity of TDH, PDC and PDH is based on conversion of NAD^+ in NADH; the latter compound has a specific absorption at 340 nm. This absorption can be used to follow this conversion spectrophotometrically.

The composition of the incubation mixtures used to assay the four enzymes is presented in table 2.1. For each assay, 2 ml incubation mixture was used. After addition of 10-100 µl enzyme, the volume was adjusted to 3 ml with distilled water. The increase in the absorption was measured in a quartz cuvette with a light path of 10 mm in a Zeiss spectrophotometer (PMQ II). In this PMQ II, an exit resistance was removed allowing the direct connection of a logarithmic Vitatron recorder (Vitatron UR-100). Below an absorption of 0.8. the difference between the spectrophotometer measurement and the reorder registration was less than 0.005 absorption units.

Table 2.1

Incubation mixtures for the assay of TDH PDC and PDH activity

TDH	PDC	PDH
50 mM K-carbonate pH 9.5	50 mM K-phosphate pH 8.5	50 mM K-carbonate pH 9.0
15 mM Tropic acid (K-salt)	15 mM Tropic acid (K-salt)	--
1.2 mM NAD^+	1.2 mM NAD^+	1.2 mM NAD^+
375 mM Hydrazine-HCl pH 9.5		

The increase in absorption was measured at 25 ± 1^0; the incubation mixture was placed in a thermostat bath prior to the measurement. The bath kept the cuvette house and the cuvette holder of the spectrophotometer on the same constant temperature as well.

The molar absorption coefficient of NADH at 340 nm is 6.22 x 10^3 $M^{-1}cm^{-1}$. When 1 µmol NADH is produced in a 3 ml cuvette with a light path of 1 cm, the A_{340} will increase with 2.07. The production of NADH in µmol /min can be calculated from the increase in absorption per unit of time. The incubation mixture itself has an A_{340} of ± 0.05. Where needed, a correction was made for this back ground value.

The standardized assay of TDH activity was carried out as described above. The A_{340} was registered during minimal 4 min. In the investigation of the enzymological properties of the TDH (chapter 6 and 7), the hydrazine was omitted from the incubation mixture, unless stated otherwise. All experiments were corrected for the increase of A_{340} in the absence of substrate.

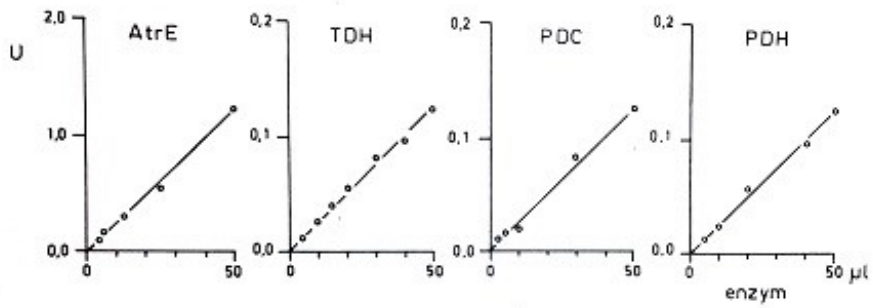
In the assay of PDH activity, 20 µl 20 mM phenylacetaldehyde (in acetone) was added to the NAD^+ incubation mixture in the cuvette. Next enzyme solution and water was added to a final volume of 3 ml. The A_{340} was registered during at least 4 min. Acetone in the amount used has no effect on the PDH assay. The increase of A_{340} was corrected for the increase in absence of substrate except in those cases where phenylacetaldehyde was added in order to stabilize the enzyme.

In the assay of PDC, 0.8 U TDH was added to the 2 ml incubation mixture. Next, distilled water was added to a final volume of 3 ml. The solution was incubated in the spectrophotometer at 25^0. The A_{340} was registered. Once this absorption had reached a value of about 0.25, PDC sample volume of 10-50 µl was added. The absorption was followed for another 5 min. In a control experiment, the increase of absorption was measured in the absence of PDC. PDC activity was calculated from the difference between these 2 observations. The unit of PDC is the amount of enzyme that under these conditions an extra increase in absorption effectuates of 2.07/min. In case the PDC sample contained PDH as well, the sample was incubated prior to the PDC assay at 55^0 during 15 min in order to inactivate the PDH. Denatured protein, as far it was present, was removed by centrifugation. The SA was calculated on basis of the initial protein concentration.

The relation between enzyme activity measured and the amount of enzyme added is directly proportional over a long range in case of the enzymes AtrE, TDH, PDC and PDH. (See fig 2.2).

Fig 2.2

Relation between the added amount of enzyme (µlitre) and the activity U measured



2.10 ASSAY OF THE SPECIFIC ENZYME ACTIVITY IN EXTRACTS OF PMBL-1 AND MUTANTS

The specific activity of the four tropic acid enzymes was investigated in bacteria grown in synthetic medium (chapter 2.4) with 0.08% phenylglyoxylic acid as carbon source and inducer (see chapter 10.5).

The bacteria were cultivated overnight with 0.1% succinic acid and 0.025% phenyglyoxylic acid. The next morning, an inoculum of the culture was transferred in synthetic medium with 0.08% phenylglyoxylic acid. An estimation was made of the doubling time. In the evening, the actual culture was started. A calculated amount of bacteria was used as the inoculum so the end of the logarithmic growth phase would be reached the next morning (A_{700} ± 0.9). The cultures were cooled with ice to 0-4^0 30 – 90 min after reaching the stationary growth phase and centrifuged at 7000 x g during 10 min. Next, the bacteria were suspended in 1 mM EDTA, 1 mM ME, 0.05 M SDS in 5-20 ml 50 mM K-phosphate pH 7.0.

The enzymes were extracted by means of an ultrasonic treatment during 5 Min with a MSE 100 Watt Ultrasonic (no 7100) adapted with a double walled reactor compartment that kept the temperature on 0-4^0. During this procedure, the A_{700} was reduced to less than 5% of the initial value.

The effect of the duration of the ultrasonic treatment on the yield of extraction was investigated as well as the inactivation of the enzymes as the result of this treatment. In case of the enzymes AtrE, TDH, PDC and PDH, 3 min extraction appeared to be sufficient. The enzymes resisted the ultrasonic treatment during 5-10 min.

The extract was centrifuged at 105.000 x g during 60 min. The enzyme activities and protein concentration, were measured in the supernatant, taken

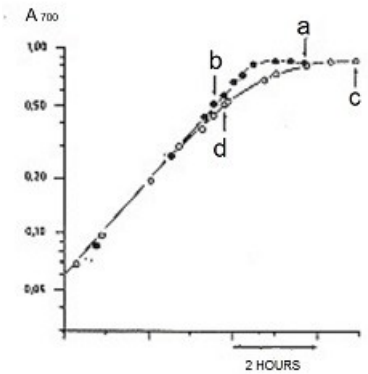
by pipette from the upper part of the centrifuge tube. The specific enzyme activities were calculated from these data. The assay of the enzyme activities was carried out as soon as possible following the extraction.

Not-centrifugated extract contains a NADH-oxidase that causes disturbance of the spectrophotometric assay of TDH, PDC and PDH. Hegeman (1966) has reported the binding of the NADH-oxidase in Pseudomonas ATCC 12633 to a particulate fraction. This has been confirmed for PMBL-1: the NADH oxidation activity is negligible after centrifugation.

The preparation of the extract, the assay of the enzyme activities and the assay of the protein concentration are reliable and have no larger variation than 5% in the quantification of the tropic acid enzymes. The conditions during the phase of growth at the time of harvesting have a significant effect on the specific activity. This is shown in the experiments shown in figure 2.3 and table 2.4. The specific activities in the logarithmic growth phase and the stationary growth phase were compared. The effect of less favorable aeration was studied as well.

Fig 2. 3 and Table 2.4

Effect of the growth phase and aeration on the specific activities of the tropic acid enzymes.



Culture	AtrE	TDH	PDC	PDH
a	1.09	0.365	0.530	0.190
*	1.06	0.350	0.540	0.205
b	0.66	0.285	0.440	0.125
*	0.53	0.250	0.345	0.100
c	1.67	0.450	0.700	0.250
*	1.51	0.435	0.600	0.240
d	0.99	0.380	0.570	0.155
*	0.93	0.365	0.505	0.160

A_{700} : Growth of PMBL-1 in synthetic medium with 0.2% phenylglyoxylic acid.
•— • with extra air conducted over the culture *specific activity of tropic acid
o — o without extra aeration enzymes in duplicate

The specific activity of the enzymes in the cultures harvested at ”a” and “c” (stationary growth phase) and at “b” and “d” (logarithmic growth phase) is shown in table 2.4.

The specific activity is maximal if the cultures are harvested under sub-optimal aeration conditions in the stationary growth phase.

The specific activity in independently cultivated cultures of PMBL-1 in duplicate gives an impression of the reproducibility of these assays. In similar experiments it has been shown that the specific activity in the stationary growth phase remains nearly constant during 60-120 min. Thereafter, it starts to decrease gradually. In the determination of the specific activity of the tropic enzymes, the cultures have been harvested, for this reason, 30-90 min after the start of the stationary growth phase; extra air was conducted over the culture, because this is more reproducible in practice compared with cultivation under sub-optimal aeration conditions.

2.11 PURIFICATION OF THE TROPIC ACID ENZYMES

All purification steps were carried out at 0-4^0. Purification was carried out using about 100 g wet weight Pseudomonas bacteria (2.4).

2.11.1 Purification of AtrE, Precipitation with streptomycin sulfate

Extraction: the bacteria were thawed and suspended under vigorous stirring in 900 ml 0.1 M potassium phosphate pH 7.0 (HMP). This suspension was treated by ultrasonic oscillation in portions of 50 ml in the 10 kcs Raytheon Oscillator (Waltham U.S.A.) during 5 min. The extract was centrifuged two times at 10.000 x g during 10 min and thereafter in the Spinco L-2 preparative ultracentrifuge (rotor 30) at 80.000 x g during 60 min. Enzyme activity and protein concentration were measured in the supernatant (2.9)

Nucleic acid material was removed by precipitation with streptomycin; this was added dropwise as a concentrated solution under vigorous stirring to a final concentration of 2%. Sediment was removed by centrifugation 14.000 x g 10 min.

Fractionated precipitation with ammonium sulfate

Precipitation was carried out with ammonium sulfate after adding K-phosphate buffer to a final concentration of 50 mM. The extract was saturated subsequently with ammonium sulfate to 40, 55 and 85% saturation respectively by addition of 24.3, 9.7 and 22.9 g powdered ammonium sulfate per 100ml.

After each addition, the pH was readjusted to 7.0. About 30 minutes later, the extract was centrifuged at 14.000 x g during 20 min. The AtrE precipitated mainly as the consequence of the increase of the saturation from 55 to 85 %.

The 85%-precipitate was collected and refractionated by suspension in a 50% saturated ammonium sulfate solution during 30 min. In this step, most of the AtrE was dissolved whereas only less than half of the protein dissolved. After centrifugation, AtrE was precipitated with ammonium sulfate, resuspended in a minimal amount of buffer and dialyzed against 15 L 15 mM tris - HCl pH 8.1 during 48 hours. The dialysis buffer was refreshed after 24 hours. The dialysis tube was open at the upper end during the dialysis.

Column zone electrophoresis

After dialysis, the sample volume was reduced to 50% by lyophilization. After addition of 0.005% saponin, the sample was ready for preparative column zone electrophoresis according to Flodin (1956) and further designed by at the MBL by Dr. F. Berends.

Electrophoresis was carried out in a glass column 2.9 x 100 cm (660 ml), fitted at the lower end with a sintered glass filter. Cellulose powder was mixed in vacuo with electrophorese buffer: 30 mM tris 15 mm HCl 0.005% saponin pH 8.1. The column was filled with cellulose powder under 3 m water pressure. The cellulose has a stabilizing function only. It had been treated by the supplier in order to minimize the adsorption of protein.

The column was placed in a 8x100 cm column filled with buffer and connected with the anode compartment. The upper end of the inner column was connected to the cathode compartment. Differences in salt concentration and pH that could occur during prolonged electrophoresis were prevented by continuous mixing of buffer in the anode and cathode compartment. Once the sample was applied on the cellulose column, it was moved to halfway the column with ± 250 ml electrophoresis buffer. Then a voltage of 1500V was applied (±11 V/cm cellulose bed) during 40-48 hours; thereafter, the column with electrophorese buffer was eluted (30-40 ml per hour); the eluate was collected in fractions of 5 ml. The enzyme activity and protein concentration of each fraction was measured. Fractions with a relative high specific activity were collected and without further treatment used for the next chromatography.

DEAE cellulose chromatography.

The anion exchange material DEAE-cellulose was washed, equilibrated with HMP and poured into a glass column of 3 x 8 cm. After the sample was applied, the non-bound material was removed by elution using HMP. Next the column was eluted with 600 ml HMP with a linear salt gradient of 0.03 M KCl to 0.1M KCl. The rate of elution (30-40 ml/hour) was controlled using a pump. The AtrE was eluted at a salt concentration of ± 0.06 M KCl. The eluate was collected in fractionsof 5 ml.

Fractions with high specific activity were combined, dialyzed against HMP buffer and adsorbed in a small DEAE-cellulose column (2 x 2.5 cm). This small column was eluted with 0.4 M KCl, resulting in a concentrated sample (15-20 ml).

Gel filtration with Sephadex G-100

A glass column (diameter 6 cm) was filled with Sephadex G-100 to a height of ±30 cm (column volume ± 1050 ml), according to the instruction by Pharmacia Inc. The column was equilibrated with the elution buffer 0.1 M KCl in HMP. Protein-protein and protein-Sephadex interactions were prevented by the high salt concentration. These could give rise to a less efficient separation and considerable losses. The concentrated sample was applied, eluted (40 ml/h) and collected in 4.8 ml fractions. The purified enzyme sample was composed of fractions with a high specific enzyme activity of more than 500 U/mg protein.

2.11.2 Purification of THD, PDC and PDH

The purification of the enzymes TDH, PDC and PDH was started by the extraction and purification with streptomycin-sulfate identical to the start of the purification of AtrE.

The TDH was precipitated by raising the ammonium sulfate saturation from 45 to 55%. The TDH was then purified by gel-filtration using Sephadex G-100 and chromatography using DEAE-cellulose. These procedures made use of the same columns and elution fluids as those used for AtrE. The TDH was only partially separated from AtrE in case it was present as well.

The PDC was precipitated by bringing the ammonium sulfate saturation from 50 to 60%; the enzyme was eluted from DEAE-cellulose with a linear salt gradient 0.1-0.4 KCl in HMP. It was nearly completely separated from TDH if still present (chapter 7.2).

The PDH was purified in the presence of 50 mM K-phosphate pH 7.0, 1 mM ME, 1 mM EDTA, 0.05 mM SDS and 1 mM phenylacetaldehyde. The nucleic acid material was precipitated with streptomycin sulfate and precipitated by raising the ammonium sulfate saturation from 40 to 50%. In the chromatography over DEAE-cellulose, the enzyme was eluted with a 0 – 0.25 M KCl gradient in the buffer mentioned.

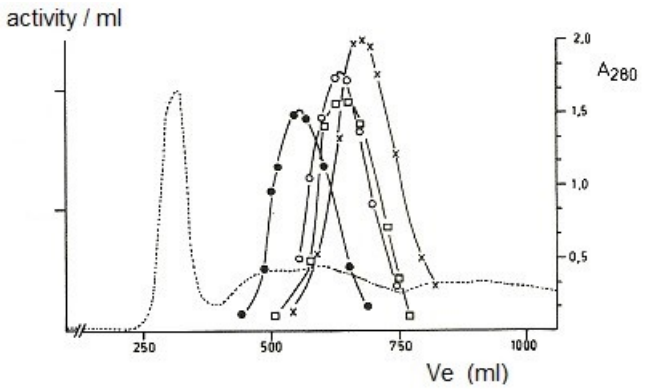
2.11.3 Estimation of the molecular weight of the tropic acid enzymes

The volume used to elute a protein during gel filtration is within certain limits linearly proportional with the negative logarithm of the molecular weight (MW);

this holds for globular proteins which are neither adsorbed by the column, nor for another reason as for their size are excluded by the Sephadex gel filtration column (Janson, 1967). This method has been used to estimate the MW of the enzymes AtrE, TDH, PDC and PDH. The gel filtration was carried out as described for the AtrE (2.11.1) with a sample that contained the 4 enzymes after fractionation with ammonium sulfate. The separation is shown in fig 2.5.

Fig 2.5

Separation of the tropic acid enzymes by gel filtration.



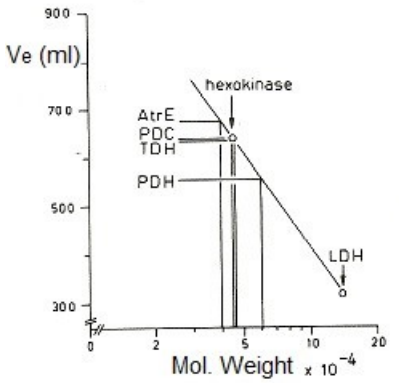
The enzyme sample (10 ml ammonium sulfate fraction 40-80% was applied on a Sephadex G-100 column and eluted with 0.1 M KCl in HMP. Enzyme activities in arbitrary units (on left Y axis) were assayed according to 2.9
Ve = elution volume (ml)

x----x	AtrE	(Ve = 675)
o----o	TDH	(Ve = 635)
□---□	PDC	(Ve = 640)
•-----•	PDH	(Ve = 560)
……..	A_{280}	(protein)

The enzymes lactic acid dehydrogenase (MW 140.000) and hexokinase (MW 45.000) (Schachman, 1963) were added as protein references (not shown). The LDH was detected after gel filtration by the NADH dependent hydrogenation of pyruvic acid; the hexokinase was assayed titrimetrically in the presence of ATP and Mg^{2+} according to Moor *et al.* (1968). The elution volumes (mean value from 3 experiments) were used to estimate the molecular weight of the enzymes AtrE, TDH, PDC and PDH on 39.000, 46.000, 45.000 and 60.000 respectively (fig. 2.6).

Fig 2.6

Estimation of the molecular weight from the elution volumes in gel filtration



The elution volumes V_e (ml) of the reference proteins (hexokinase and LDH) have been plotted against the logarithm of their molecular weight x 10^{-4} (X- axis). Elution volumes of the tropic acid enzymes (fig 2.5) have been used to estimate their molecular weights.

2.12 SPECTROSCOPIC AND CHEMICAL ANALYSIS

The Chemical Laboratory of the National Defense Organization, Rijswijk, The Netherlands provided excellent support in the spectroscopic and chemical analysis. Infrared spectroscopy for the elucidation of the structure of 2-phenylmalonic semi-aldehyde (pma, see chapter 3) was carried out by Drs. F.H. Meppelder and H.C. Beck.

The gas chromatographic assay of phenylacetaldehyde was performed using a Becker Gas Chromatograph no. 2558 by A. Verwey. The stationary phase was a column of 0.4 x 180 cm with 20% OV-17 on Chromosorb W-AW 60-80 mesh; the mobile phase was N_2 (pressure at the injection point 0.8 kg/cm^2) and H_2 (0.2 kg/cm^2); the temperature at injection was 210^0, the column temperature 130^0; flame ionization detection. Under the conditions mentioned, benzaldehyde, phenylacetaldehyde and 2-phenylpropanal have retention times of 5.8, 8.8 and 11.8 min. respectively.

Elementary analysis was done by N. Kramer. Freshly distilled organic solvents were supplied by F. A. A. Mitzka. For the recording of UV spectra, a Beckman DK-2 spectrophotometer was used. Melting points were assessed using a Büchi melting point microscope: heating speed max 0.5^0 per min.

2.13 ESTIMATION ENOL CONTENT OF PHENYLMALONIC SEMI-ALDEHYDE

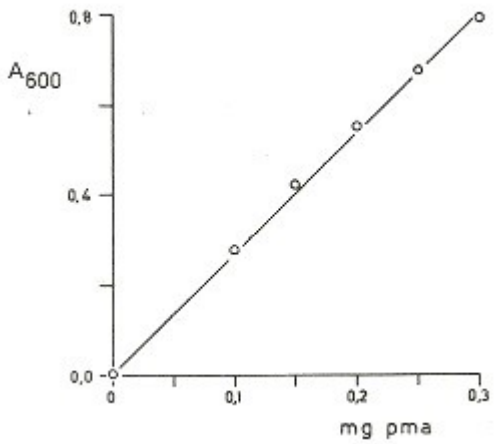
This assay is a variant of the Kaufmann and Richter method (1925) based on the colorimetric quantification of the complex of the enol compound with $FeCl_3$.

In a cuvette (light path 10 mm), 2.55 ml ferric chloride reagent (methanol with 1% $FeCl_3$) was mixed with 50-400 µL pma and adjusted to 3 ml with distilled water. The ferric chloride complex with pma was quantified by its absorption at 600 nm. The absorption was not constant however: usually a decrease was observed of 1-2 % per min. Therefore, the absorption was registered during 5 min. The absorption immediately after mixing of the enol-pma and the reagent was obtained by extrapolation.

A solution of pma in anhydrous diethyl ether was used in the assay to correlate the A_{600} and the amount of enol-pma. In this solvent pma is completely present in the enol form, as was concluded from the comparison with pma in carbon tetrachloride, that is for 100% in the enol form as apparent from spectroscopic analysis. Ether was used as a solvent because carbon tetrachloride was incompatible with enzymological experiments (see chapter 6 and 7).

The absorption at A_{600} is directly proportional to the amount of enol-pma added (fig. 2.7). The assay is not disturbed by 400 µl 0.5 M tris-HCl buffer pH 7.5 or by 400 µl water; phosphate buffer caused $FeCl_3$ to precipitate and was not used for this reason. The assay was carried out at 25^0. The reagent was brought on that temperature prior to the assay.

Fig 2.7 Assay of the enol content of a pma solution



Various amounts of pma dissolved in 0.4 ml diethyl ether were mixed with 2.55 ml ferric chloride reagent. The absorption at 600 nm was recorded during 5 min. The absorption after extrapolation to time t = 0 is plotted against the amount of enol-pma added.

CHAPTER 3

SYNTHESIS AND PROPERTIES
2- PHENYLMALONIC SEMI-ALDEHYDE

3.1 INTRODUCTION

During the study of the metabolism of atropine, indications were obtained for a role of 2-phenylmalonic semi-aldehyde (pma, 3-oxo-2-phenyl propanoic acid) (see fig. 3.1 V) as intermediate in this breakdown. It appeared highly desirable to have the disposal of this compound. However, in the large handbooks one will search in vain for references with information about the properties and synthesis of this compound in spite of its rather simple structure. The methyl and ethyl ester of pma have been synthesized but the acid in free form would be not stabile and was therefore considered to be unknown (Rodd 1956).

An attempt to synthesize the pma by oxidation of tropic acid using alkaline potassium permanganate resulted in a product which was able to induce the tropic acid enzymes. Further analysis revealed that not pma but phenylglyoxylic acid was synthesized. By this lucky coincidence, a gratuitous induction of the tropic acid enzymes was discovered, that will be discussed in chapter 10.

Later in this research, one single reference was found in the literature which describes the synthesis of pma (Strukov 1952). The paper provides only limited information on the nature of this product. It mentions the melting point and the spontaneous decomposition in water in phenylacetaldehyde and carbon dioxide.

Synthesis of pma has been carried out according to this recipe. The reaction product was identified as pma in the enol form (enol-pma).

The keto-enol tautomerism of pma appeared to have a major role in studies of the effect of this compound on the enzymatic dehydrogenation of tropic acid. Therefore, the tautomeric properties of pma have been studied more in detail. A quantitative colorimetric assay of enol-pma was designed. It was possible to demonstrate that the pma disappears in ethanol at 0^0 C. whereas infrared analysis showed the simultaneous formation of the keto form of pma (keto-pma). The tautomeric rearrangement has been studied in aqueous conditions as well. These results have been very relevant to explain the kinetics of the enzymes TDH and PDC.

3.2 THE SYNTHESIS OF THE 2-PHENYLMALONIC SEMI-ALDEHYDE

The pma (V) is synthesized starting from phenylacetic acid according to the reaction scheme in fig 3.1. Properties of intermediate compounds and of the final product are listed in Table 3.2

Fig.3.1
Synthesis of 2-phenylmalonic semi-aldehyde (pma)

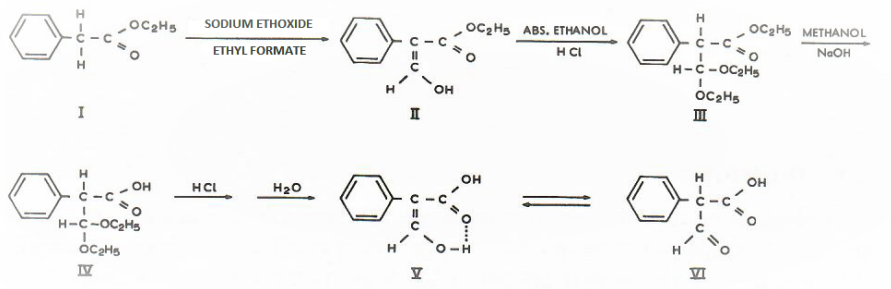


Table 3.2
Properties of the products in the synthesis of pma
b.p. = boiling point m.p = melting point

Compound	Experimental	Literature or calculated value
II ethyl ester of pma	b.p. 124-126^0 / 12 mm n_D^{23} 1.5312	b.p. 139-140^0 / 16 mm n_D 1.5322 (Beilstein)
III diethyl acetal of ethyl ester of pma	b.p 142-144^0 / 6 mm n_D^{24} 1.5318	b.p. 166-168^0 / 20mm (Strukov, 1952)
IV diethyl acetal of pma	m.p, 127.5-128 ^{0} C-H-O analysis 65.50% C 7.58% H 27.21% O	m.p. 121-123^0 (Strukov 1952) C-H-O analysis 65.53% C 7.61% H 26.86% O
V pma	m.p. 85-94^0 (decomposition) C-H analysis 66.02% C 5.02% H	m.p. 101-103^0 (decomposition) (Strukov 1952) C-H analysis 65.85% C 4.91% H

Phenylacetic acid was converted in its ethyl ester (I); the ethyl ester of pma (II) was made by condensation with ethyl formate in sodium ethoxide, according to the method of Friedman and Gladych (1956). The product was purified by extraction and distillation.

Compound II mixed with ferric chloride dissolved in methanol reacted with a violet color that disappeared after the addition of bromine water. This indicated that compound II was present in enol-form either totally or partially.

Further synthesis was according to Strukov (1952). Compound II dissolved in dry ethanol was converted in the diacetal of the ethyl ester of pma by perfusion using dry hydrochloric vapor. After neutralization with alcoholic lye, compound III was isolated by distillation under reduced pressure. Next the ester bond was hydrolyzed using 2 N NaOH in 80% methanol. The diethyl acetal of pma (IV) was sedimented after acidification, recrystallized from toluene and identified based on the melting point and elementary analysis.

The pma was obtained by stirring compound IV in concentrated HCl at 0^0, followed by addition of an equal volume distilled water. The product was collected on a filter, thoroughly washed with ice water, washed with petroleum ether (boiling point 40-60^0) and dried in vacuum.

3.3 IDENTIFICATION OF 2-PHENYLMALONIC SEMI-ALDEHYDE

The elementary analysis of the synthesized product (table 3.2) correlates well with the theoretically expected values. The compound reacts with $FeCl_3$ in ethanol forming a violet complex. This is an indication that the compound is completely or partially in the enol-form. In water decomposition occurs under the formation of the characteristic odor of phenylacetaldehyde.

The final identification of the pma was based on the analysis of the infrared and nuclear magnetic resonance spectra, on the gas chromatografic identification of the decomposition product as phenylacetaldehyde and biochemical evidence (this thesis chapter 6 and 7) The melting point, the only property described by Strukov was not suitable for characterization (3.3.1).

3.3.1 The decomposition range

The crude reaction product decomposes at the melting point (101-103^0) into phenylacetaldehyde and carbon dioxide (Strukov 1952). The product synthesized in this thesis project appeared to decompose at 85-94^0. After three recrystallizations using carbon tetrachloride as solvent, nice needle-form crystals were obtained, but the decomposition range remained 10-15^0 below the literature value. Small crystals appeared to decompose much earlier compared to the larger ones. It seemed that the crystal size could have a significant effect on the range of the decomposition. This was confirmed: a part of a large crystal was pulverized to

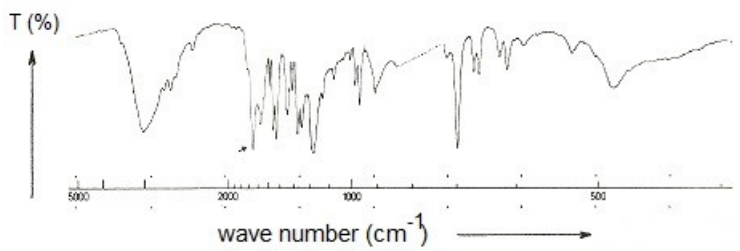
powder. The decomposition range observed for the crystal and the powder was 92-93^0 and 58-67^0 respectively.

It is clear that the melting or decomposition range of pma is not suitable as a parameter for its characterization; the discrepancy between the experimental values and those found in literature does not exclude that the pma compound synthesized by Strukov and the one made in this study are identical. However, Strukov's crystals must have been of respectable dimensions.

3.3.2 Infra-Red and Nuclear Magnetic Resonance spectroscopy

The results of the infra-red and nuclear magnetic resonance spectroscopy have been interpreted by Dr H. Meppelder. He concluded that the product synthesized is indeed the enol form of 2-phenylmalonic semi-aldehyde. His conclusion is based on the following observations. Fig 3.3 shows the infra-red spectrum of the recrystallized product dissolved in carbon tetrachloride. The spectrum of the solid compound is identical.

Fig 3.3
Infra-red spectrum of pma in carbon tetrachloride
X-axis: wavenumber (cm^{-1}); Y axis: transmission T (%)



If pma would have been synthesized as the keto form one would expect the characteristic absorption of the stretch vibration of the aldehyde C=O at 1740-1720 cm^{-1} and for the C=O of the carboxyl group at 1725-1700 cm^{-1}. Both absorptions are totally absent in the spectrum.

For pma in the enol-structure one expects to find the stretch vibration of the carbonyl C=O at an absorption of about 1648 cm^{-1}, which has been found by Friedman and Gladych (1956) for the C=O of the tropine ester of pma. The spectrum of pma shows a strong band at 1653 cm^{-1} and is nicely in agreement with the literature data mentioned. This frequency is abnormally low, because the normal value for an $\alpha-\beta$ non-saturated acid is about ± 1700 cm^{-1}: (the structurally related atropic acid C_6H_5-C(=CH_2)-COOH has a value of 1694 cm^{-1}).

The bathochromic shift could have been caused by the formation of an intramolecular hydrogen bridge (see fig 3.1 V), for which the hydroxyl group of the enol form is available.

The C=C in the side chain of atropic acid absorbs at 1620 cm^{-1}; for an enol structure of pma in which the hydroxyl group is involved in a hydrogen bridge, one may expect a lower value for the absorption of the C=C as well. So, the absorption at 1574 cm^{-1} can be attributed to the C=C bond. Therefore, the infra-red spectrum is not compatible with the keto form of the pma, but can be interpreted for that compound in the enol form.

The nuclear magnetic resonance spectrum lacks the characteristic absorption of the aldehyde group. It shows absorptions corresponding with the presence of an ethylene- and a hydroxyl- proton. A full confirmation of the enol structure of pma was obtained by comparison of its NMR spectrum with that of atropic acid and by a study of the effect of trifluoracetic acid.

3.3.3 Gas Liquid Chromatography

More proof of the identity of pma was obtained by the observation that During thermic decomposition phenylacetaldehyde is formed. This was investigated by gas liquid chromatography. A solution of pma in ether was injected in the gas chromatograph with the injection block at a temperature of 200^0. Only one product was found under condition as described in chapter 2.12; it had a retention time of 8.8 min, identical to that of the reference phenylacetaldehyde.

3.3.4 Biochemical indications

The enzyme tropic acid dehydrogenase is able to accelerate dehydrogenation of tropic acid as well as hydrogenation of pma. After enzymatic hydrogenation of pma, tropic acid was detected as product by thin layer chromatography (see chapter 6).

3.4 TAUTOMERIC REARRANGEMENT OF 2-PHENYLMALONIC SEMI-ALDEHYDE

3.4.1 Quantitative assay of the enol form

A quantitative assay for enol-pma was developed to explain the role of the tautomeric rearrangement of pma in the interpretation of the kinetics of the tropic acid dehydrogenase TDH. This is based on the quantitative enol assay, reported by Kaufman and Richter (1925) making use of the color reaction of an

enol-compound with $FeCl_3$ in methanol. The experimental conditions have to be controlled very well to make the method usable for quantitative purposes. The assay could be disturbed by a shift in the keto-enol rearrangement by the formation of acetal or by oxidation of the enol by $FeCL_3$. This can be corrected as described in chapter 2.13 by registration absorption of the $FeCl_3$-enol complex at 600 nm during a short time period and by extrapolation to time = 0.

3.4.2. Shift of the keto-enol equilibrium in ethanol at 0^0.

The stability of enol pma in various solvents has been investigated using the $FeCl_3$-enol assay. The enol concentration remains constant at last during several hours if the pma is dissolved in dry diethyl ether. In ethanol a decrease of the enol concentration is observed. The decrease proceeds at 0^0 by a first order reaction with a rate constant of 0.2 - 0.3 $hour^{-1}$;about half amount of the enol-pma is converted in about 3 hours. The most logical explanation for this decrease is the shift of the keto-enol equilibrium in favor of the keto-form but a decomposition of enol- or keto-pma into phenylacetaldehyde might occur as well. The decrease might also be explained by the formation of the diethyl acetal of pma, by which keto-pma would be withdrawn from the keto-enol equilibrium.

In chapter 6 a quantitative enzymatic assay is reported for the total amount of pma (keto + enol); this assay does not react on the diethyl acetal or on phenylacetaldehyde. Using this assay, it was concluded that the total amount of pma in ethanol at 0^0 did not decrease more than 5% during 3 hours. Therefore, the decrease of enol-pma must have been accompanied by the formation of keto-pma.

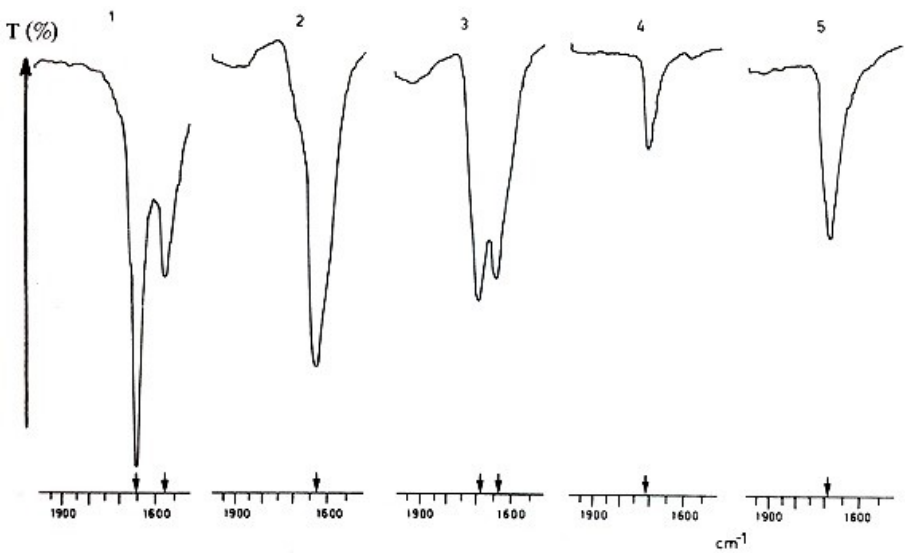
Direct indications for the formation of keto-pma have been obtained using infra-red spectroscopy. Spectra of pma were recorded in the range of 2000-1500 cm^{-1} with carbon tetrachloride or ethanol as solvent in a refrigerated infra-red cell. Usually stretching vibrations of carbonyl groups in an individual aldehyde or carboxylic groups are accompanied by absorptions at 1740 and 1700 cm^{-1}. The enol form does not show absorption at these frequencies (see this chapter 3.2.2) but for the keto-form absorption maxima are expected in this region of the spectrum.

Fig 3.4.1 shows a part of the spectrum of enol-pma dissolved in carbon tetrachloride with absorption maxima at 1653 and 1574 cm^{-1}. Fig. 3.4.2 is the spectrum of pma dissolved in ethanol at 0^0 and measured immediately. This spectrum shows the well known broadening of the absorption maxima and a shift to a lower wavelength as the result of interaction with the solvent. The absorption maximum was found at 1635 cm^{-1}. Fig 3.4.1 shows the spectrum of in ethanol and stored at 0^o for 4 hours. A considerable peak absorption is observed at 1690 cm^{-1}.

The absorption at 1635 cm $^{-1}$ has decreased. In fig. 3.4 numbered 4 and 5 show the spectra of phenylacetaldehyde and of the diacetal of pma respectively in amounts that can be formed maximally from pma. The position and the size of these absorption maxima in the spectra in fig 3.4.3 do not suggest the conversion in one of these two compounds to some extent. In addition, the total amount of pma in the cuvette was hardly decreased as it was shown by the enzymatic assay mentioned. One may conclude that the decrease of the concentration of enol-pma, as observed with the $FeCL_3$ assay is accompanied with the formation of a compound with a maximum absorption at 1690 cm^{-1}. Since an absorption in that area is expected for the carbonyl group van keto-pma in ethanol, this provides direct evidence for the shift of the keto-enol equilibrium in a pma solution in ethanol at 0^0 in favor of the keto form.

Fig 3.4

Spectral data indicating the formation of keto-pma



The infra-red spectrum with 100% transmission T on the Y axis is recorded for 2000-1500 cm^{-1} (5.0-6.6 μ) at 0^0 for the following samples (concentration 0.2 M).

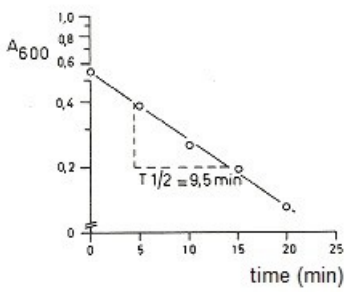
pma in ether
pma in ethanol at 0^0 (measured directly)
same (measured after 4 hours)
phenylacetaldehyde in ethanol
diethyl acetal of pma in ethanol

3.4.3 Rate of the tautomeric rearrangement in aqueous solvent

The speed of the conversion of enol-pma into keto-pma has been studied in aqueous solvent under conditions of the assay of the TDH and PDC enzymatic activity, using the quantitative enol assay. The buffer was tris-HCl because phosphate buffer reacts with the $FeCl_3$ in the assay. During fixed periods of time, tubes containing 0.40 ml 0.2 M tris-HCl buffer (pH7.5) and 20 µL enol-pma in ether (10 mg/ml) were incubated at 25^0 C. Then 2.55 ml ferric chloride regent (2.13) was added. The amount of enol-pma was calculated from the absorption at 600 nm. Fig. 3.5 shows the enol concentration plotted semi-logarithmically against the time. The enol-pma is converted according to a first order reaction with a reaction constant k = 0.073 min^{-1}. Half-life T1/2 = 9.5 min. The data presented in chapter 6 will show that also in this solvent the disappearance of enol-pma is accompanied with the formation of an equal amount of keto-pma. Therefore, the reaction constant mentioned regards the rate of the tautomeric rearrangement in aqueous condition.

Fig. 3.5

Effect of incubation in buffer on the enol content of pma



Enol-pma was incubated in 0.4 ml (0.2M) tris-HCl pH 7.5 during various periods of time. Thereafter 2.55 ml $FeCl_3$ was added and the enol content assayed according to chapter 2.13.

CHAPTER 4

BREAKDOWN OF ATROPINE AND TROPIC ACID

IN PSEUDOMONAS PMBL-1

4.1 INTRODUCTION

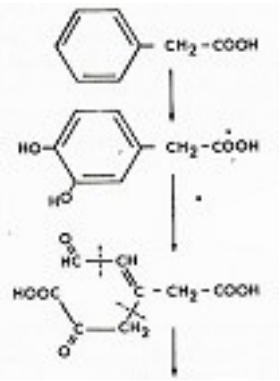
The genus Pseudomonas has the striking ability to metabolize many aromatic compounds and to use those as source of energy. For a survey, see review papers by van der Linden and Thijsse 1965 and Gibson 1968. Typical examples are benzoic acid and 6-chloroxylenol used on a large scale as preservative and disinfectant respectively.

The breakdown of aromatic compounds in Pseudomonas species may start by a hydroxylation of the aromatic ring, whereas in other cases the side chain is broken down firstly, either partially or completely.

The degradation of the aromatic ring by hydroxylation (fig 4.1) results in the formation of the di-phenol, followed by insertion of an oxygen molecule to produce an unsaturated more basic fatty acid. Finally, decomposition products

Fig 4.1

Possible metabolic pathway in Pseudomonas for breakdown of phenylacetic acid through 3,4 dihydroxyphenylacetic acid into puruvic,acid, acetoacetic acid, formic acid.



are formed consisting of 2-4 carbon atoms, which can be used by the bacterium as building blocks for the synthesis of essential components of the cell. The positioning of the hydroxyl groups and the oxygen molecule in the aromatic ring appears to be different for various Pseudomonas species; fig. 4.1 shows only one of the possibilities. This type of breakdown has been shown for the aromatic compounds benzoic acid, phenylacetic acid, 3-phenylpropionic acid and thymol (2-hydroxy-3-isopropyl toluene) (Chamberlain and Dagley 1968).

In cases of breakdown of the side chain first, this occurs usually by oxidation and decarboxylation. For example, in another Pseudomonas putida, mandelic acid (2-hydroxyphenylacetic acid) is converted into benzoic acid (Gunsalus et al. 1953 a and b; Stanier et al. 1953); n-butylbenzene is oxidized to phenylacetic acid.

Both patterns have been observed in the breakdown of p-cresol (p-hydroxy-toluene) (Chapman and Hopper 1968); some Pseudomonas species oxydize this compound to p-hydroxyphenylacetic acid (degradation of the side chain), whereas in other Pseudomonaceae p-cresol is converted by hydroxylation of the phenyl ring into 2.3 dihydroxytoluene.

There is no connection between the chemical structure of a compound and its metabolic pathway in a certain type of bacterium. Therefore, it was not possible to predict the most probable breakdown of tropic acid in the Pseudomonas strain isolated by Rörsch and Berends (MBL).

In the research described in this chapter, the adaptation of PMBL-1 to some aromatic carboxylic has been studied to determine whether these compounds are closely related to the metabolism of tropic acid. In addition, it has been tried to identify degradation products of tropic acid in the growth medium of wild type and mutants of PMBL-1. The results obtained suggest that in the metabolism of tropic acid the aliphatic side chain is broken down firstly.

4.2 CARBON SOURCES FOR PMBL-1

A number of compounds has been studied as possible carbon source in order to get some idea on the capacity of Pseudomonas PMBL-1 to metabolize aromatic and atropine-like compounds. Pseudomonas PMBL-1 was cultivated in a synthetic medium with 0.1% glucose or succinic acid and 0.025% of the compound to be investigated. This culture was used to inoculate fresh synthetic medium with 0.025% of the compound under study and 0.005% succinic acid. This culture was incubated at 30^0. After 18 hours, the concentration of the bacteria was assayed according to chapter 2.4.

The following compounds can be used as carbon source:

atropine	phenylacetic acid	phenylglyoxlic acid
homatropine	p-hydroxyphenylacetic acid	phenylacetaldehyde
tropine	m-hydroxyphenylacetic acid	benzaldehyde
tropic acid	phenylpyruvic acid	

These compounds can serve as carbon source in higher concentrations as well, except phenylacetaldehyde and benzaldehyde (toxic above 0.025%).

None or very limited growth was observed with the following compounds:

2-phenylacrylic acid	3-phenylacrylic acid	2-phenylpropanol
2-phenyl-2-hydroxypropionic acid	2-phenylpropionic acid	3-phenylpropanal
2-hydroxy-3-phenylpropionic acid	2-phenylpropanal	2-phenylethanol
2-hydroxyphenylacetic acid	benzyl alcohol	

An extended list of compounds that can or cannot be metabolized see table 10.3.

4.3 THE ADAPTATON OF PMBL-1 FOR AROMATIC ACIDS

The adaptation of Pseudomonas PMBL-1 has been studied for tropic acid and several other aromatic acids. One of the aspects investigated concerned the simultaneous adaptation to tropic acid and other compounds. In this way it could be possible to observe relationships between the metabolism of these aromatic acids without actual knowledge of their breakdown pathway in the bacterium.

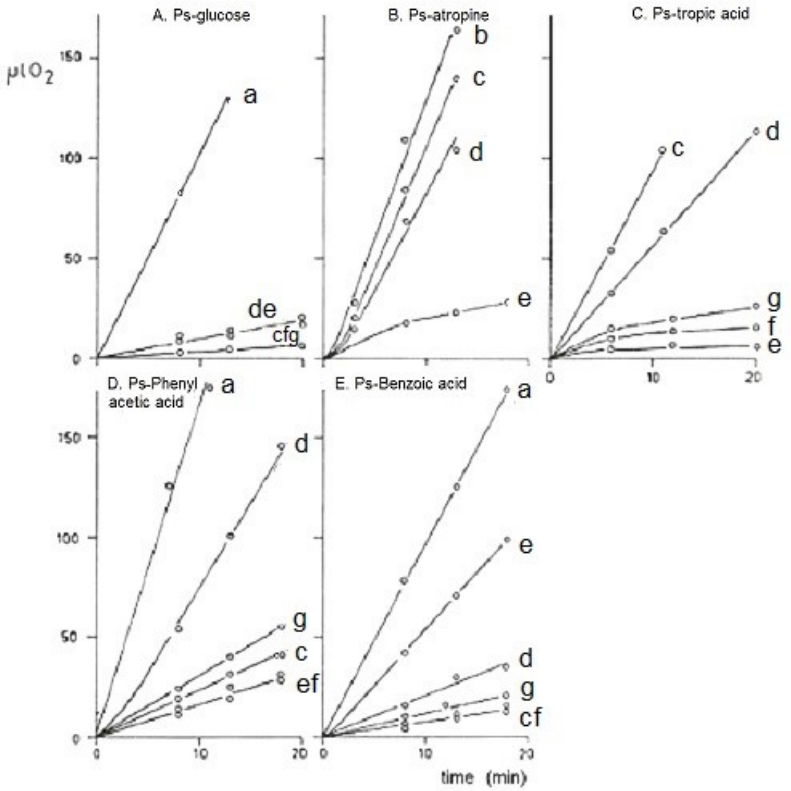
In such a study, one can use the feature of bacteria, which have been adapted to a certain carbon source, to be able to metabolize this compound immediately upon its addition to the medium. Non-adapted bacteria need an adaptation period before the metabolism can proceed. A precise distinction between metabolizing and non-metabolizing cells can be made for obligate aerobic organisms by measuring the uptake of oxygen as a function of time (Stanier 1950).

This method has been used to study adaptation of PMBL-1 (obligate aerobic see chapter 2.3) for the following carbon sources: glucose, atropine, tropic acid, phenylacetic acid, p-hydroxyphenylacetic acid and m-hydroxyphenylacetic acid, benzoic acid and glucose. The uptake of oxygen gas has been quantified using the manometric technique according to Warburg (chapter 2.6).

The results are presented in fig. 4.2.A - E. The oxygen consumption observed after addition of the carbon source specified (at time t=0) is plotted against time. Fig.4.2 shows that Pseudomonas cultivated with glucose as carbon source (Ps-glucose) can metabolize only glucose right away.

Fig 4.2 A-E

Oxygen uptake by PMBL-1



A. glucose
B. atropine
C. tropic acid
D. phenylacetic acid
E. benzoic acid

a. glucose
b. atropine
c. tropic acid
d. phenylacetic acid
e. benzoic acid
f. p-hydroxyphenylacetic acid
g. m-hydroxyphenylacetic acid

PMBL-1 grown on various carbon sources A-E. Ps-glucose is PMBL-1 grown with glucose as carbon source etc. On time t=0 various compounds a-g were added. Oxygen consumption was measured. Data were corrected for the spontaneous oxygen consumption (0.5-1.5 µl/min). Experimental details are documented in chapter 2.6.

Pseudomonas PMBL-1 cultivated with atropine as carbon source (Ps-atropine) starts to consume oxygen immediately after addition of atropine, tropic acid and phenylacetic acid, but not after addition of benzoic acid (fig. 4.2 B). According to expectation, Ps-atropine is adapted for tropic acid. Ps-atropine appears to be adapted to phenylacetic acid as well, suggesting either phenylacetic acid is metabolized by the same enzymes or phenylacetic acid is an intermediate in the breakdown of atropine.

The oxygen consumption by Ps-tropic acid (fig.4.2.C) confirms the experiment with atropine. These bacteria show adaptation not only to tropic acid but to phenyl acetic acid as well. These bacteria show no or only a little metabolic activity immediately after addition of benzoic acid, m-hydroxy- or p-hydroxyphenylacetic acid.

Fig. 4.2.D shows the oxygen uptake by Ps-phenylacetic acid: the bacteria have been adapted for phenylacetic acid and for glucose, but incubation with tropic acid, m-hydroxy- and p-hydroxyphenylacetic acid does not result in immediate oxygen consumption. The results shown in fig. 4.2.E suggest that after growth with benzoic acid as carbon source only this compound and glucose are metabolized straight away. The oxygen uptake after addition of glucose in this and the previous culture suggest that the enzymes involved in the breakdown of glucose are present in the cells irrespective the presence of glucose in the medium used for cultivation of the bacteria.

These adaptation studies show that the system for the breakdown of atropine and tropic acid is only present in Ps-atropine and Ps-tropic acid. Ps-atropine and Ps-tropic acid are fully adapted for phenylacetic acid. However, tropic acid is not immediately metabolized by Ps-phenylacetic acid. This suggests that the adaptation of Ps-tropic acid for phenylacetic acid is not due to the use of the same enzymes, but due to phenylacetic acid being an intermediate in the breakdown of atropine and tropic acid.

4.4 INVESTIGATION WITH MUTANTS, BLOCKED IN THE BREAKDOWN OF TROPIC ACID.

Parallel to the study of the adaptation of PMBL-1, a study of the metabolism of tropic acid was undertaken in PMBL-1 and in mutants by a search for the presence of degradation products in the cultivation medium. This was done after cultivation in synthetic medium with tropic acid as sole carbon source.

After cultivation, the medium was extracted and analyzed (chapter 2.7) by thin layer chromatography. An experiment with the wild type PMBL-1 failed. Thin layer chromatography only confirmed the consumption of tropic acid by the bacteria. In a next step, mutants were isolated from PMBL-1, not able to breakdown tropic acid anymore. Several dozens of mutants of PMBL were

obtained unable to grow with atropine or tropic acid as sole source of carbon. These mutants were still able to metabolize tropine, phenylacetic acid or glucose: phenotype Atr^{-} Tro^{-} Trp^{+} Pac^{+} Glu^{+}.

It seemed not unreasonable to assume that amongst these mutants some still are able to carry out a partial breakdown of tropic acid. Some might even be able to excrete an intermediate in the tropic acid breakdown when cultivated in the presence of tropic acid with glucose as carbon source.

The mutants were cultivated in a synthetic medium with 0.2% glucose and 0.2% tropic acid. The cultures were shaken at 30^0 during 3 days. Thereafter, the growth medium was analyzed using thin layer chromatography. However, none of the mutants showed accumulation of degradation products of tropic acid in the cultivation medium to such an extent that those products could be detected.

4.5 MUTANTS, BLOCKED IN THE METABOLISM OF PHENYLACETIC ACID AND p-HYDROXYPHENYLACETIC ACID

Since the investigation of Tro^{-} mutants did not result in new information on the breakdown of tropic acid, it was tried to isolate mutants unable to breakdown phenylacetic acid. The breakdown of this compound is related to that of tropic acid as concluded from the adaptation study (4.3). So, a study of the metabolism of Pseudomonas blocked in the metabolism of phenylacetic acid (Pac^{-}) might provide information about the breakdown of tropic acid.

PMBL-1 was treated with a mutagenic agent (chapter 2.5). The treated bacteria were selected for the phenotype Pac^{-}. Simultaneously, mutants were selected blocked in the metabolism of p-hydroxyphenylacetic acid (Php^{-}).
Amongst about 10.000 colonies isolated, a total of 52 mutants were found. Their phenotypes have been listed in table 4.3. The table shows that all Pac^{-} mutants have lost the ability to metabolize tropic acid.

Table 4.3
Pac and Php mutants of PMBL-1

Phenotype			Number of mutants per 10^4 colonies
Pac^{-}	Tro^{+}		0
Pac^{-}	Tro^{-}	Php^{+}	25
Pac^{-}	Tro^{-}	Php^{-}	4
Pac^{+}	Tro^{+}	Php^{-}	23

A mutant of the phenotype $Tro^{+}Pac^{-}$ has not been found, even not later in this research. This points to a role of phenylacetic acid in the tropic acid metabolism. These mutants have been subject of further study see 4.6.

The metabolism of p-hydroxyphenylacetic acid is much less related to that of tropic acid. Mutants with the phenotype $Tro^{+}Pac^{+}Php^{-}$ have been found. This makes it less plausible that p-hydroxyphenylacetic acid is an intermediate in the breakdown of tropic acid or phenylacetic acid.

4.6 INVESTIGATION OF MUTANTS BLOCKED IN THE METABOLISM OF PHENYLACETIC ACID

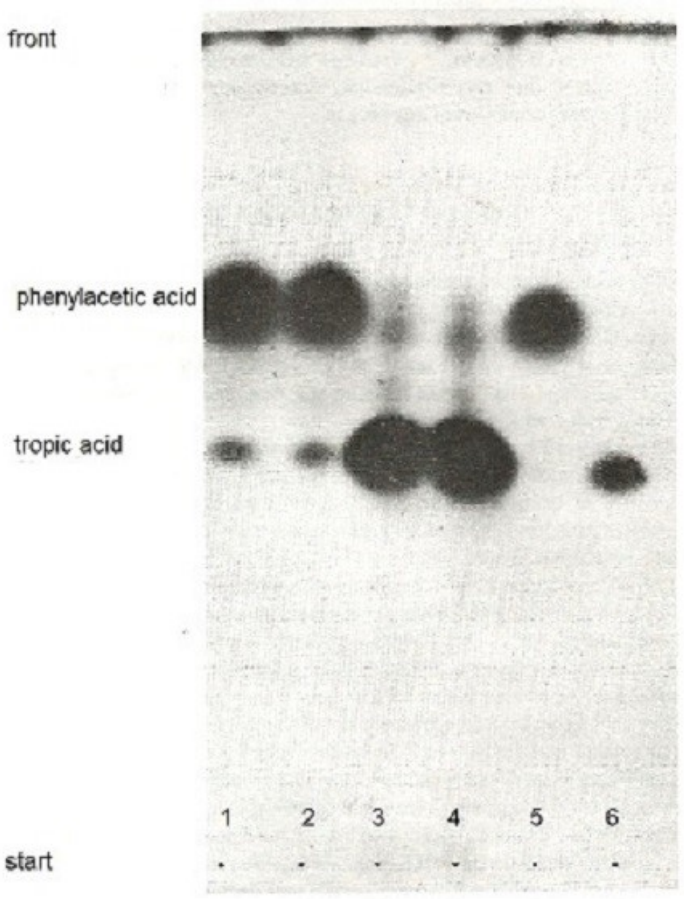
All mutants blocked in the metabolism of phenylacetic acid are no longer able to utilize tropic acid. The cultivation medium of these Pac^{-} mutants was analyzed in the search for the presence of tropic acid metabolites. The rationale for this experiment was the idea that the chance for a partially intact tropic acid metabolic pathway in Pac^{-} is larger than in mutants selected for the phenotype Tro^{-}.

The mutants were cultivated in a synthetic medium with 0.2 % tropic acid and 0.2% glucose as carbon source and shaken at 300 during 3 days. The bacteria were removed by centrifugation. The growth medium was acidified, extracted and analyzed by thin layer chromatography (chapter 2.7). The result for 2 Pac^{-} mutants (PMBL-114 and PMBL-107) is shown in fig 4.4. PMBL-114 has converted tropic acid nearly completely in a compound with the chromatographic behavior of phenylacetic acid. Under the same conditions PMBL-107 is able only to convert a small portion of the tropic acid. The conversion as found in the medium of PMBL-114 has been found later for 18 other Pac^{-} mutants.

In order to confirm the identity of the conversion product, the experiment with PMBL-114 has been repeated on a larger scale. The mutant was cultivated in 6 L synthetic medium containing in total 12 g tropic acid. The medium was subsequently treated as described above. About 5g of the converted product was obtained. The product recrystallized from petroleum ether 40-60^{0} C as tiny leaves. The compound was identified as phenylacetic acid: it behaves in thin layer chromatography as phenylacetic acid after elution with the solvents EMX and BEM. Its ultraviolet and infrared spectrum (Davies 1951) are identical to that of phenylacetic acid. In elementary analysis 70.75 % carbon and 6.01% hydrogen were found; in theory, one expects for phenylacetic acid 70.57% and 5.92%. The product isolated melts at 77.5^{0}, phenylacetic acid melts at 77,0^{0}, the melting point of the mixture is 77.0^{0}. These observations prove that tropic acid can be converted in phenylacetic acid by mutants of Pseudomonas PMBL-1.

Fig 4.4

Conversion of tropic acid in phenylacetic acid by pseudomonas mutants



Thin layer chromatogram of the extract of the cultivation medium of PMBL-114 (1 and 2) and PMBL-107 (3 and 4), cultivated in 0.2% glucose and 0.2% tropic acid during 3 days. Reference compounds phenyl acetic acid (5) and tropic acid (6). Elution fluid was EMX, detection by H_2SO_4-HNO_3 (1:1).

4.7 BREAKDOWN OF PHENYLACETIC ACID

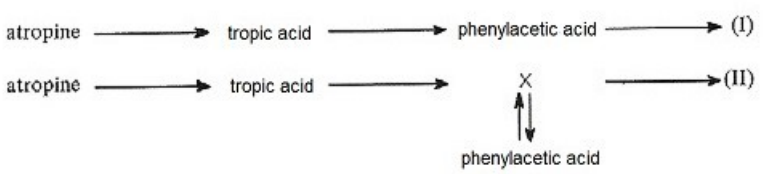
Phenylacetic acid is a rather normal carbon source for Pseudomonas (Stanier 1960). It is broken down by hydroxylation and oxygenation. Although the metabolism of phenylacetic acid in PMBL-1 has not been studied in detail, this study has provided some indications for the course of the breakdown of this compound. During the study of the mutants of the phenotype $Tro^{-}Pac^{-}$, one of these mutants (PMBL-112) appeared to convert tropic acid in a compound with an R_f value slightly higher than that of tropic acid.

Using preparative thin layer chromatography, a small amount of the compound was isolated in a chromatographically pure condition. The UV-spectrum showed the characteristic absorption of o-hydroxy- or m-hydroxyphenylacetic acid: a maximum at 271 nm and a shoulder at 277 nm. In further chromatography the o-hydroxy compound could be excluded. The compound with the higher R_f is therefore most probably m-hydroxyphenylacetic acid. The spectral and chromatographic data were not compatible with p-hydroxyphenylacetic acid neither with any of the other compounds in annex 1.

So probably, the breakdown of phenylacetic acid takes places through hydroxylation into m-hydoxyphenyl acetic acid.

4.8 DISCUSSION

Pseudomonas PMBL-1, cultivated in the presence of tropic acid is adapted to metabolize phenylacetic acid. Pseudomonas mutants can convert tropic acid in phenylacetic acid. Both findings confirm an intense metabolic relationship between these two aromatic acids. Nevertheless, one should not draw the immediate conclusion that phenylacetic acid is an intermediate in the breakdown of atropine and tropic acid (scheme1). It cannot be excluded that phenyl acetic acid is generated from the real intermediate X by a reversible reaction (scheme II) (Adelberg 1953).



In the breakdown according to scheme II, one expects to find mutants of the phenotype Pac^{-} Tro^{+}. However, all mutants selected for the phenotype Pac^{-} showed to be blocked in the breakdown of tropic acid. Mutagenic treatments

which resulted in the isolation of about 70 mutants with a specific mutation in the metabolism of atropine and tropic acid, did not yield mutants with the phenotype Pac^{-} Tro^{+}. Scheme II therefore is improbable. The conclusion is justified that the breakdown of atropine and tropic acid takes its course through a conversion into phenylacetic acid.

This conclusion is of major importance for the elucidation of the metabolic pathway of atropine and tropic acid and for the identification of the enzymes involved in this breakdown in Pseudomonas.

CHAPTER 5

THE ATROPINE ESTERASE

5.1 INTRODUCTION

Rörsch and Berends (1965) obtained first indications for the role of atropine esterase in the breakdown of atropine in Pseudomonas PMBL-1. These scientists did enzyme tests on an extract of this bacterium cultivated with atropine as carbon source. The extract appeared to have a strong catalytic effect on the rate of hydrolysis of atropine. The catalytic effect was not observed any longer after incubation of the extract with proteolytic enzymes, nor after a short thermic treatment at 80^0. The bacterium seemed to have at its disposal an atropine esterase, able to accelerate the hydrolysis of the ester atropine.

The properties of the esterase (AtrE) are described in this chapter. The enzyme will be characterized by the identification of tropic acid and tropine as the products of the enzyme reaction. Investigation of the substrate and stereo-specificity revealed the specificity of this enzyme for (-)atropine and some atropine-like compounds. Next, a protocol will be presented for the purification of the atropine esterase. This allows for a purification of the enzyme 500-600 times as compared with the crude bacterial extract. The investigation of the properties of the atropine esterase has been carried out for the major part in collaboration with Dr. F. Berends and Dr. R.A. Oosterbaan (MBL)

5.2 QUANTITATIVE ASSAY OF THE ACTIVITY OF THE ATROPINE ESTERASE

Tropic acid is formed during the hydrolysis of atropine. The production of acid is being used to measure enzyme activity. In the laboratory, acid production is quantified by keeping the pH constant in a non-buffered system by addition of NaOH. In this way, the tropic acid formed is titrated continuously in a so-called pH-stat: the amount of alkali needed to keep pH constant is registered as a function of time. Routinely, the assay is carried out in an incubation mixture of 0.4 mM (-) atropine, 0.1 M KCl and 0.2% saponin at pH 7.0 and 25^0. These conditions are optimal for the assay of the activity of the atropine esterase. The choice of pH and the substrate concentration will be motivated in 5.4.

In the assay of purified atropine esterase samples, the production of the acid appeared not to be directly proportional to time, but to decrease persistently. This decrease can be prevented by adding the natural detergent saponin during the enzyme assay. The cause of this phenomenon and the effect of saponin is unclear;

maybe saponin prevents the formation of enzyme aggregates or the attachment of the enzyme to the glass wall of the incubation vessel.

5.3 THE PURIFICATION OF THE ATROPINE ESTERASE (AtrE)

The result of the procedures described in chapter 2.11 is listed in table 5.1. The enzyme was purified 610 times relative to the extract at the start with a yield of 19%. This result is representative for enzyme purifications carried out in this way.

The extract was prepared by ultrasonic treatment of the bacteria, followed by centrifugation. Subsequently, the major part of the nucleic acid material was precipitated using streptomycin analogous to the protamine precipitation according to Linn (1965). This precipitation is obligatory to prevent the disturbance of the next fractionation with ammonium sulfate due to the presence of nucleic acids.

The fractionated precipitation using ammonium sulfate resulted in an enzyme sample with a specific activity (SA) of 6 U per mg protein. After refractionation, a SA of 15 was obtained.

In the next step, sample was purified using column-zone-electrophoresis. In this procedure, conditions such as the type of the buffer, the pH and the electric field strength were chosen such that the migration of the AtrE in the direction of the anode is substantially compensated by the counter-osmosis operating in the opposite direction. Under this condition, the enzyme remains in a narrow zone in the column, whereas the majority of the protein leaves the column during electrophoresis. This results in a high resolution of this purification step. The yield is high as well (± 90%). A disadvantage is the long duration of the electrophoresis.

Table 5.1
Purification of the atropine esterase

	TA	yield	SA	purification
Extract	27.0	100%	1.05	1 x
After streptomycin precipitation	22.0	81%	-	-
After ammonium sulfate reprecipitation	11.0	41%	15.4	15 x
After column zone electrophoresis	10.1	37%	136	130 x
After DEAE-cellulose chromatography	6.7	25%	363	308 x
After Sephadex G-100 gel filtration	5.1	19%	641	610 x

Enzyme activity and protein concentration were assayed according to 2.9.2;
TA in 10^3 U; SA in U/mg protein.

The enzyme was purified with an additional factor of 4 by DEAE cellulose anion exchange chromatography and Sephadex G-100 gel filtration. The enzyme was eluted from DEAE-cellulose using a salt gradient. This technique also has a high resolution and a high yield (60-70%). After gel filtration, the enzyme has a specific activity of 500-600; samples with this degree of purification have been used to explore the properties of the atropine esterase.

5.4 PROPERTIES OF THE ATROPINE ESTERASE

5.4.1 Analysis of the products of the enzyme reaction

Analysis of the products of the enzyme reaction revealed that in the action of the AtrE on atropine the products tropic acid and tropine were formed. This was demonstrated by incubation of 0.4 mM (±)atropine with purified enzyme in the pH-stat (pH 7.0, 25^0) during 40 min. Hydrolysis was terminated by inactivation of the enzyme at 80^0. The incubation mixture was analyzed by thin layer chromatography. It turned out that atropine was partially converted by the AtrE and that two compounds had been formed with the chromatographical behavior of tropic acid and tropine respectively (fig 5.2.1 and 2).

5.4.2 Stoichiometry

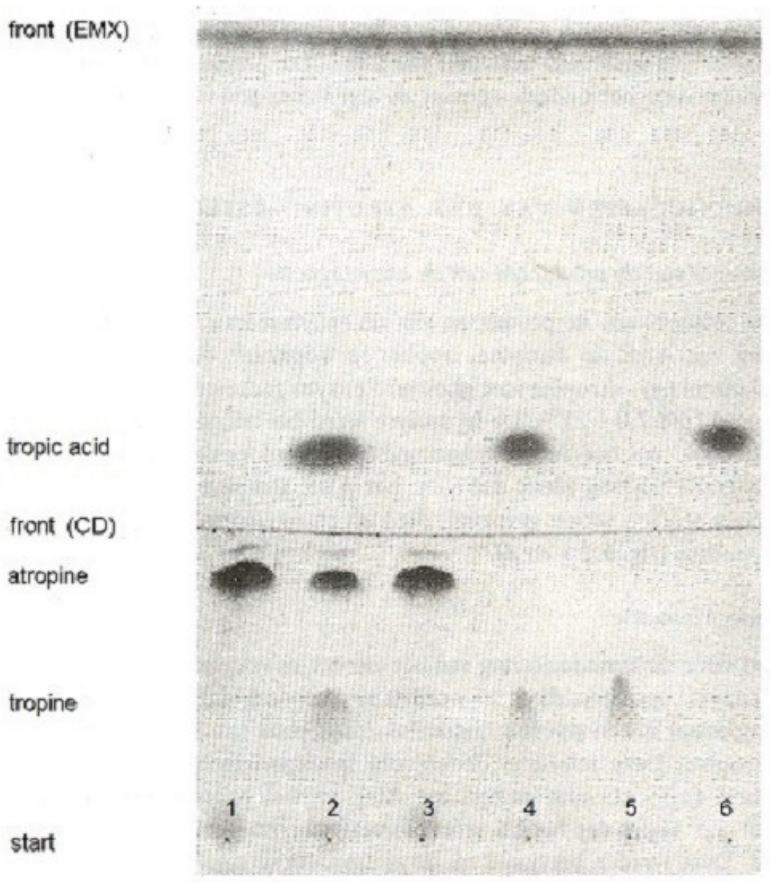
Both for the characterization of the enzyme as for the quantitative assay of the enzyme activity, one has to show that the amount of tropic acid produced and the amount of hydrolyzed atropine have a stoichiometric relation. This has been investigated in experiments with a limited amount of (-)atropine being nearly completely hydrolyzed using the AtrE. Table 5.3 shows that stoichiometric amounts of tropic acid were formed. That amount of tropic acid was calculated from the acid production as result of the enzymatic hydrolysis. In addition, the tropic acid formed was assayed using an enzymatic conversion to be described in chapter 6.

5.4.3 Substrate optimum

The enzyme activity as a function of the concentration of (-)atropine does not show the expected maximal value at high substrate concentrations. At low substrate concentrations the activity increases with an increase of the concentration, but above a certain value further increase results in a decrease of the enzyme activity (fig 5.4). The substrate optimum is at 0.2-0.4 mM (-)atropine. The purified AtrE can accelerate the hydrolysis of the methyl ester of tropic acid as well. The inhibition by excess substrate does not occur in this case; at high substrate concentration a maximal value is observed.

Fig 5.2

Thin layer chromatogram of the products of enzymatic conversion of atropine



Atropine was incubated during 40 min according to 2.9.2 in the absence or presence of 10 U AtrE. After enzymatic treatment, the incubation mixture was heated at 70^0 during 10 min and analyzed by thin layer chromatography. Elution by EMX, followed by drying and a second elution with CD. Detection H_2SO_4 /HNO_3.

1	(±)atropine (control, no AtrE)	40 min incubated at 25^0
2	(±)atropine + AtrE	40 min incubated at 25^0
3	(–)atropine (control, no AtrE)	40 min incubated at 25^0
4	(–)atropine + AtrE	40 min incubated at 25^0
5	tropine	
6	tropic acid	

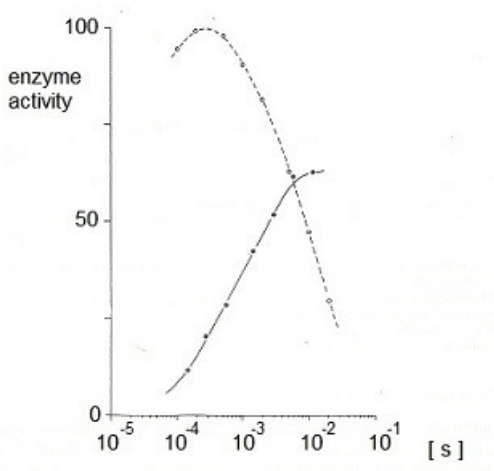
Table 5.3
Stoichiometry of the enzymatic hydrolysis of atropine

ATROPINE (µmol)	TROPIC ACID (µmol)	H^+ (µgeq)
0.1	0.099	-
0.2	0.197	-
0.3	0.291	-
0.4	0.388	-
1	-	0.81
2	-	1.93
3	-	2.96
4	-	4.16

(-)Atropine in the amounts indicated was incubated according to 2.9. As soon as the reaction was completed, the amount of tropic acid formed was quantified according to 6.4.1; the amount of acid formed was measured titrimetrically.

Fig 5.4

The effect of the substrate concentration on the activity of AtrE.

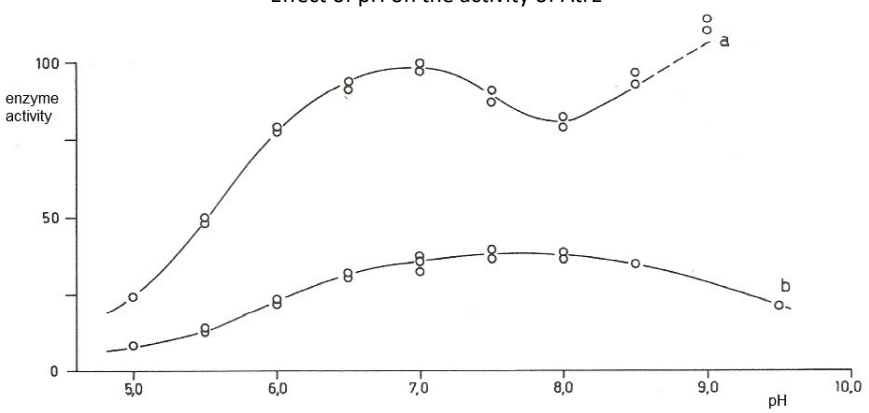


A constant amount of purified AtrE was assayed for its enzyme activity according to 2.9.2 with (-)atropine (0-0-0) and the methyl ester of (±)tropic acid (•-•-•) as substrate. Substrate concentration [s] in M. Enzyme activity with 0.4 mM (-)atropine set on 100%.

5.4.4 The pH-optimum

The activity of the AtrE enzyme as a function of the pH is shown in fig 5.5. This dependence was measured with the substrates 1 mM (-)atropine and 2 mM methyl ester of (±)tropic acid. With 1 mM (-)atropine, an optimum in activity is observed at pH 7.0 and a relative minimum at pH 8,0. The measurements at pH>9.0 are less accurate because the tropine residue starts to behave as a buffer above this pH, disturbing the titrimetric assay. With 2 mM methyl ester of (±)tropic acid as substrate, a simple bell-shaped curve is found as graphical representation of the pH dependence. The difference in the shape of the curves might be caused at least partially by the effect of the pH on the inhibition of enzyme activity by excess substrate in the case of (-)atropine.

Fig.5
Effect of pH on the activity of AtrE

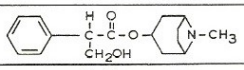
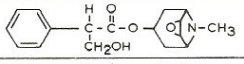
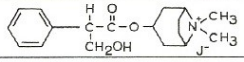
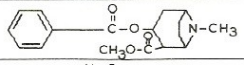
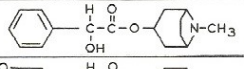
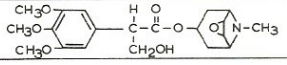
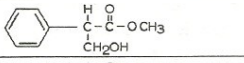
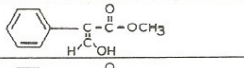
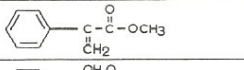
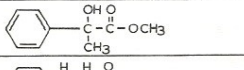
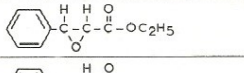
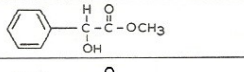
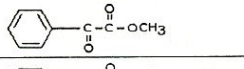
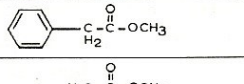
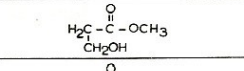
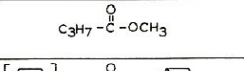
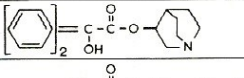
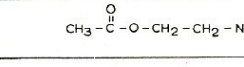


The effect of pH on the enzyme activity was investigated by adjusting the settings of the pH stat equipment (2.9.2). The reaction mixture contained the substrate and 0.02% saponin in 0.1M KCl. The activity for atropine pH 7.0 was set on 100.
1 mM (-)atropine
2 mM methyl ester van (±)tropic acid

5.4.5 The substrate specificity

The substrate specificity of AtrE was investigated with the ester compounds, listed in table 5.6. All compounds were tested in a concentration of 0.4 mM, the optimal concentration for (-)atropine. The enzyme activity of the AtrE sample, measured with (-)atropine as substrate, is set on 100. The enzymatic hydrolysis of the other substrates was compared with this activity.

Table 5.6

Substrate specificity of the AtrE			Activity	Km (M)
	(-) (+/-) (+)	atropine	100 59 4	$\pm 10^{-5}$
	(+/-)	scopolamine	65	
	(-) (+/-)	N-methyl atropine	74 48	$1.7 \cdot 10^{-5}$
		cocaine	0	
	(+/-)	homatropine	163	
		R_0 - 3 - 1158	0	
	(+/-)	tropic acid methyl ester	18	$7 \cdot 10^{-4}$
		2-phenylmalonic semi-aldehyde methyl ester	0	
		atropic acid methyl ester	0	
	(+/-)	atrolactinic acid methyl ester	6.5	
		2,3-epoxy-3-phenyl propionic acid ethyl ester	0	
	(+/-)	mandelic acid methyl ester	13	$6 \cdot 10^{-4}$
		phenylglyoxylic acid methyl ester	7	
		phenylacetic acid methyl ester	0.5	
		3-hydroxypropionic acid methyl ester	0	
		n-butyric acid methyl ester	0	
		3-chinuclidinyl benzilate	0	
		acetylcholine	1	

Activity with (-)atropine as substrate is 100% All assays at pH7.0 and 25^0 ,substrate conc. 0.4 mM not in the table: α-naftylacetate and o-nitrophenyl acetate. Relative activity 0.5 and 1 respectively.

Racemic (±)atropine and (±)scopolamine are equivalent substrates in the concentrations used, but are hydrolyzed at a much lower speed in comparison with (-)atropine. (+)Atropine is only hydrolyzed very slowly. (-)N-methyl-atropine is a good substrate and is hydrolyzed faster compared with the racemic compound. This shows a strong preference of the enzyme for the (-)enantiomer. Introduction of three methoxy groups in the aromatic ring of scopolamine renders the compound unsuitable as substrate. Homatropine, the ester of tropine and mandelic acid is hydrolyzed 1.5 times faster as compared with (-)atropine.

The methyl ester of tropic acid and mandelic acid methyl ester were hydrolyzed as well, although much slower. The other compounds including acetyl choline, the tropa-alkaloid cocaine and the atropine analogue quinuclidinyl benzilate are hydrolyzed very slowly or not at all.

In case of four substrates the Michaelis constant K_M is mentioned. This constant for (-)atropine cannot be assessed accurately. Due to the phenomenon of substrate inhibition, experiments have to be done at very low concentrations of the substrate. The method described in 2.9.2 is not suitable to do this. The K_M values for the methyl esters of tropic acid and mandelic acid are about 50 x larger in comparison with the K_M for (-)atropine.

5.4.6 The stereo specificity

The higher rate of hydrolysis of (-)atropine compared with that of (+)atropine (table 5.6) indicated that the AtrE has a preference for the (-) isomer. This has also been demonstrated very clearly by Berends (1969) who observed that during the initial hydrolysis of (±)atropine by the AtrE nearly exclusively (-)tropic acid is released. This has been proven by measuring the optical rotation of the product formed. Also data by thin layer chromatography confirm the stereo specificity of the enzyme. The experiment described in 5.4.1.has been carried with (-)atropine, instead of (±)atropine. The substrate was incubated during 40 min with AtrE while the pH was held constant. The (-)atropine appeared to have been hydrolyzed completely in contrast with the partial hydrolysis of (±)atropine under the same conditions (fig 5.2 c and d).

5.4.7 Stability of the enzyme

The enzyme samples are usually stable at 4^0. The effect of temperature on the stability was investigated for a sample that contained 0.18 mg protein per mg and was dialyzed against HMP (pH 7.0). The sample was incubated at various temperatures during 30 min. Temperatures up to 40^0 did not cause loss of enzyme activity; at 45^0 about half of the activity was lost, whereas above 50^0 the enzyme was inactivated nearly completely.

The same sample was also used to study the effect of pH on the stability of the enzyme, incubated at various pH values at 25^0 during 30 min. The esterase maintained its full activity at pH 5-10, but was inactivated outside this pH range.

5.4.8 Miscellaneous

Atropine esterase does not need a cofactor for its catalytic activity; the activity is not stimulated by addition of metal ions and is not inhibited by 1 mM EDTA. The esterase is not inhibited by 1 mM tropic acid or phenylacetic acid. Tropine is a competitive inhibitor with a K_i of 0.8mM (Berends 1969), but has no effect in concentrations formed during titration.

5.5 DISCUSSION

The assay of the activity of atropine esterase, as presented, is simple, reliable and directly proportional with the amount of enzyme added (fig 2.2). This datum, combined with the stability of the enzyme and the opportunity to purify the enzyme with a good yield, designates the enzyme as an enzymologically well-to-handle object.

Inhibition by excess substrate found for (-)atropine and the normal substrate saturation pattern for the methyl ester of tropic acid suggest a role of the positively charged tropine-ion in this inhibition. Inhibition by excess substrate is also known for the acetylcholine esterase and has been extensively investigated for this enzyme. It has been explained by the assumption of an anionic site in the enzyme, important for the attachment of the positively charged substrate molecule. During the enzyme action, the acyl group of the ester is transferred to the enzyme under formation of an acyl enzyme. At high substrate concentration, a second substrate molecule is bound to the anionic site of this acyl-enzyme, resulting in an inhibition of the de-acylation of the enzyme and therefore of the enzymatic hydrolysis (Krupka 1963). It might be that the inhibition of AtrE by excess substrate is caused by interaction with an anionic site as well. An extended discussion of this phenomenon and of the unusual pH dependence of the enzyme can be found in Berends et al (1967).

The AtrE of Pseudomonas is definitely not an esterase with a broad working spectrum, in contrast to the enzyme of rabbit serum (see chapter 1). The investigation of the substrate specificity of AtrE has revealed that atropine and esters much alike atropine as N-methylatropine, scopolamine and homatropine are good substrates for the enzyme. Homatropine is even faster hydrolyzed as compared with (-)atropine. One might suggest the name homatropine esterase for the enzyme. However, the enzyme studied here has a much closer relationship to the metabolism of atropine compared with homatropine, because mandelic acid - the acid component in homatropine - is not metabolized

by the PMBL-1 (chapter3) and because homatropine is hardly able to induce the tropic acid enzymes (chapter 10). The more compounds differ in structure from that of atropine, the lower the rate of their enzymatic hydrolysis by AtrE. The preference observed for the (-)enantiomer of atropine puts stronger emphasis on the specificity of the atropine esterase.

For the sake of completeness, findings will be summarized obtained by Dr. R. Oosterbaan and Dr. F. Berends (MBL) in their investigations of the enzyme atropine esterase.

Atropine esterase is inhibited by organophosphorus compounds diïsopropyl phosphorofluoridate (DFP) and Soman (pinacolyl methylphosphono fluoridate). In case of the inhibition with ^{32}P-Soman it has been demonstrated that this was due to a reaction with only one specific serine residue in the enzyme ("the active serine"). The amino acid sequence around this active serine has been shown to be – histidyl – seryl – methionyl – glycyl –.

The activity per catalytic center is about 30.000 molecules (-)atropine per min. The molecular weight of the enzyme 30.000 has been calculated from the amino acid composition and the sedimentation constant. Based on the elution volume in Sephadex G-100 gel filtration, the molecular weight is estimated to be 39.000 (see chapter 2.11 13).

Protein assay for the nearly pure enzyme using the method of Lowry and the A_{280} (see chapter 2.9.1.) indicates a considerable higher protein content than that follows from the amino acid analysis. The specific activity of enzyme samples was always measured using one of the first two methods. The most purified samples were found to have a SA of 500-600 U/mg protein. If for the calculation of the SA the real protein content is used, based on the amino acid composition, the SA values found are 900 - 1000. From the activity per active center and the molecular weight, one can calculate a SA of about 1000 for the complete purified enzyme. This is in accordance with the observation that samples assayed by Lowry's method and with a SA 600, are nearly homogenous in sedimentation analysis and polyacrylamide gel electrophoresis.

An antiserum has been prepared by immunization of a rabbit with purified AtrE; this can be used to recognize enzymatically non-active AtrE from Pseudomonas mutants.

As stated at the introduction of this thesis, the atropine esterase should be specific for atropine in order to be suitable as a limited model of the atropine receptor. This condition is met (table 5.6). In addition, various compounds with an

atropine-like action spectrum in vivo inhibit the AtrE in vitro. Quinuclidinyl benzilate appears to be a reversible competitive inhibitor with $K_i = 3 \times 10^{-6}$ M: this indicates a high affinity of the enzyme for the inhibitor.

In summary, atropine esterase from PMBL-1 meets the conditions mentioned in the introduction. The enzymological properties, the data regarding the inhibition by organophosphorus compounds and the specificity of enzyme induction (chapter 10) characterize the atropine esterase as a specific serine-esterase involved in the breakdown of atropine in Pseudomonas.

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):

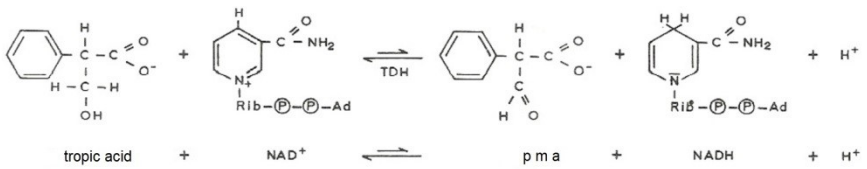


Fig 6.1

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

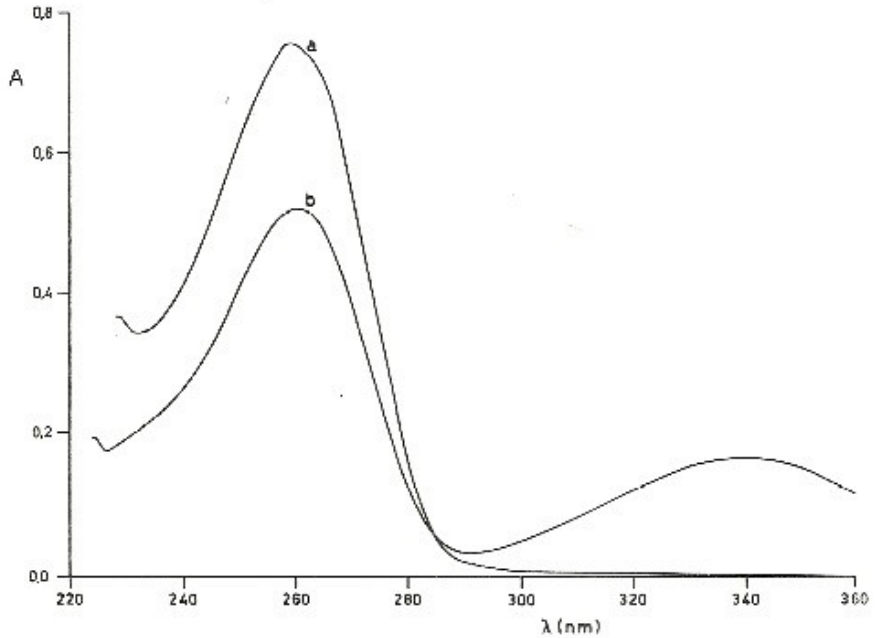
6.4.1 The analysis of the reaction products and the stoichiometry of the reaction

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.

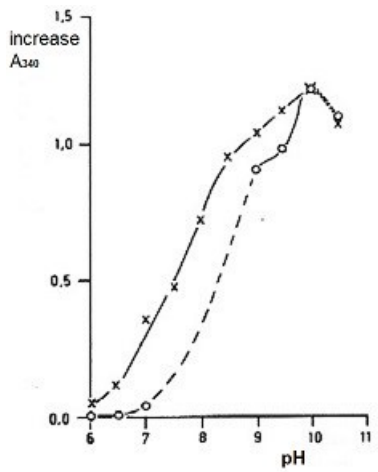
Due to its instability, the identification of pma, the conversion product of tropic acid, has been carried out in combination with the analysis of the product of the decarboxylase which will be discussed in the next chapter (7.2). In that experiment, the conversion of tropic acid in phenylacetaldehyde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

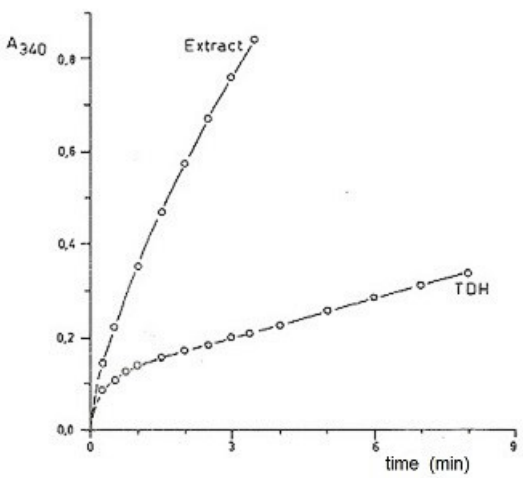
6.5 ENZYMATIC DEHYDROGENATION OF TROPIC ACID IN NEUTRAL ENVIRONMENT

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.

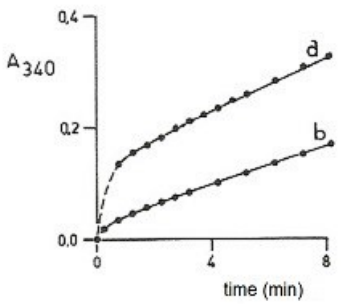
course of the NADH production: the initial absorption increase is faster in the presence of this large amount of enzyme, but the absorption at which the slow-down occurred and the slow NADH production thereafter were not affected.

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

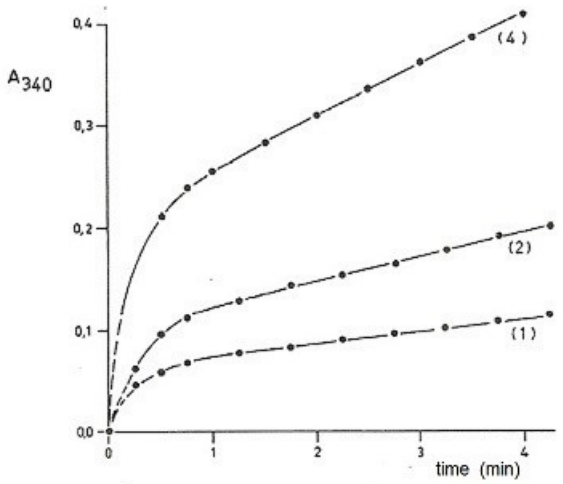
curve b: same, but now in the presence of 0.6 mM NADH; the curve is corrected for the absorption of the NADH added.

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.

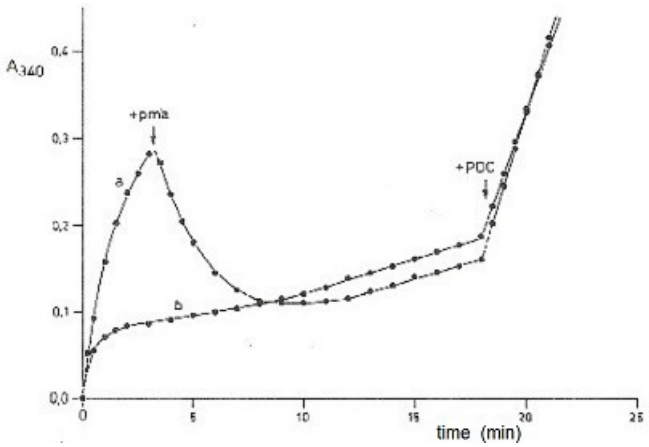
Addition of pma during the dehydrogenation of tropic acid (fig 6.9, curve a) resulted not only in the inhibition of the dehydrogenation, but even in a decrease of the absorption. After some time, a minimum value was reached after which the absorption slowly increased. Adding the same amount of pma at 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



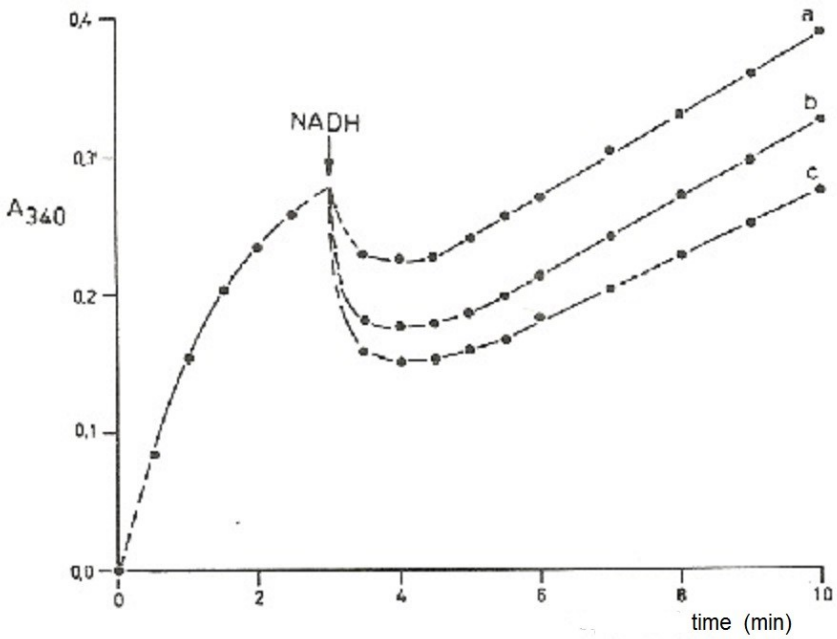
NAD^+ (3.33 mM), tropic acid (50 mM) and TDH (0.8 U) were added to 3 ml 33mM K-phosphate pH 7.5. The pma dissolved in diethyl ether was added in a final concentration of 1 mM on time = 3 min 15 sec (curve a) and on t=0 (curve b). The effect of PDC is discussed in chapter 7.

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.

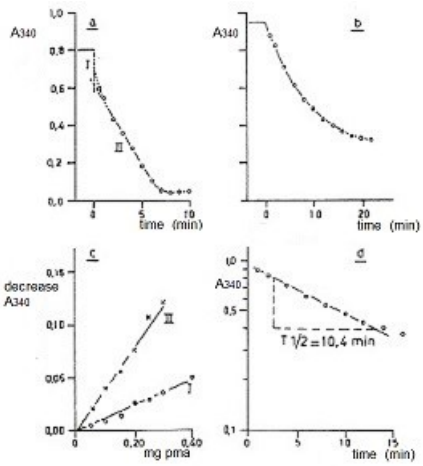
The decrease in absorption observed during incubation of pma , NADH and TDH is attributed to the enzymatic hydrogenation of pma, the so-called back reaction:

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.

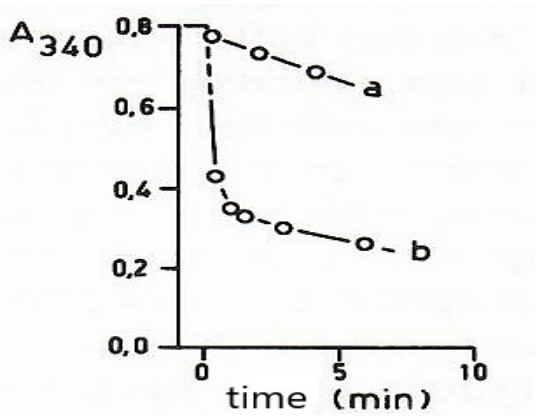
Since enol-pma in ethanol at 0^0 is converted slowly in the tautomeric keto-form, it has been possible to verify the assumption keto-pma being the actual substrate for the back reaction.

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

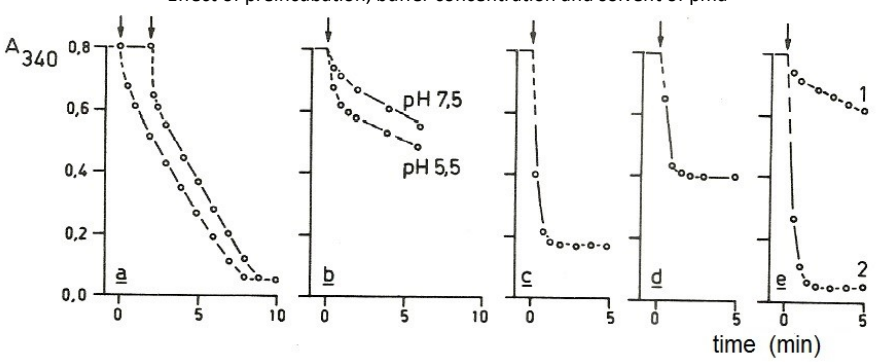
absorption A_{340} 0.62 = 0.30 µmol NADH). Due to the stoichiometry of this conversion, a quantitative assay for pma became available for further research.

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

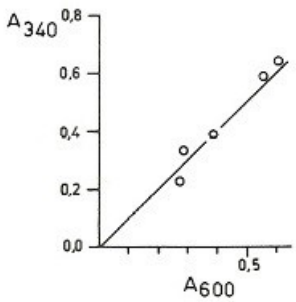
A quantitative explanation for phase I was obtained by taking samples during the rearrangement in ethanol at various moments at 0^{o}. Approximately, the following relationship was found:

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.

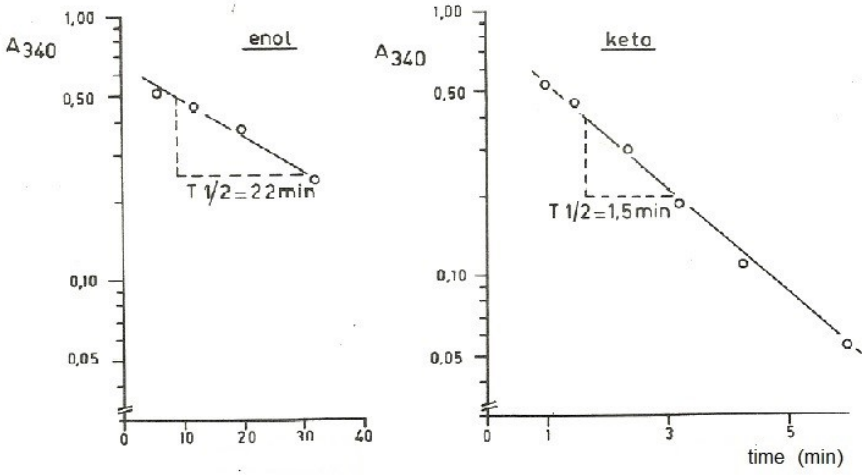
In order to measure the speed of decomposition of keto-pma, 2.5 mg pma was dissolved in 50 µL ethanol, water was added up to 1 ml. Samples of 40 µL of this keto-pma solution were preincubated with 0.4 ml 33 mM K-phosphate pH 7.5 during various periods of time. Thereafter, NADH and TDH were added in 33 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).

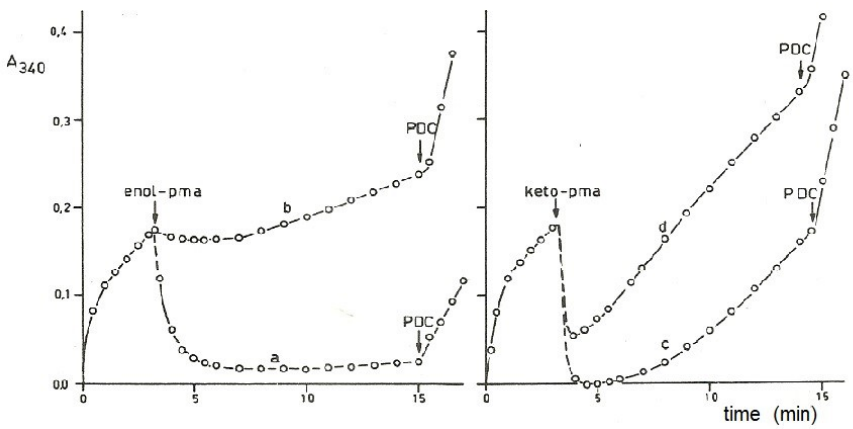
These values are in good agreement with the decomposition constant of keto-pma, as calculated from the experiments earlier in this chapter. One may conclude that the NADH production after establishment of the equilibrium is controlled by the decomposition rate of the keto-pma. It cannot be explained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

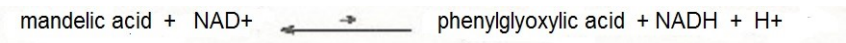
Table 6.17
Equilibrium constants for the NAD^+ dependent dehydrogenation of tropic acid

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.

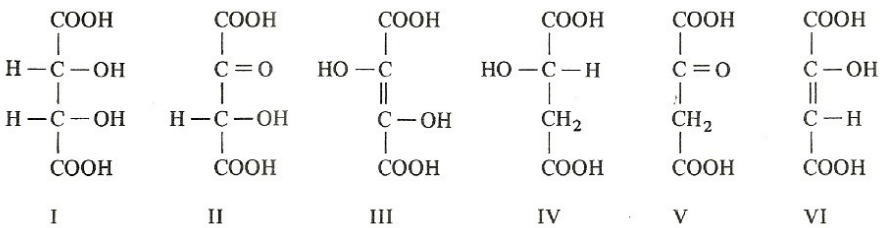
In the Pseudomonas ATCC 12633, mandelic acid (2-hydroxyphenylacetic acid) is oxidized to phenylglyoxylic acid by means of a mandelic dehydrogenase. This enzyme seems to be unable to use NAD^+ as hydrogen acceptor (Kennedy 1968). One could imagine that in the hypothetical reaction :



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.

CHAPTER 7

THE 2-PHENYLMALONIC SEMI-ALDEHYDE DECARBOXYLASE

7.1 INTRODUCTION

Third enzyme in Pseudomonas PMBL-1 involved in the conversion of atropine into phenylacetic acid is the 2-phenylmalonic semi-aldehyde decarboxylase (PDC). It is not possible to measure the activity of PDC in a simple way (for instance by measuring CO_2 production), because the decarboxylation of 2-phenylmalonic semi-aldehyde (keto-pma) takes place spontaneously in aqueous environment (half life time about 90 sec pH 7.5 25^0).

To measure the activity and to investigate the properties of this enzyme, use has been made of the effect of PDC on the enzymatic dehydrogenation of tropic acid in the presence of purified TDH in neutral environment. In this process, the rate of the NADH production after establishment of the equilibrium is determined by the speed of withdrawal of the keto-pma from the equilibrium. Since this decarboxylation is accelerated by the PDC, it is possible to use this NADH production as a measure for the activity of the decarboxylase.

This chapter deals with the identification of the enzyme (7.2), description of its assay (7.3), the partial purification (7.4) and properties (7.5) of the purified enzyme. In the discussion (7.6) the question is raised why an enzyme is present in Pseudomonas to catalyze a reaction that proceeds spontaneously very well.

7.2 INDICATIONS FOR THE EXISTENCE OF A DECARBOXYLASE

In the preceding chapter, it has been shown that the dehydrogenation of tropic acid by purified enzyme in neutral environment proceeds through the establishment of an equilibrium followed by a rate limiting non-enzymatic decarboxylation of the keto-pma formed.

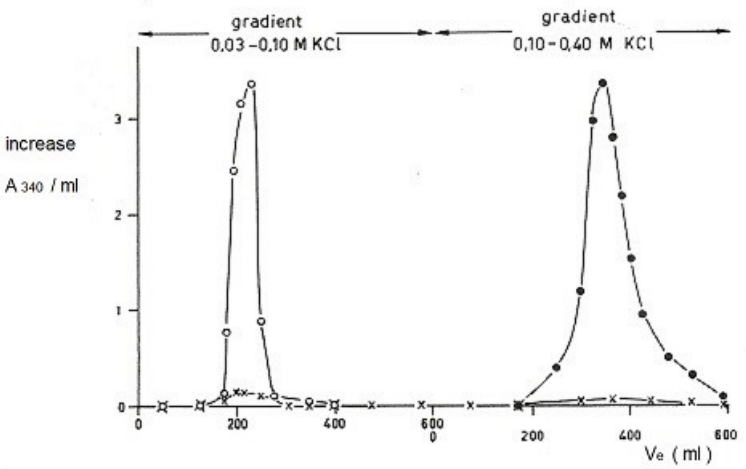
The establishment of the equilibrium was not observed using non-purified TDH samples. This suggested that in the crude TDH another component could be present able to accelerate the decarboxylation of keto-pma, so a terminating reaction would result instead of a slowly shifting equilibrium.

Chromatography of non-purified TDH

The establishment of the equilibrium was only noticed using TDH purified with DEAE cellulose chromatography. Therefore, it was conceivable that the presumed component mentioned above was separated from the TDH. This suggestion was confirmed in the following experiment.

A DEAE cellulose column was used to absorb a non-purified TDH sample. The column was eluted first with 600 ml HMP with an increasing salt concentration 0.03 - 0.1 M KCl and thereafter with 600 ml HMP with an increasing salt concentration of 0.1 - 0.4 M KCl (fig 7.1). The eluate was investigated for the presence of TDH with the activity assay at pH 7.5. Measured in this way, small activity x – x – x was present in fraction 40-50 (elution volume 200-250 ml).

Fig 7.1
Fractionation of a TDH sample on DEAE cellulose



The column was eluted with 600 ml salt gradient 0.03 - 0.1 M KCl in HMP (fraction 1-120) and subsequently with 600 ml 0.1 - 0.4 M KCl in HMP (fraction 121-240). On the ordinate: the TDH activity in the fractions (after adding to tropic acid and NAD^+ in 33 mm K-phosphate pH 7.5) as the total increase in absorption, during the first 3 min calculated for 1 ml eluate.
x – x – x increase absorption measured without addition
• – • – • increase of absorption measured with 50 µL fraction 46 added
o – o – o increase of absorption measured with 50 µL fraction 192 added
Fraction volume 5 ml. More data on chromatography please see chapter 2.11

Following the suggestion above, one could imagine that in the other fractions a component was present able to stimulate the NADH production by purified TDH at pH 7.5. The NADH production by TDH fraction 46 was tested for a possible effect on any of the other fractions. Curve x – x – x represents TDH activity without addition, curve • – • – • TDH activity of fractions in presence of fraction 46.

The fractions 160 – 240 (elution volume 200-600 of the second salt gradient) showed at pH 7.5 a much larger NADH production in the presence of fraction 46 than expected on basis of the fractions separately. In these combined fractions

different kinetics were observed as well: whereas with fraction 46 only NADH production quickly decreased (establishment of the equilibrium), fraction 46 showed in the presence of any of the fractions 160-240, kinetics of a continuous NADH production as it was observed with non-purified TDH preparations at this pH. The fractions 160-240 might contain a component able to withdraw the pma from the equilibrium.

In the same way, "fraction 192" was investigated. Named after the most active fraction, "fraction 192"is the collective name for the combined fractions 180-200 (100 ml; elution volume 300-400 of the second salt gradient); the effect of "fraction 192" was studied on the NADH production in the presence of fractions with TDH activity at pH 7.5. The NADH production by the fractions 40-50 was stimulated considerably by fraction 192. Also in this case the NADH production shifted from a biphasic process into a more continuous formation of NADH.

Samples of fraction 192 treated with proteolytic enzymes (subtilisin, pronase) or heated at 100^0 for 5 min lost their stimulating activity; therefore, the responsible component was most probably a protein.

Effect of fraction 192 on the equilibrium catalyzed by TDH

The change in kinetics is illustrated in fig 7.2. Purified THD taken from fraction 46 was incubated with tropic acid and NAD^+ at pH 7.5. After establishment of the equilibrium, 100 µL of fraction 192 was added. This resulted instantly in a NADH production with the kinetics of the non-purified enzyme.

A similar result was obtained by adding an amount of hydrazine instead of fraction 192. Hydrazine is known to react with aldehydes; it might be able to withdraw the pma from the equilibrium. The resemblance of the effect of the protein fraction and the hydrazine supports the assumption that the effect of fraction 192 has to be ascribed to a component that catalyzes conversion of pma.

Effect of fraction 192 on the stability of keto-pma

Direct proof that the protein in question catalyzes the conversion of pma – and not affects the enzymatic dehydrogenation in another way - was obtained by study of the effect of this protein on the stability of keto-pma; the enzymatic hydrogenation reported in 6.6 was used for the assay of keto-pma.

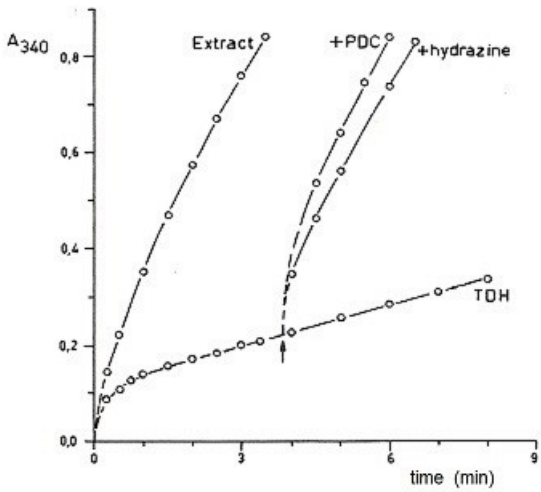
The effect of the protein on the stability of pma was assessed by quantifying the amount of pma that was decomposed in 33 mM K-phosphate pH 7.5 during 30 sec, compared with the amount of pma converted in the presence of fraction 192 under comparable circumstances.

The results reported in table 7.3 show that in the absence of the fraction 192

protein 21% of the pma is converted spontaneously after 30 sec. In the presence of the protein fraction 192, pma is converted completely in the same amount of time.

Fig 7.2

Effect of PDC and hydrazine on the enzymatic dehydrogenation of tropic acid at pH 8.0



Tropic acid and NAD^+ were incubated with non-purified TDH ("extract") or with purified TDH ("TDH" fraction 46 fig 7.1). After 3 min and 45 sec were added: 50 µL fraction 192 ("PDC") and 0.25 M hydrazine pH 8.0 respectively. Correction has been made for the change in volume. Further details see fig 6.6

Fig 7.3

Effect of the protein fraction on the stability of pma

incubation time (sec)	"fraction 192"	concentration pma mM
0	–	0.067
30	–	0.053
30	+	<0.001

Keto-pma (in ethanol-water 1-20) was incubated with 0,3 µmol NADH in 3 ml 33 mM K-phosphate pH 7.5 at 25^0 in absence or presence of 20 µL fraction 192. On time = 0 and time = 30 sec, the pma concentration was measured by addition of TDH (see 6.6).

It is obvious to suggest that the conversion of pma by the protein component, will be the decarboxylation of pma and the protein thus a decarboxylase. This has been investigated by analysis of the products formed.

Effect of fraction 192 on the production of phenylacetaldehyde

During the enzymatic decarboxylation one expects the formation of CO_2 and phenylacetaldehyde, but these compounds will also be formed spontaneously during the fast decarboxylation of keto-pma. If one wants to discriminate between enzymatic decarboxylation and this spontaneous decomposition, special precautions have to be taken. For instance, a very short incubation time with a large amount of enzyme, or a system which offers a low concentration of keto-pma to a relatively high amount of enzyme during a prolonged period of time. The latter was realized by incubating tropic acid, NAD^+ and TDH in the presence of a NADH → NAD^+ regenerating system: pyruvic acid as hydrogen acceptor and lactic acid dehydrogenase. This system converted the NADH formed under consumption of pyruvic acid in NAD^+ and lactic acid. The NADH concentration remained low. The dehydrogenation was not inhibited by the NADH formed (6.4.5 and 6.5).

The conversion of tropic acid in this system was investigated in presence and absence of fraction 192. In the presence of the fraction, a considerable amount of phenylacetaldehyde was formed during incubation at 25^0 during 60 min. This was visualized by thin layer chromatography, elution fluids BEM (fig 7.4), EMX.

In the absence of fraction 192, only a small amount of phenylacetaldehyde was formed (data not shown). This experiment proofs that the protein fraction is involved in the formation of the product of decarboxylation: phenylacetaldehyde. This conclusion in combination with the direct effect of the enzyme on keto-pma is a strong indication that the protein fraction 192 contains the 2-phenylmalonic semi-aldehyde decarboxylase (PDC).

The reaction product was identified by treatment of a diethyl ether extract of the incubation mixture with 2.4dinitrophenylhydrazine, followed by analysis by thin layer chromatography. The formation of the 2.4 dinitrophenylhydrazon of phenylacetaldehyde was demonstrated: with the elution fluid benzene: petroleum ether $40\text{-}60^0$ (75:25 v/v); the hydrazon of the product had a R_f value of 0.27; R_f of the hydrazon of reference compound phenylacetaldehyde 0.28.

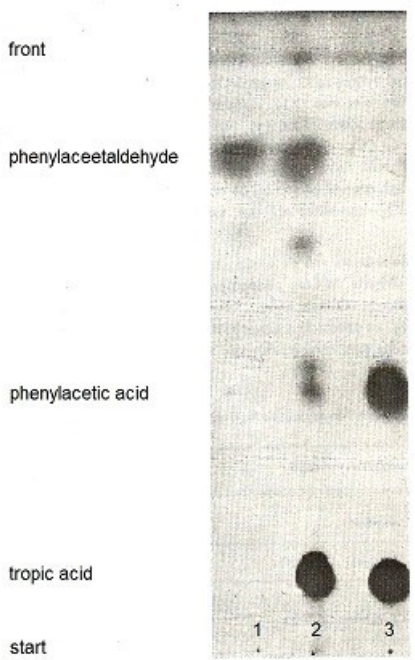
In gas liquid chromatography, the primary product of the conversion had a retention time of 8 min, equal to that of the reference phenylacetaldehyde.

Effect of fraction 192 on the production of CO_2

The formation of the reaction product CO_2 and the effect of fraction 192 on this formation was studied by the Warburg manometric method (2.6).

Fig 7.4

Thin layer chromatography of the incubation mixture of tropic acid, TDH and PDC



The reaction mixture consisted of tropic acid (300 µmol), NAD^+ (10 µmol), TDH 20 U and fraction 192 (1 ml) in 15 ml 0.1 M K-phosphate pH 8.5. A NAD^+ regenerating system (450 µmol pyruvic acid and 0.3 mg LDH) was added. Incubation at 25^0 during 60 min. The reaction mixture was extracted with diethyl ether and analyzed by chromatography with elution fluid BEM. Detection H_2SO_4/HNO_3

1. phenylacetaldehyde
2. incubation mixture
3. tropic acid and phenylacetic acid

Pyruvic acid (R_f 0.12) can not be made visible with the detection method used.

Tropic acid, NAD^+, TDH and fraction 192 were deposited in the main compartment of the Warburg vessel and incubated in the presence of the NAD^+ regenerating system. The incubation was carried out at pH 8.4. CO_2 if formed would remain as bicarbonate in solution. After 60 min, H_2SO_4 was added from the side compartment; from the subsequent change in pressure, the amount of µliter CO_2 formed during the incubation was calculated.

Table 7.5 shows the considerable amount of gas released from the complete incubation mixture. With KOH in the side compartment, there is no gas production; therefore, the developed gas is CO_2. The CO_2 production did not occur in the absence of TDH or fraction 192. The large effect of this fraction on the CO_2 production is a further confirmation of the role of this enzyme in the breakdown of tropic acid. Thiamine pyrophosphate and pyridoxal phosphate, common cofactors in enzymatic decarboxylation do not have an effect on the CO_2 development; there was no effect of Mg^{++} or EDTA.

This confirms the direct role of protein fraction 192 in the decarboxylation of pma into phenylacetaldehyde and CO_2. It concerns here the 2 phenylmalonic semi-aldehyde decarboxylase. By the identification of phenylacetaldehyde and CO_2 two products have been identified indirectly formed in the dehydrogenation of tropic acid.

Fig 7.5

CO_2 production during the conversion of tropic acid by TDH and PDC

Incubation mixture	mM	µL CO_2
complete	–	209
middle compartment with KOH	–	9
no TDH	–	4
no PDC (fraction 192)	–	6
+ thiamine pyrophosphate	0.15	195
+ pyridoxal phosphate	0.15	210
$MgCl_2$	0.5	219
EDTA	0.5	198

The incubation mixture in the main compartment of the Warburg mini vessel contained 13 µmol tropic acid, 1 µmol NAD^+, 0.8 U TDH, 0.2 ml fraction 192 and the NAD^+ regenerating system (0.3 mg LDH and 18 µmol pyruvic acid) in 2.7 ml 40 mM K-phosphate pH 8.4. In the inner compartment, 0.1 ml water or 0.1 ml 0.5 N KOH was deposited; in the side compartment, 0.2 ml 2 N H_2SO_4. After 60 min incubation at 30^0, the sulfuric acid was added and the CO_2 released measured.

7.3 THE QUANTITATIVE ASSAY OF THE PDC ACTIVITY

The assay of PDC (see 2.9.3) is based on the effect of this decarboxylase on the enzymatic dehydrogenation of tropic acid. The enzyme assay was carried out as follows: 10 mM tropic acid, 0.8 mM NAD^+ and 0.8 U TDH were incubated in phosphate buffer pH 8.5 until the rate of NADH production was controlled by the spontaneous decarboxylation of pma. Next, PDC was added and the further NADH production registered. The PDC activity was calculated from the difference of the NADH production before and after the addition of PDC.

To prevent that in the presence of PDC the shift in the equilibrium became rate controlling, excess TDH (0.8 U) was used. The tropic acid and NAD^+ concentration and the pH are based on the results of introductory experiments, in which these parameters were varied. At a higher pH or at a higher substrate or NAD^+ concentration is it not possible to measure the effect of PDC accurately due to the high absorption at 340 nm as the result of the establishment of the equilibrium under these conditions. Choosing for a lower pH or a reduced tropic acid or NAD^+ concentration lowers this absorption, but the increase of absorption registered after the addition of PDC was under these circumstances not directly proportional with the time, preventing an accurate assay of this enzyme.

The conditions described allow well for an accurate assay of PDC. The enzyme activity measured is directly proportional with the amount added (fig. 2.2).

This enzyme assay is not affected by TDH that might be present in PDC samples because this enzyme is already added in excess. But the PDC assay is disturbed by the next enzyme in the metabolic pathway of tropic acid, the phenylacetaldehyde dehydrogenase PDH. Under the conditions described, the product of the decarboxylation (phenylacetaldehyde) is converted in phenylacetic acid under production of NADH. This can be repressed by heating the enzyme sample (0.5 mg/ml) on 55^0 during 15 min; this results in complete inactivation of the PDH while the PDC keeps nearly its full activity.

7.4 PARTIAL PURIFICATION OF THE PDC

The result of the purification of the decarboxylase enzyme (2.11.2) is reported in table 7.6. The enzyme has been purified 85 x comparing with the crude bacterial extract. Yield 29%. The enzyme was nearly completely separated from the other tropic acid enzymes.

The behavior during gel filtration over Sephadex G-100 allows an estimation of the molecular weight on 45.000 (2.11.3); this treatment did not result in further purification.

Table 7.6

Partial purification of the PDC from PMBL-1

Preparation	TA	yield	SA	purification
Extract	7	100%	0.76	1x
After streptomycin precipitation	6.9	99%	–	–
After ammonium sulfate precipitation	3.9	56%	3.5	5x
After DEAE chromatography	2.0	29%	64.2	85x

Enzyme activity and protein concentration were measured according to 2.9. TA is reported in 10^3 U. SA in U/mg protein.

The isoelectric point of the enzyme is 4.6 as determined by electrophoresis in a gradient of LKB 'Ampholytes'.

7.5 PROPERTIES OF THE PURIFIED ENZYME

7.5.1 Substrate specificity

The assay for the activity of the PDC is based on the decarboxylation of the pma, formed as the result of the enzymatic dehydrogenation of tropic acid. Therefore, it is not possible to use this test (7.3) to study the PDC substrate specificity.

The ability of aromatic acids to serve as substrate for PDC has been investigated by incubating about 20 µmol of these compounds with the enzyme (2 U) at pH 7.0 and 25^0 during 1 hour. The incubation mixture was acidified and extracted with di-ethyl ether. The extract was analyzed for the presence of metabolites using thin layer chromatography with elution fluids EMX and BEM.

Detection by H_2SO_4-HNO_3, 2,4-dinitrophenylhydrazine and rhodamine B, an agent for universal detection of UV absorbing compounds. Incubation without PDC served as control.

The pma was decarboxylated into phenylacetaldehyde, both spontaneously and in the presence of PDC. However, none of the following compounds was decarboxylated by the PDC:

phenylglyoxylic acid	2-phenyl-2-hydroxypropionic acid
phenylpyruvic acid	phenylacetic acid
phenylmalonic acid	2-phenylpropionic acid
tropic acid	2-hydroxy-phenylacetic acid

In principle, this method can be used to show decarboxylation. This is concluded from similar experiments in which phenylglyoxylic acid was incubated with the phenylglyoxylic decarboxylase that can be isolated from Pseudomonas ATCC 12633 (9.5); in this case, a considerable conversion in benzaldehyde was demonstrated.

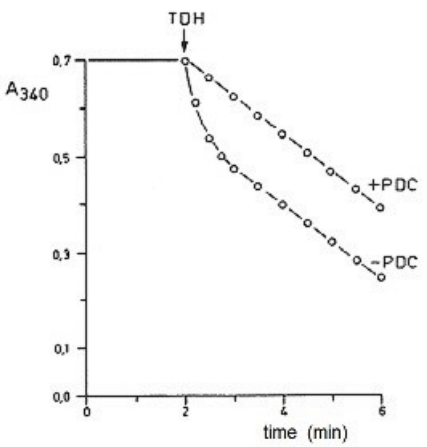
The inability of tropic acid, phenylmalonic acid and phenylacetic acid to serve as a substrate for PDC was also confirmed by manometric experiments. During incubation of these compounds, production of CO_2 was not observed in the presence of PDC.

7.5.2 Specificity for the keto-form of pma

As reported (7.2) decarboxylase has a strong catalytic effect on the decarboxylation of keto-pma. In a similar experiment, enol-pma was incubated in a solution of NADH in presence and absence of PDC during 2 min. Then, TDH was added and the absorption decrease at 340 nm registered (fig 7.7)

Without PDC, the kinetics of the decrease of the absorption observed was as expected. First, a fast initial decrease (phase I) as the result of the hydrogenation of the amount of keto-pma, present at the time of addition of the TDH. Next, a slow decrease (phase II) controlled by the rate of conversion of the remaining enol-pma into the keto-form.

Fig 7.7 Effect of PDC on enol-pma



Curve – PDC: enol-pma (1.5µmol) was incubated with 0.11 mM NADH in 20 mM K-phosphate pH 7.5 at 25^0 during 2 min. Then, 5 U TDH was added.
Curve + PDC: as above but 0.2 U PDC was added on time t=0.

TDH was added to a solution of enol-pma, preincubated with PDC. In this case, the initial fast decrease of the A_{340} was not observed. The decrease of absorption in phase II occurred with exactly the same speed as the in previous experiment. Apparently, the preincubation with PDC results exclusively in the decarboxylation of the keto-pma, whereas enol-pma is not converted by this enzyme.

This shows that PDC is specific for keto-pma. In this experiment, a large amount of TDH and only a small amount of PDC was used. This was in order to prevent that the PDC would affect noticeably the decrease of absorption in phase II by decarboxylation of keto-pma, in this phase formed by the tautomeric rearrangement.

7.5.3 The stability of the enzyme

The stability of the PDC has been investigated at various temperatures using a purified sample, dialyzed against HMP and containing 0.18 mg protein/ml.

The PDC appeared to be much more stable at higher temperature compared with the other tropic acid enzymes. It was not inactivated by an incubation at 65^0 during 30 min; the enzyme was inactivated 50% by incubation at 85^0 30 min.

The stability of PDC during incubation at various pH values has been studied with the same preparation. The enzyme was stable at 25^0 during 30 min in the pH range of 5-10, but was inactivated outside this pH range.

7.5.4 Miscellaneous

The assay of PDC described in this chapter does not allow to establish the pH optimum for this enzyme, because changes in the pH have effect on the TDH and the establishment of the equilibrium as well. But the effect of some additions could be studied that do not affect the tropic acid dehydrogenation in neutral environment. The metal ions: Mg^{++}, Mn^{++} and Ca^{++} (1 mM), EDTA (1 mM), phenylacetaldehyde and phenylacetic acid (1 mM) did not have an effect on the enzymatic decarboxylation.

In the experiments with the technique according to Warburg (7.2), any effect of the cofactors thiamine pyrophosphate and pyridoxal phosphate was not observed either.

Finally, it should be mentioned that the activity of the PDC can be demonstrated titrimetrically as well, at pH 5.0 in a pH-stat. This is possible because the decarboxylation at this pH is accompanied by acid consumption: the CO_2 does not go in solution as bicarbonate. During incubation with pma, acid consumption was observed in this system, which increases after addition of PDC with a factor of 2.5. However, this method is not suitable for an activity assay of the enzyme.

7.6 DISCUSSION

The action of the PDC has been demonstrated in various ways. Production of NADH is accelerated during the enzymatic dehydrogenation of tropic acid in neutral environment after establishment of the equilibrium (see also fig. 6.9 and fig. 6.16). The enzyme stimulates the formation of phenylacetaldehyde and CO_2 and has a direct effect on the stability of keto-pma. These observations lead to the conclusion that it concerns indeed the 2-keto-phenylmalonic semi-aldehyde decarboxylase.

Hydrazine has a similar effect on the equilibrium established in the presence of TDH. Probably this is related as well to the withdrawal of pma from the equilibrium. This could be caused by a reaction of pma with hydrazine to form the hydrazone; however, it is also possible that hydrazine accelerates the decomposition of pma.

Because of the indirect assay of the PDC activity, it is not possible to determine the pH-optimum. The enzyme does not need a cofactor like thiamine pyrophosphate or pyridoxal phosphate. The enzyme has a remarkable resistance against higher temperatures unlike various decarboxylases for other aromatic acids, including the 2,3-dihydroxybenzoic acid decarboxylase (Subba Rao 1967) and the phenylpyruvic acid decarboxylase (Asakawa 1968). These enzymes are very unstable.

Thin layer chromatography has been used to study the substrate specificity of PDC; none of the aromatic acids tested except for pma is converted in the presence of PDC. The PDC does not catalyze the decarboxylation of enol-pma either. It seems probable that PDC is specific for the substrate keto-pma. The specific function of this enzyme in the bacterium is confirmed by studying the induction of PDC to be reported in chapter 10.

Due to the instability of the keto-pma in neutral aqueous environment, it seems on first sight strange that an enzyme with the function to catalyze the decarboxylation of pma is produced in Pseudomonas PMBL-1.

The presence of the decarboxylase could be understood if one assumes that under physiological circumstances - due to the position of the equilibrium catalyzed by TDH – the steady state concentration of the keto-pma is very low. As a consequence, the spontaneous decomposition of pma results in the production of a very low amount of phenylacetaldehyde per unit of time, too low to support the normal growth with tropic acid as sole source of carbon.

The necessity of the presence of the decarboxylase is evident in mutants that have lost exclusively the genetic information for PDC. These are not able any longer to grow with atropine or tropic acid as sole source of carbon (10.7)

In the metabolic pathway of tropic acid, the first step is a NAD^+ dependent dehydrogenation, followed by an enzymatic decarboxylation. From a theoretical point of view, this is very interesting with regard to the use of NAD^+ as cofactor. The position of the equilibrium suggests that the redox potential of NAD^+/NADH is considerably lower as compared with that of tropic acid/pma. NAD^+ as such is therefore not very suitable as hydrogen acceptor for this dehydrogenation. If the TDH would use a hydrogen acceptor with a higher redox-potential, the dehydrogenation as such would proceed easier. However, such a hydrogen acceptor is less favorable for the energy supply of the cell.

Apparently, Pseudomonas PMBL-1 can use NAD^+ as hydrogen acceptor for the dehydrogenation of tropic acid. This might be feasible because in the next irreversible step pma is continuously removed by decarboxylation. This means that the large decrease in free energy as result of the decarboxylation enables the cell to use a hydrogen acceptor of low redox potential in the preceding step.

This is of course very beneficial for the energy supply in the cell.

CHAPTER 8

THE PHENYLACETALDEHYDE DEHYDROGENASE

8.1 INTRODUCTION

The enzymatic dehydrogenation of phenylacetaldehyde is the last reaction in PMBL-1 in the conversion of atropine into phenyl acetic acid. The enzyme involved is called phenylacetaldehyde dehydrogenase (PDH). The enzyme is present in extract of PMBL-1 cultivated in the presence of atropine or tropic acid. PDH like tropic acid dehydrogenase can make use of the cofactor NAD^+. However, it distinguishes itself in many aspects from the TDH. Initially, the investigation of PDH was seriously hampered due to the low stability of the enzyme during dialysis and in diluted suspensions. In an especially composed buffer, inactivation of the PDH (8.2) could be prevented. It appeared possible to set up a procedure for the partial purification of the enzyme (8.3). In the presence of the enzyme, phenylacetaldehyde is converted into phenylacetic acid under stoichiometric consumption of NAD^+. As far as could be ascertained, the reaction in vitro is not reversible.

The dehydrogenase is specific for phenylacetaldehyde and has a great affinity for its substrate. PDH is absent in PMBL-1 cultivated with phenylacetic acid or glucose as source of carbon. Probably, it concerns an inducible enzyme as well.

8.2 ACTIVITY ASSAY AND STABILITY OF THE ENZYME

The quantification of the PDH is based on the same NADH method as described for TDH (2.9.3). The amount of NADH formed during the reaction is measured spectrophotometrically. The system used is composed of 0.13 mM phenylacetaldehyde and 0.8 mM NAD^+ in 33 mM K-carbonate buffer pH 9.0 (see 8.4). Both substrate and NAD^+ are present in sufficient concentration to allow the enzymatic conversion to proceed as a zero order reaction. Inhibition by excess substrate was not observed.

Initially, the ultrasonic extraction of the bacteria (2.11.1) was carried out in 10 mM K-phosphate buffer pH 7.0. However, the enzyme appeared not to be stable in such extracts. In less concentrated samples (1-4 mg protein/ml), the activity of the PDC was reduced to 70 % of the value found immediately after extraction in several hours. After 18 hours, activity was only 10-30%. The activity of more concentrated samples (20-50 mg protein/ml) was not constant either, although the enzyme activity decreased less rapidly. After dialysis against 10 mM tris HCl pH 8.0 during 48 hours at 0^0, the PDH was completely inactivated.

The inactivation was independent of the protein concentration. The instability was a serious complication for the purification and further research on the PDH. Therefore, the stability of a non-purified sample was investigated in detail during dialysis and storage in diluted solution. It was tried to find conditions under which the inactivation did not occur or was less.

8.2.1 Stability during dialysis

The PDH was inactivated irreversibly by dialysis against 10 mM tris-HCl pH 8.0 (48 hours at 4^0) (table 8.1). Reactivation could not be achieved neither by addition of concentrated dialysis buffer nor by adding a heat-denatured enzyme sample. The inactivation could not be undone by addition of the ions: Mg^{2+}, Zn^{2+} or Mn^{2+} and not by the cofactors NAD^+ or $NADP^+$.

A partial stabilization could be realized during dialysis of a more concentrated sample by adding 100 mM KCl to the dialysis buffer (see table 8.1). Even more protection was obtained by addition of 1 mM EDTA. The salts NH_4Cl, $MgCl_2$ and Na_2SO_4 could not replace the KCl. Dialysis against 100 mM K-phosphate pH 8.0 and 1 mM EDTA resulted in even less inactivation. The activity after dialysis against 100 mM K-phosphate pH 7.0 and 1 mM EDTA was about the same as that of a non-dialyzed sample stored at 4^0 during 48 hours. Apparently, the enzyme is protected by 100 mM K^+ ions against inactivation by dialysis. However, both samples contained 40% less enzyme activity compared to the value found immediately after the preparation of the extract.

Table 8.1

Effect of the composition of the dialysis buffer on the stability of PDH

Buffer	Enzyme activity
10 mM tris-HCl pH8.0	0.01
10 mM tris-HCl pH8.0 + 100 mM KCl	0.15
10 mM tris-HCl pH8.0 + 100 mM KCl + 1 mM EDTA	0.40
100 mM K-phosphate pH 8.0 + 1 mM EDTA	0.67
100 mM K-phosphate pH 7.0 + 1 mM EDTA	0.95
direct after extraction	1.62
after storage 48 hours at 4^0	1.01

A non-purified PDH sample (24 mg protein/ml; 5 ml) was dialyzed against 2 litres buffer as specified during 48 hours. Enzyme activity was assayed according to 2.9.3.

8.2.2 Stability in diluted samples

The use of 100 mM K-phosphate pH 7.0 and 1 mM EDTA could not prevent loss of activity during the ultrasonic treatment, especially in diluted samples (1-4 mg protein/ml). Therefore, the effect of various compounds on the stability of PDH after dilution has been further investigated.

Starting point was a very concentrated extract (37 mg protein/ml), containing 3.1 U/ml. This was diluted 20 times in 50 mM K-phosphate pH 7.0 and 0.I mM EDTA supplemented with one or more other compounds. Enzyme activity was assayed directly after dilution and after storage at 4^0 during 48 hours. As shown by table 8.2, several compounds are able to prevent the inactivation partially: mercapto ethanol (ME), Na lauryl sulfate (SDS), the substrate phenylacetaldehyde and the cofactor NAD^+ in the concentrations used.

Later it was found that 0.01% bovine serum albumin and the SH compounds as cysteine, dithiothreitol and glutathione (1 mM) do not give protection. The same holds for the surface active compounds Triton X-100 and saponin (0.01%), the cofactor flavin-adenine-dinucleotide (1 mM), ammonium sulfate (1%) and aceton (5%).

Na-lauryl sulfate, mercapto ethanol and phenylacetaldehyde in combination offer a good protection for the spontaneous inactivation of PDH (table 8.2). After dialysis against 50 mM K-phosphate pH 7.0, 0.1 mM EDTA, I mM ME, 0.05 mM SDS and 1 mM phenylacetaldehyde, even a slight increase in enzyme activity was found. It was possible to obtain extracts with a reproducible PDH content by using this buffer during the preparation of extracts.

Table 8.2
Effect of some compounds on the stability of PDH in a diluted solution

	mM	activity immediately after dilution (U/ml)	activity after 48 h at 4^0 (U/ml)
1.no addition	–	0.165	0.025
2.mercapto-ethanol	1	0.165	0.047
3.Na-lauryl sulfate	0.05	0.160	0.120
4.phenylacetaldehyde	1	0.160	0.126
5.NAD^+	10	0.180	0.076
2 + 3 +4 in combination	–	0.165	0.208

A non-purified PDH sample (3.1 U/ml; 37 mg protein/ml) was diluted 20 x with 50 mM K-phosphate buffer and 0.1 mM EDTA (pH 7.0). Compounds named in the table were added. Enzyme activity was assayed according to 2.9.3.

The activity remained constant also in extracts of small amounts of bacteria, so allowing a reliable assay of the enzyme content.

In the further investigations of the stabilizing effect of various compounds, a solution containing 50 mM phosphate was used and not 100 mM, as was present in the buffer with the good results on dialysis. This is because a salt concentration of 100 mM disturbs various purification techniques like the precipitation with streptomycin sulfate and DEAE cellulose chromatography.

In the assay of PDH in non-purified samples with a low PDH activity, increase of absorption had to be corrected for the absorption increase in the absence of substrate. This correction was not possible when phenylacetaldehyde was present in the enzyme sample. In these cases, the phenylacetaldehyde was omitted from the stabilizing buffer; the PDH assay was then carried out directly after the preparation of the extract.

8.3 PARTIAL PURIFICATION OF THE PDH

Purification of the PDH was carried out in the presence of the stabilizing buffer (see 8.2) en according the method described in 2.11.2. An enrichment of 52x relative to the crude extract was obtained with a yield of 15%, although the presence of phenylaceetaldehyde did not contribute to the elegance of the procedures. The overview of the purification in table 8.3 is representative for 3 purification procedures carried out in this way. The most purified sample had a specific activity of 22.5. U/mg protein; it still contained detectable amounts of AtrE and was considerably contaminated with TDH. The preparation had no PDC activity.

Stabilizing buffer has been used as much as possible. Nonetheless, it could not be prevented that losses occurred in some cases, for example during chromatography. Apparently, the stability of partial purified PDH samples is dependent on some unknown factors.

Table 8.3

Purification of PDH of PMBL-1

Sample	TA	Yield	SA(U/mg)	Purification
Extract	790	100%	0.10	1 x
Streptomycin sulfate precipitation	726	92%	–	–
Ammonium sulfate precipitation	370	47%	0.54	5.4 x
DEAE cellulose chromatography	118	15%	5.2	52 x

Protein concentration and enzyme activity assayed according 2.9

8.4 SOME PROPERTIES OF THE PURIFIED PDH

8.4.1 Analysis of the products of the enzymatic conversion

The conversion of phenylacetaldehyde was investigated by incubation of the compound with PDH in the presence of NAD^+ and a NAD^+ regenerating system at 30^0 during 60 min (7.2). After the incubation, the reaction mixture was extracted and analyzed by thin layer chromatography. The sample did not contain phenylacetaldehyde anymore but instead a compound which behaved like phenyl acetic acid during chromatography with the elution fluids BEM (fig 8.4) and EMX.

Indications for the formation of NADH during the dehydrogenation of phenylacetaldehyde are the same as found for the dehydrogenation of tropic acid (chapter 6.4.1). The absorption spectrum of a reaction mixture of phenylacetaldehyde, NAD^+ and PDH show during the incubation a decrease of the absorption at 260 nm and the specific increase of absorption at 340 nm.

8.4.2 Stoichiometry of the reaction

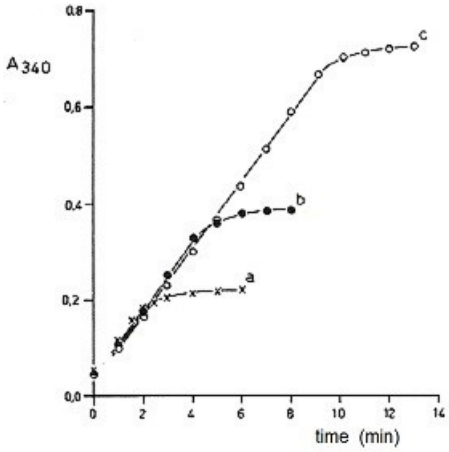
In this experiment, the amount of NADH was determined, which was formed at the complete conversion of a limited amount of the substrate. Since the reaction under the standard condition of the activity assay (2.9.3) proceeds quantitatively, there was no need for special precautions like the high pH in the similar experiment with TDH. In the conversion of 0.08, 0.16 and 0.32 µmol phenylacetaldehyde, an increase of absorption at 340 nm was observed corresponding with the formation of 0.08, 0.16 and 0.32 µmol NADH respectively (fig 8.5).

8.4.3 The pH optimum

The pH dependence of the PDH activity is shown in fig 8.6 curve a. The observed activity of the PDH increased at higher pH, had a relative maximum at pH 9.0 followed by a sharp increase above pH 10. The increase in absorption at 340 nm at high pH appeared to be caused by a spontaneous reaction of the phenylacetaldehyde (aldol-condensation?) resulting in products with high absorption (fig 8.6 curve b). The maximum at pH 9.0 appeared to be the absolute pH optimum for the enzyme after correction for this non enzymatic conversion of phenylacetaldehyde (fig 8.6 curve c).

Fig. 8.5

Effect of the substrate concentration on the formation of NADH



Phenylacetaldehyde was incubated with 0.8 mM NAD^+ and 0.035 U PDH in 3 ml 33 mM K-phosphate pH 9.0. For 0.08, 0.16 and 0.32 µmol phenylacetaldehyde the total absorption increase at 340 nm was 0.165 (curve a), 0.335 (curve b) and 0.67 (curve c) respectively.

It was not possible to measure the activity of PDH above pH 10 due to the spontaneous reaction of phenylacetaldehyde.

8.4.4 Specificity of PDH

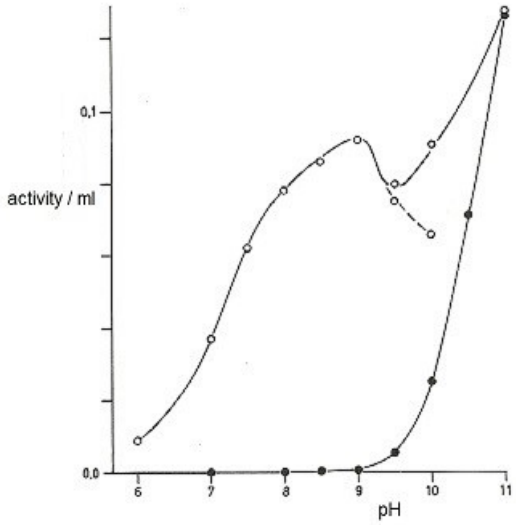
Substrate specificity

Also PDH, the fourth enzyme in the metabolic pathway of atropine and tropic acid showed a high substrate specificity like the enzymes AtrE and TDH. In table 6.5.2 (page 71, right column), a survey is presented of the ability of a number of compounds to serve as substrate for PDH. Benzaldehyde, benzaldehyde derivatives and 2-phenylpropanal show at pH 9.0 a large spontaneous increase in absorption. Therefore, these compounds have been investigated as substrate at pH 8.0 and compared with phenylacetaldehyde at this pH.

Out of all compounds in table 6.5.2, only the phenylacetaldehyde is dehydrogenated by PDH. Even compounds with a large structural resemblance with phenylacetaldehyde are not suitable as its substrate: 2-phenylpropanal, the methyl ester of pma, benzaldehyde and benzaldehyde derivatives do not show activity with PDH.

Fig 8.6

The effect of the pH on the activity of PDH



The activity of the PDH enzyme has been investigated in the range of pH 6 – 9 in 33 mM K-phosphate buffer; in the range of pH 9 – 11 in 33 mM K-carbonate buffer.
At pH 9.0 the activity was nearly the same in both buffers.

a. o ——— o observed increase A_{340} in presence of PDH
b. •———• spontaneous increase A_{340} (no PDH added)
c. o – – – o enzymatic activity corrected for spontaneous increase A_{340}

Phenylpyruvic acid that could be decarboxylated into phenylacetaldehyde did not produce NADH on incubation with NAD^+ and purified PDH enzyme or crude extract. Acetaldehyde was not dehydrogenated either.

Affinity of PDH for its substrate is large. Lowering the phenylacetaldehyde concentration from 0.12 to 0.03 mM does not affect the rate of the NADH production (see fig 8.5). The rate of NADH production is constant until the substrate has been almost completely converted.

From the curve for the conversion of 0.03 mM phenylacetaldehyde, the K_M of the PDH can be estimated. The rate of conversion decreases once the substrate concentration is below 6×10^{-6} M. This suggests a K_M with the substrate phenylacetaldehyde smaller than 6×10^{-6} M.

Suitability of some compounds as hydrogen acceptor

The cofactor $NADP^+$ is not able to replace NAD^+ as hydrogen acceptor. The activity with $NADP^+$ under standard conditions (2.11.2) is less than 1% of the activity with NAD^+. Dichlorophenol-indophenol-sodium (DCIP) nor potassium ferricyanide are suitable as hydrogen acceptor for the purified enzyme (see also 6.4.3).

A reduction of DCIP was observed with purified PDH samples in the absence of NAD^+. This change had to be fully ascribed to a reaction of DCIP with mercapto ethanol, added to the reaction mixture to stabilize the PDH.

8.5 CONVERSION OF PMA BY A PARTIALLY PURIFIED PDH SAMPLE.

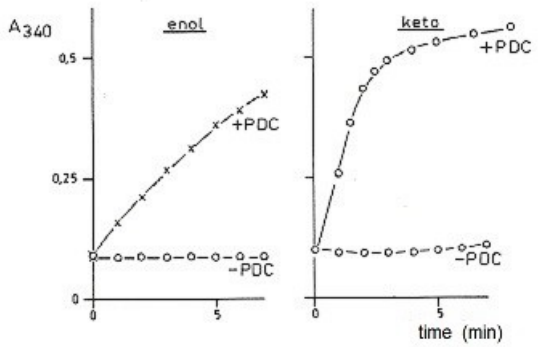
The pma formed from tropic acid by the TDH might be not a suitable substrate for PDH. However, a number of complications arise to prove this point. A priori this seems difficult to prove due to the limited stability of keto-pma; in aqueous environment, this compound is converted nearly completely in phenylacetaldehyde, the substrate of PDH in a short time (half-life 90 sec). One would expect that during incubation of keto-pma with NAD^+ and PDH a certain amount of NADH will be formed. Surprisingly, this was not observed during incubation of 0.3 µmol keto-pma with NAD^+ and a partial purified PDH sample (fig 8.7, right curve - PDC).

The fact that NADH production fails to appear can be explained by the relatively high TDH content of the PDH sample. As a consequence, the NADH formed in the dehydrogenation of phenylacetaldehyde by PDH was consumed again in the back reaction of keto-pma into tropic acid. (fig 8.8). Apparently, this back reaction proceeds fast, compared with the spontaneous decarboxylation of pma and the conversion of phenylacetaldehyde in phenylacetic acid. Therefore, the NADH concentration does not increase significantly during the first minutes. According to this mechanism, two molecules of keto-pma are converted in one molecule tropic acid and on molecule phenylacetic acid in the first phase of the reaction. After some time, the absorption will increase slowly. This is caused by a shift in the by TDH established equilibrium to the right by decarboxylation of pma. At pH 7.5, this reaction is slow due to the low concentration of pma at equilibrium.

This explanation is experimentally confirmed by studying the effect of PDC. The presence of PDC (fig 8.7, curve at the right) accelerates the conversion of keto-pma in phenylacetaldehyde. This reaction is faster than the enzymatic dehydrogenation of phenylacetaldehyde.

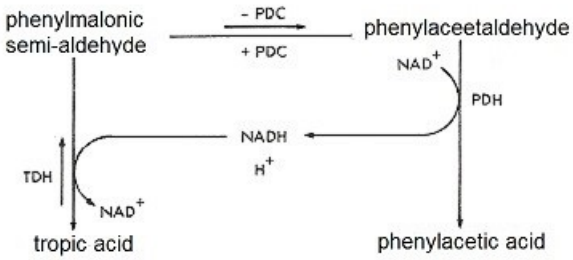
Fig 8.7

Effect of PDC on the conversion of pma by PDH



In 0.2 M tris-HCl pH 7.5 were incubated: 0.3 µmol enol-pma or keto-pma, 2.4 µmol NAD^+ and 0.3 ml of a PDH sample, producing an increase in absorption of 0.190 per min under the conditions of the experiment with phenylacetaldehyde as substrate. The PDH sample contained per ml 0.26 U TDH. The experiments + PDC were carried out in presence of 0.8 U PDC. Total volume 3 ml.

Fig 8.8



Conversion of two molecules of keto –pma: one via in phenylacetaldehyde into phenylacetic acid under production of NADH and CO_2, the other hydrogenated into tropic acid under consumption of NADH.

Net reaction: 2 pma -> phenylacetaldehyde + CO_2 + tropic acid. No NADH produced.

This leads to the situation that the keto-pma will have been largely decarboxylated at the moment that only a small part of the phenylacetaldehyde has been dehydrogenated, that is needed to generate NADH required for the back reaction. So only a small part of the keto-pma will be used for the back reaction and not much NADH will be consumed in that way. Finally, all phenylacetaldehyde formed will be dehydrogenated, resulting in a considerable amount of NADH.

A similar experiment with enol-pma (fig 8.7 curve to the left) is in agreement with the properties described for enol-pma in chapter 6. In absence of PDC, enol-pma produces spontaneously an amount of phenylacetaldehyde but this is considerably less than in the experiment with keto-pma. The dehydrogenation by PDH and the hydrogenation by TDH will be fast compared with the tautomeric rearrangement of the enol-pma and the rate of decomposition of keto-pma. All NADH produced will be consumed for the back reaction. The addition of PDC has much less effect compared with the experiment with keto-pma, because not more keto-pma can be decarboxylated than spontaneously is formed from enol-pma.

From the effect of PDC on NADH production during incubation of pma, NAD^+, PDH and TDH, one can conclude that decarboxylation of keto-pma precedes the dehydrogenation and that pma is not dehydrogenated by PDH or very sparsely.

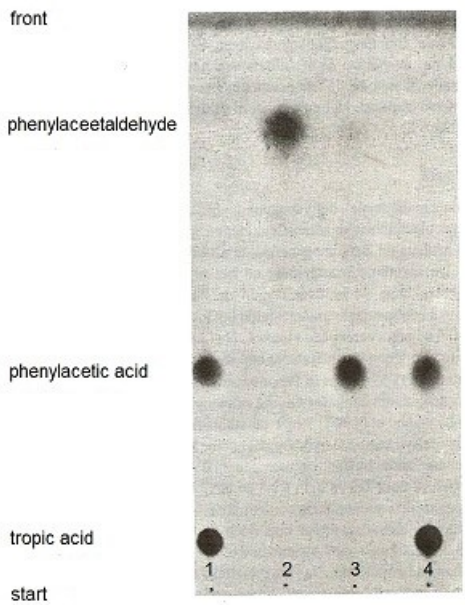
8.6 MISCELLANNEOUS

The enzymatic dehydrogenation of phenylacetaldehyde is an irreversible process in vitro. A decrease of absorption is not observed during incubation of phenylacetic acid with PDH and NADH, even not at lower pH (pH 7.0). The product phenylacetic acid does not act as an inhibitor of PDH; in the presence of I.0 mM of this compound, the dehydrogenation proceeds undisturbed. The dehydrogenation is inhibited by NADH, probably by competition with NAD^+. This inhibition does not play a role under the conditions for the assay of activity of PDH as was demonstrated by the experiment shown in fig 8.5. NADH was produced with in a constant speed until the substrate is converted nearly completely.

The effect of some additions was investigated using a purified PDH, dialyzed against 10 mM tris-HCl and 100 mM KCl pH 7.0. The dehydrogenation of phenylacetaldehyde was inhibited by addition of 0.25 M hydrazine. Probably, the substrate is bound by the hydrazine. This has been used in the assay for TDH (6.2). PDH is not inhibited or stimulated by 0.1 mM adenosine diphosphate, adenosine triphosphate or 10 mM K-phosphate when the activity is measured in an otherwise phosphate-free incubation mixture. This dehydrogenation is probably not directly coupled to a phosphorylation.

Fig 8.4

Conversion of phenylacetaldehyde in phenylacetic acid by the PDH



Phenylacetaldehyde (80 µmol), purified PDH (3U), NAD^+ (10 µmol) and the NAD^+ regenerating system (fig 7.4) were incubated in 33 mM K-phosphate buffer pH 8.0 at 25^0 during 60 min. The incubation mixture was acidified and extracted. The extract was analyzed by thin layer chromatography using elution fluid BEM; detection was carried out with H_2SO_4 – HNO_3 (2.7)

1. tropic acid + phenyl acetic acid
2. not incubated
3. incubated at 25^0 during 60 min
4. tropic acid+phenyl acetic acid

The following metal ions in a concentration of 0.5 mM have no effect on the enzyme activity: Ca^{++}, Mg^{++}, Ni^{++}, Co^{++} and Mn^{++}. PDH is sensitive for SH-inhibitors Cu^{++}, Hg^{++} and p-chloro-mercuri-benzoic acid: the enzyme is completely inactivated by 0.01-0.05 mM during 2 min. Iodoacetic acid, iodoacetamide or Na-arsenite had no effect in a concentration of 1 mM.

A PDH sample with a protein concentration of 0.18 mg protein/ml and dialyzed against the stabilizing buffer (8.2) was tested for its stability at various temperatures and pH values. The activity was completely lost in a treatment at 55^0 for 30 min. Such an incubation at 40^0 did not result in inactivation.

The effect of pH on the stability of PDH was studied by incubation the enzyme at 25^0 during 30 min followed by standard enzyme assay (2.9.3). The enzyme was inactivated completely during incubation at pH < 5 and pH >9.

8.7 DISCUSSION

The enzyme PDH accelerates conversion of its substrate phenylacetaldehyde in phenylacetic acid. The enzyme makes use of NAD^+ as hydrogen acceptor, which is reduced in stoichiometric quantities during the dehydrogenation. The spontaneous inactivation and the loss of enzyme activity during dialysis have initially hampered the study and the purification of the PDH. The use of a specially composed buffer 50 mM K-phosphate pH 7.0 with added EDTA, mercapto-ethanol, Na-laurylsulphate and phenylacetaldehyde made it possible to design a quantitative assay and a procedure for partial purification.

Gunsalus (1953 a) has detected two dehydrogenases for benzaldehyde in ATCC 12633 Pseudomonas putida which could not be purified due to their instability. Stachow (1967) has investigated the stability of a $NADP^+$ dependent benzaldehyde dehydrogenase. The enzyme was losing enzyme activity during dialysis against diluted buffer solutions (10 mM), but was protected by 0.1 M K^+, Rb^+ or NH_4^+ ions. Stachow made the assumption that irreversible conformation changes can be prevented at higher ionic strength. The benzaldehyde dehydrogenase was purified by Stachow in the presence of 0.2 M KCl and 1 mM dithiothreitol.

The stabilizing effect of Na-laurylsulfate (SDS) on PDH may have a connection with the results of Chilson (1965) who investigated the effect of SDS on maleic acid dehydrogenase from pig heart mitochondria. It turned out that inactive enzyme aggregates with a large sedimentation constant were formed in dilute solution. In the presence of SDS this aggregation did not occur; the enzyme activity remained constant.

Phenylacetaldehyde was compared with other aromatic aldehydes as substrate for the enzymatic dehydrogenation by PDH. This enzyme appears to be very specific for phenylacetaldehyde. The low Michaelis constant indicated that the enzyme has a great affinity for this substrate. These properties of the PDH strongly suggest that it concerns an enzyme specifically present in Pseudomonas for the dehydrogenation of phenylacetaldehyde.

CHAPTER 9

THE METABOLIC PATHWAY OF ATROPINE

9.1 INTRODUCTION

The tropic acid enzymes have been identified as the biocatalysts involved in the breakdown of atropine into phenylacetic acid in Pseudomonas PMBL-1. These enzymes have been described in the preceding chapters. This chapter deals with the mutual relationships of these enzymes in the atropine metabolism. The possibility is then discussed that - in addition to these – other enzymes are also induced especially for the metabolism of atropine. Indications will be discussed that the metabolism of atropine in other Pseudomonas PMBL strains proceeds according to the same metabolic pathway as in PMBL-1. Finally, the tropic enzymes will be compared with the enzymes involved in the breakdown of mandelic acid in Pseudomonas putida ATCC 12633.

9.2 SEQUENCE OF ACTION OF THE TROPIC ACID ENZYMES

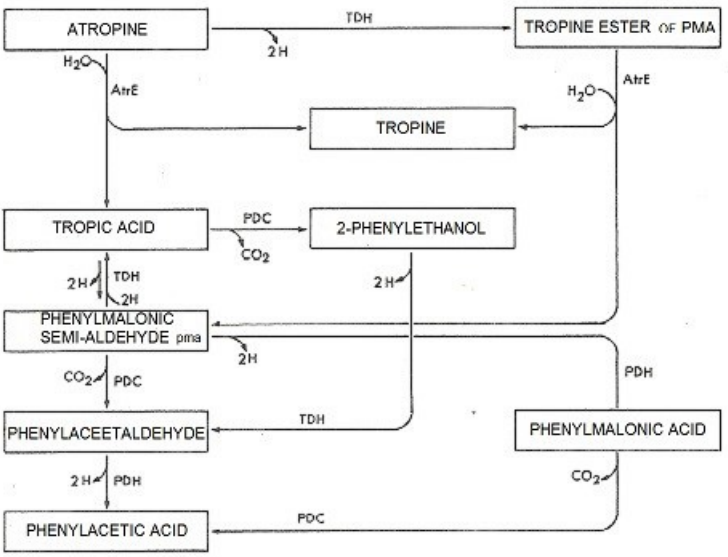
The metabolic pathway of atropine into phenylacetic acid (annex 1) is based on the substrate specificity of the tropic acid enzymes and on the identification of the products formed by these enzymes.

One could question whether these four tropic acid enzymes might operate in another sequence with the same final result. Other relevant other options are visualized in fig 9.1.

A first option is atropine as substrate for TDH and the formation of the tropine ester of pma. This compound might be converted by AtrE in pma and tropine. This option can be excluded since atropine cannot be dehydrogenated by TDH. This has been shown in the following experiment: The AtrE still present in a purified TDH sample was inhibited completely by treatment with the organophosphate Soman (pinacolyl methylphosphonofluoridate). The dehydrogenating capacity of this sample was tested with atropine, tropic acid and the methyl ester of tropic acid as substrate and compared with that of the same TDH sample not treated with Soman. The results presented in table 9.2 show clearly that neither atropine, nor the methyl ester of tropic acid were dehydrogenated by TDH. This also excluded other reaction schemes that form tropine ester of pma as first product.

Fig 9.1

Alternatives for breakdown of atropine into phenylacetic acid by the tropic acid enzymes.



A second possibility, the breakdown of tropic acid into phenylacetaldehyde through 2-phenylethanol can be excluded as well. The formation of CO_2 was not observed during the incubation of tropic acid with PDC, or with an extract of PMBL-1 cultivated in the presence of tropic acid (7.5.1). The compound 2-phenylethanol cannot act as a substrate for TDH and PDH (6.4.3). This compound is not suitable as carbon source (4.2) whereas the tropic acid enzymes were not induced in bacteria grown in a mix of succinic acid and 2-phenylethanol (10.3).

A third possibility is the conversion of pma by PDH in phenylmalonic acid, followed by a decomposition by PDC. The latter reaction however, did not happen; it was shown by thin layer chromatography that PDC is not able to convert phenylmalonic acid in phenylacetic acid (7.5). In addition, growth of PMBL-1 in the presence of phenylmalonic acid does not result in the induction of the tropic acid enzymes (10.3). Moreover, pma was not dehydrogenated by PDH (8.5).

On basis of these data, the conclusion is justified that breakdown of atropine occurs according to the scheme in annex 1 and not according to an alternative metabolic pathway.

Table 9.2

Substrate	Tropic acid dehydrogenase activity (U/ml) without Soman	0.2 mM Soman
Atropine	0.205	0.005
Methyl ester of tropic acid	0.120	0.000
Tropic acid	0.565	0.615

Atropine, the methyl ester of tropic acid and tropic acid as substrate for TDH in absence and presence of 0.2 mM Soman; THD activity assayed according to 2.9.3

9.3 OTHER ENZYMES POSSIBLY INVOLVED IN THE BREAKDOWN OF ATROPINE

9.3.1 Breakdown of tropine

Tropine is formed during hydrolysis of atropine by AtrE. It is obvious to check that tropine, which is produced during the hydrolysis of atropine by AtrE, will also be utilized by the bacterium. A clear indication is the usefulness of tropine as sole carbon source by PMBL-1. Moreover, tropine is not accumulated during the growth with atropine as carbon source, as appeared in the analysis of the growth medium using thin layer chromatography.

Niemer and Bucherer (1961) found in Corynebacterium “belladonnae” tropine to be dehydrogenated by an enzyme that uses NAD^+ as hydrogen acceptor. PMBL-1 cultivated in the presence of atropine was found to contain a tropine dehydrogenase which, however, transfers the hydrogen to the cofactor $NADP^+$.

This enzyme is absent in an extract of Pseudomonas grown with tropic acid as carbon source. Moreover, this dehydrogenase is apparently independent of the tropic acid enzymes and has not been studied in detail.

9.3.2 Uptake of substrate from the medium

Uptake of compounds by bacteria from a medium is often an active process. In a number of cases, transport proteins have been identified for the uptake of, amongst others, sulfate and sugars. For a recent overview see Pardee (1968).

Active transport of metabolites can be investigated by measuring the uptake of radioactivity if the compound with a radioactive label is presented to the bacterium. This has been carried out for Pseudomonas using ^3H-tropic acid, as described in 2.8. Wild type PMBL-1 cultivated in the presence of tropic acid

Table 9.3 Uptake of tritium by PBML-1 and mutants

Pseudomonas	Lacking enzyme	Medium	^{3}H-uptake
PMBL-1	—	A	3090 dpm
PMBL-1	—	B	210 dpm
PMBL-1	—	A	3090 dpm
PMBL-174	TDH	A	0 dpm
PMBL-176	TDH	A	575 dpm
PMBL-194	PDC	A	137 dpm
PMBL-192	PDC	A	805 dpm

Bacteria (1 ml; A_{700} = 1.5), cultivated in synthetic medium with 0.1% tropic acid + 0.1% phenylglyoxylic acid (A) or with 0.1% phenylacetic acid + 0.1 % phenylglyoxylic acid (B) were incubated with 1.6 x 10^5 dpm tropic acid during 60 min. The recorded radioactivity was corrected for the uptake by a control non- incubated culture (250-350 dpm) and is the average of 2 experiments.

(Ps-tropic acid) of phenylacetic acid (Ps phenylacetic acid) was incubated with ^{3}H-tropic acid. The amount of radioactivity taken up was measured in disintegrations per min (dpm) after 60 min (table 9.3). Ps-tropic acid appeared to take up tritium, Ps-phenylacetic acid did not. This could mean that Ps tropic acid has a transport system for tropic acid. The difference could also be explained by the fact that Ps tropic acid was metabolically adapted to tropic acid and thus could accumulate and convert this compound, resulting in an uptake of radioactivity.

In order to distinguish between these possibilities, the uptake of ^{3}H-tropic acid was assessed in 4 mutants, which could not produce either TDH or PDC anymore (10.7) as the result of mutagenic treatment. Since only one of the tropic acid enzymes was absent in these mutants, it was reasonable to expect that the damage brought about was limited. It seemed unlikely that in each of these mutants the information for a possible transport protein was lost as well.

These mutants were cultivated in a synthetic medium supplied with 0.1% tropic acid and 0.1% phenylglyoxylic acid as carbon source and thereafter incubated with the ^{3}H-tropic acid. The mutants showed only 0-25% of the uptake observed for the wild type under comparable conditions (table 9.3). These results make it is unlikely that a special transport system is in place for the uptake of tropic acid in Pseudomonas. This uptake is most probable a consequence of the capacity to convert tropic acid, since the uptake of tritium by the wild type coincides with an active metabolism of this compound.

The possibility cannot be ruled out that the bacterium has an uptake system at its disposal for atropine. However, similar experiments could not be carried out due to the insufficient radiochemical purity of the available ^{3}H-atropine.

9.3.3. Racemase

Pseudomonas fluid cultures with 0.05% (±) atropine as the sole source of carbon reached about the same optical density as with 0.05% (-) atropine. Apparently both optical isomers were metabolized. Since AtrE has a strong preference for (-) atropine, it does not seem unreasonable to expect a role of an atropine-racemase in the metabolism of the (+) isomer.

This enzyme should be present in an extract of Ps-atropine but not in an allmost pure sample of AtrE. One would expect the hydrolysis of (+) atropine could be more rapidly in the presence of the extract compared with hydrolysis by the purified enzyme. Such a difference was not observed. Therefore, it is unlikely that a racemizing enzyme is involved in the breakdown of the (+) enantiomer.

Although the bacterium can utilize both (-) and (+) tropic acid as sole source of carbon, it does not seem necessary to suppose the presence of a tropic acid racemase. TDH does not show a clear preference for either of the stereo-isomers of tropic acid. This is remarkable in view of the large substrate specificity of the enzyme (6.4).

This inconsistency could be overcome if tropic acid during the enzyme action (at pH 9.5) would racemize spontaneously. This was investigated using a polarimeter. The sponta neous racemizing appears to proceed too slowly to explain the dehydrogenation of (+) tropic acid by a stereospecific TDH. It would also be possible that a tropic acid racemase exists and is still present in the TDH samples used. This cannot be excluded in advance because the TDH is only partially purified in the method described (6.3). The racemization of (+) and (-) tropic acid in the presence of purified TDH has been investigated by polarimetry. Supporting evidence was not obtained however. It is therefore unlikely that a racemase is involved in the dehydrogenation of (+) or (-) tropic acid.

The discrepancy between the observed substrate specificity and lack of stereo specificity remains unexplained.

9.4 BREAKDOWN OF ATROPINE IN OTHER PMBL STRAINS

Rörsch (personal communication) has isolated in addition to PMBL-1 eight other micro-organisms from soil, able to metabolize atropine. These strains have

Table 9.4

Specific activity of the tropic acid enzymes in a number of Pseudomonas strains

Strain	Classified as	AtrE	TDH	PDC	PDH
PMBL-1	putida biotype A	2.2	2.2	1.9	0.22
PMBL-2001	putida biotype A	±0.6	± 1.0	0.77	± 0.3
PMBL-4001		3.6	0.73	0.57	0.09
PMBL-4501	putida biotype A	1.3	1.1	1.1	0.15
PMBL-5001		3.0	0.65	0.51	0.10
PMBL-5501	putida biotype B	+ 1.6	0.35	0.25	0.07
PMBL-6001		3.0	0.67	0.50	0.13
PMBL-6501		± 1.1	0.51	0.75	0.07
PMBL-7001	fluorescens	2.3	0.82	1.0	± 0.4

The strains were cultivated in synthetic medium with 0.1% tropic acid and 0.1% succinic acid. The specific activity is assayed in the extract according to 2.10 and reported as the number of Units per mg protein.

been deposited in the collection of the Medical Biological Laboratory under the PMBL numbers 2001, 4001, 5001, 5501, 6001, 6501 and 7001. Five of these strains have been classified by Wensinck (1969) as Pseudomonas putida or fluorescens, the other strains cannot be classified under the existing types but are definitely Pseudomonaceae.

The strains have been cultivated in a synthetic medium with tropic acid and analyzed for the presence of AtrE, TDH, PDC and PDH activity. In table 9.4, the specific activities have been listed. The total activities are in line with these values. The strains PMBL-4001, PMBL-5001 and PMBL-6001 contain a relative high amount of AtrE. These esterase activities are inhibited by organophosphates. This holds for the other strains as well. The greatest activity of TDH and PDC was found in PMBL-1. In this comparison, one has to keep in mind that the reaction conditions for the enzyme assay have been set up for PMBL-1. These might be suboptimal for the corresponding enzymes in the other strains. It is therefore not certain that PMBL-1 has indeed the largest amount or the most active TDH and PDC.

9.5 RELATIONSHIP WITH THE BREAKDOWN OF MANDELIC ACID AND PHENYLPYRUVIC ACID

Stanier et al (1953) have isolated the bacterium Pseudomonas putida (ATCC 12633, biotype A), able to grow with mandelic acid as sole source of carbon. The breakdown of mandelic acid in this bacterium is elucidated by Stanier (1953), Gunsalus (1953 a and b), Hegeman (1966), Kennedy (1968) and their coworkers. The metabolic pathway of mandelic acid and the mandelic enzymes involved are summarized in fig 9.5. The pattern of breakdown shows a remarkable similarity with the metabolism of tropic acid in PMBL-1. One could ask oneself whether one or more tropic acid enzymes would be identical to the corresponding enzymes from the mandelic acid metabolism.

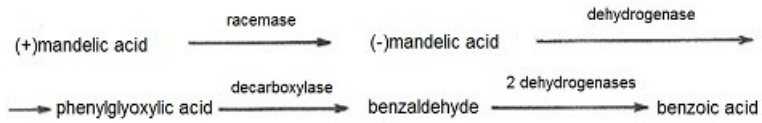


Fig 9.5 Metabolic pathway of mandelic acid in Pseudomonas ATCC 12633

This has been investigated. Pseudomonas ATCC 12633 was kindly provided by Dr. G.D.Hegeman (Berkeley, California) and deposited in the MBL strain collection under number PA-20, a notation used below.

The Pseudomonaceae PA-20 and PMBL-1 are not identical because PA-20 does not grow with tropic acid as sole source of carbon and PMBL-1 cannot metabolize mandelic acid (4.2). Moreover, it has been shown that none of the tropic acid enzymes is identical to one of the mandelic enzymes: mandelic acid, phenylglyoxylic acid and benzaldehyde are not converted by purified TDH, PDC and PDH respectively (see chapters 6 and 7 and 8). An extract of Ps tropic acid lacks the mandelic enzymes and Pseudomonas PA-20, cultivated with mandelic acid as sole carbon source, does not contain any of the tropic acid enzymes.

The difference between the metabolic pathways of mandelic acid and that of tropic acid not only concerns the substrate specificity of the enzymes involved. The equivalent for the AtrE is not present in the extract of PA-20. The hydrolysis of the methyl ester of mandelic acid is not catalyzed by the extract. Strain P-20 contains a racemase in contrast to PMBL-1. The mandelic dehydrogenase is stereospecific for (+) mandelic acid, bound to a particle fraction and sedimented by centrifugation. In vitro, the enzyme makes use of the dye 2,6-dichlorophenol-indophenol as hydrogen acceptor and does not transfer the hydrogen to the cofactor NAD^+. The phenylglyoxylic acid decarboxylase needs thiamine pyrophosphate as cofactor, whereas in dehydrogenation of benzaldehyde in PA20, 2 dehydrogenases are involved with NAD^+ and $NADP^+$ respectively as cofactors.

It is clear that the strains PA-20 and PMBL-1 have complete different enzyme systems for the breakdown of tropic acid and mandelic acid respectively.

PMBL-1 is not able to breakdown mandelic acid, but phenylglyoxylic acid is metabolized. In PMBL-1, this breakdown is analogous with the phenylglyoxylic acid metabolism in PA-20. The compound is converted by decarboxylation and dehydrogenation in benzoic acid (see annex 1).

The production of benzaldehyde by an extract of Pseudomonas PMBL-1 cultivated in the presence of phenylglyoxylic acid has been confirmed using thin layer chromatography. The pertaining decarboxylase has been demonstrated as well. This enzyme is not identical to PDC. A mutation that results in a loss of PDC has no effect on the ability to use phenylglyoxylic acid as carbon source (10.7). Reversely, PMBL-132 lacking the phenylglyoxylic acid decarboxylase and unable to metabolize phenylglyoxylic acid anymore, contained the PDC and was able to grow undisturbed with tropic acid as sole source of carbon.

In strain PMBL-1, benzaldehyde is converted into benzoic acid by a benzaldehyde dehydrogenase that needs $NADP^+$ as hydrogen acceptor. It cannot use NAD^+ for this purpose. This enzyme distinguishes itself from PDH which only can use NAD^+ as cofactor. The kinetics of the complete conversion of benzaldehyde at low substrate concentration leads to the conclusion that this enzyme has a very low Michaelis constant for this substrate. In this way, it looks like PDH. The low Michaelis constant has been used gratefully to develop an assay for phenylglyoxylic acid decarboxylase.

The benzaldehyde dehydrogenase is able to dehydrogenate small amounts of benzaldehyde nearly completely under production of the stoichiometric amount of NADH, thanks to the great affinity of the enzyme for this substrate. Therefore, this enzymatic conversion can be used for the quantitative assay of benzaldehyde, produced from phenylglyoxylic acid by decarboxylation.

As shown by Seidenberg (1962) and Asahawa (1968), phenylpyruvic acid is decarboxylated to phenylacetaldehyde and then oxidized to phenylacetic acid. This study was done in Achromobacter euridyce. The decarboxylase needs thiamine pyrophosphate and Mg^{2+} as cofactors. This enzyme is rather aspecific and takes care for the decarboxylation of indole derivatives as well. These authors did not study the dehydrogenation of phenylacetaldehyde in phenylacetic acid. In this organism, like in PMBL-1, phenylacetaldehyde is produced by enzymatic decarboxylation. There might be a relationship between the breakdown of

phenylpyruvic acid and the conversion of tropic acid. However, phenylpyruvic acid is not decarboxylated by PDC (7.5). Moreover, it appears that in mutants of PMBL-1, in which the PDC is lacking, the metabolism of phenylpyruvic acid is no disturbed. Apparently, PDC is not involved in the metabolism of phenylpyruvic acid.

The data show that in PMBL-1 different enzyme systems are available for the breakdown of tropic acid, phenylglyoxylic acid and phenylpyruvic acid. These data, especially those related to the enzymes phenylglyoxylic acid decarboxylase and the benzaldehyde dehydrogenase have been very valuable in the study of the synthesis of the tropic acid enzymes, described in the next chapter.

CHAPTER 10

REGULATION OF SYNTHESIS OF THE TROPIC ACID ENZYMES

10.1 INTRODUCTION

In the introduction of this thesis, the perspective has been mentioned that the enzymes which are involved specifically in the metabolism of tropic acid might be used to study the regulation of protein and enzyme synthesis in Pseudomonas. As a first condition for such a study, it was stated that the synthesis of these enzymes like the atropine esterase should be regulated by induction.

Nearly simultaneously with the first indications for the existence of the enzymes TDH, PDC and PDH, it became clear that these enzymes indeed meet this condition. Like AtrE, these enzymes were present in PMBL-1 cultivated with atropine or tropic acid and absent in PMBL-1 cultivated with tropine or phenyl acetic acid as carbon source. Therefore, the enzymes involved in the breakdown of tropine and phenylacetic acid are in this context not of interest. This study is limited to the enzymology and induction of the four tropic acid enzymes.

An enzyme is called an inducible enzyme when the synthesis of that enzyme is controlled by the presence of a certain compound or compounds in the medium. Such a compound is called inducer. In absence of the inducer, only a very small amount of the enzyme is synthesized and only a basal enzyme level is maintained. Bacteria cultivated in the presence of the inductor contain the induced enzyme level. This can be 2000 – 4000 times higher than the basal enzyme level.

Depending on the relation between the inductor and the induced enzyme, the following types of enzyme induction can be distinguished:

a. Induction by the substrate of the enzyme or by a metabolic precursor of the substrate;
b. Induction by the product of the enzyme reaction or its derivative. Because initially the induced enzyme is not required for the breakdown of the inducer, this type of induction is called gratuitous. Full scale induction takes place after a small amount of the substrate is converted into the product/inducer by action of enzyme produced at the basal level constitutively.
c. Induction by a compound which does not belong to the metabolic pathway or which cannot be metabolized at all. This is also called gratuitous induction.

The regulation of enzyme synthesis by induction is accomplished as follows. The segment of the bacterial chromosome that carries the information for the enzyme in question, the structural gene, is not expressed as long as it is blocked by a specific protein, the so-called repressor.

This repressor protein has a high affinity for the operator, a small segment of the chromosome that is located very close to the structural gene. The repressor binds tightly to the operator. In the absence of the inducer, the repressor prevents the structural gene to be expressed. Induction takes place once the inductor forms a complex with the repressor with the result that the repressor is no longer bound by the operator and the block for expression of the structural gene and the synthesis of the enzyme in question is lifted.

The following induction patterns can be distinguished in case of induction of more than one enzyme.

Coordinated induction*:* Here, one compound induces the synthesis of more than one enzyme by deblocking one operator. This is only possible if the operator and the structural genes for the respective enzymes are contiguous on the chromosome and form a so-called operon.

Parallel induction: One inducer can act simultaneously at different operators leading to parallel induction of more than one enzyme or protein. Every structural gene has its own operator. This form of regulation is also observed if structural genes are located on the chromosome on very short distance. This is difficult to distinguish from coordinated induction.

Sequential induction: In sequential induction, the inducing compound induces only the enzyme that catalyzes the conversion of this compound. The product formed is the inducer of the next enzyme.

In the literature, there is some uncertainty on various types of induction. There is confusion on parallel versus coordinated expression. The term sequential induction is used when products of one operon appear quickly after each other. In this thesis, these terms will be used as defined above.

This chapter reports on a study on the regulation of the tropic acid enzymes, including the mechanism of the induction, the induction specificity and the nature of the real inducers. First arguments will be given that the enzyme synthesis is really de novo synthesis and not the activation of inactive precursors.

Subsequently, a study is presented in which for a number of compounds it is checked whether the presence in the cultivation medium results in the synthesis of the tropic acid enzymes. The results suggest that the enzymes are not induced sequentially.

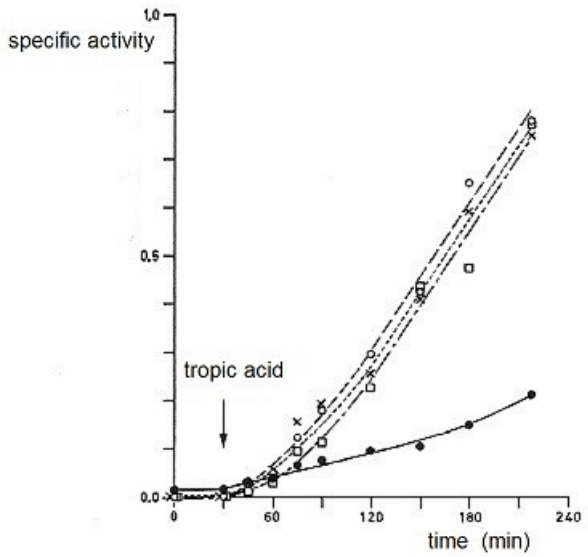
More experiments will be presented comparing the enzyme production in PMBL-1 and in a number of mutants of PMBL-1, leading to the conclusion that atropine and tropic acid are not recognized as such by the system that the regulates their production. The induction is of the gratuitous type where these compounds first have to converted into the intermediate phenylacetaldehyde.

The chapter is concluded with a discussion, which deals in greater depth with various aspects of induction of the tropic acid enzymes.

10.2 KINETICS OF THE INDUCTION: EFFECT OF CHLORAMPHENICOL

The kinetics of the induction of the tropic acid enzymes was studied by the adding tropic acid to a non-induced culture of Pseudomonas and quantifying the

Fig. 10.1
Induction of the tropic acid enzymes in PMBL-1



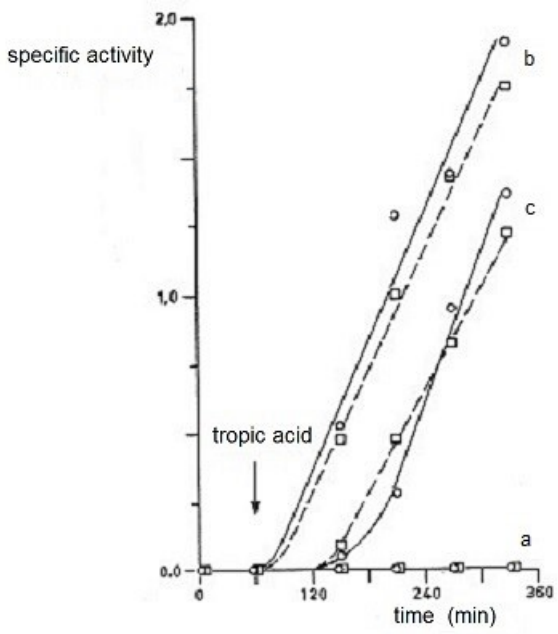
Tropic acid as added to a non-induced culture in the logarithmic growth phase on time t=30 min. Samples were taken at different times. Specific activity (U/mg protein) was measured of AtrE (x – x), TDH (o – o), PDC (□ – □) and PDH (• – •).

enzyme content as a function of time. The specific enzyme activity in the bacterial extract was used as measure for the enzyme content. The results are shown in fig. 10.1. It appeared that the specific activity of the four enzymes increased continuously during 3.5 hours after addition of tropic acid. The relative increase of the PDH activity during this period was considerably less compared with the other enzymes. There appears to be no clear differences in the time at which the specific activity of the enzymes started to increase.

Chloramphenicol is a frequently used reversible inhibitor of protein synthesis In bacteria. In order to confirm that protein synthesis is really required for the induction of the tropic acid enzymes, the effect of this antibiotic was studied on the kinetics of the induction.

Tropic acid and chloramphenicol were added to a non-induced Pseudomonas culture in the logarithmic growth phase. The induction of TDH and PDC was determined.

Fig. 10.2
Effect of chloramphenicol on the induction of TDH and PDC



Three identical cultures (a, b and c) of PMBL-1 were prepared with 0.2% succinic acid as carbon source. On time = 60 min, 0.1% tropic acid was added (culture b) or 0.1% tropic acid + 10^{-4} M chloramphenicol (cultures a and c). On time = 120 min, chloramphenicol was removed (culture c). TDH (o – o); PDC (□ – □).

A culture with only tropic acid served for comparison. The results are reported in fig 10.2. In the presence of chloramphenicol, there was no enzyme induction. Induction was reestablished after removal of the chloramphenicol. This was accomplished by centrifugation of the culture and resuspension the cells in medium with tropic acid but minus chloramphenicol. The induction of AtrE and PDH was studied in the same type of experiment with similar results.

Apparently, induction of tropic acid enzymes is inhibited by chloramphenicol. This inhibition is reversible. It can be concluded from these experiments that de novo protein synthesis is required for the induction process. The increase in specific activity is not the result of activation of inactive precursors already present.

10.3 THE SPECIFICITY OF INDUCTION OF THE TROPIC ACID ENZYMES

Ability to induce the tropic acid enzymes in PMBL-1 by a number of compounds has been investigated. The bacteria were cultivated in a synthetic medium with 0.1% succinic acid with addition of 0.025% of the compound to be investigated. Also here, the specific activity of the tropic acid enzymes in the extract was used the measure for the ability to induce. Succinic acid was chosen as general carbon source after preliminary experiments had shown that the induction of the enzymes in 0.2% succinic acid was not repressed. This medium has the advantage that also the inducing ability can be investigated of compounds which cannot be used by Pseudomonas as carbon source.

Induction with 0.025% of various compounds was, if any, not maximal. The low concentration was used to be able to investigate also compounds which in higher concentrations are toxic, like phenylacetaldehyde and benzaldehyde. Results are summarized in table 10.3. In addition to atropine and tropic acid, only phenylglyoxylic acid can induce amongst the compounds that can be used as carbon source. Phenylacetaldehyde and benzaldehyde do this to a lesser extent.

Compounds that do not act as carbon source but are able to induce the tropic acid enzymes are: 2-phenylpropanal and p-dimethylaminobenzaldehyde. Very weak induction is observed with 2-phenylpropionic acid, 3-phenyllactic acid, 3-phenylallylethanol, benzyl alcohol and epoxysterol. All other compounds do not induce. In table 10.3, pma is not listed. This compound has been investigated, appeared to be function as carbon source and to induce the tropic acid enzymes. Due to the instability of this compound, it is not possible to find out whether this has to be attributed to the compound itself or to the product of spontaneous decarboxylation: phenylacetaldehyde.

Induction specificity of the tropic acid enzymes

compound (0.025%)*	Table 10.3 0.1% succinic acid AtrE	TDH	PDC	PDH	Table 10.4 0.005% succinic acid AtrE	TDH	PDC	PDH
atropine*	1.61	0.64	0.45	0.22	2.14	0.90	1.17	0.34
tropine*	0.01	0.01	0.00	0.01	0.03	0.00	0.00	0.01
tropic acid*	1.67	0.71	0.59	0.35	2.03	0.78	0.72	0.31
phenylacetaldehyde*	0.31	0.55	0.54	0.20	1.47	0.32	0.68	0.22
phenylmalonic acid*	0.05	0.01	0.01	0.02	0.10	0.02	0.00	0.02
phenylacetic acid*	0.04	0.01	0.02	0.03	0.06	0.02	0.03	0.03
homatropine*	0.07	0.00	0.00	0.01	0.12	0.00	0.00	0.01
mandelic acid	0.04	0.00	0.00	0.01	–	–	–	–
phenylglyoxylic acid*	1.09	0.34	0.39	0.16	2.15	0.60	0.50	0.45
benzaldehyde*	0.30	0.16	0.18	0.05	2.35	0.48	0.74	0.29
benzoic acid*	0.01	0.00	0.00	0.01	0.10	0.00	0.01	0.01
2-phenylacrylic acid	0.01	0.00	0.00	0.01	–	–	–	–
2-OH-2-phenyl propionic acid	0.07	0.00	0.01	0.00	–	–	–	–
2-phenylpropionic	0.20	0.03	0.06	0.03	–	–	–	–
phenyllactic acid	0.29	0.06	0.07	0.03	–	–	–	–
phenylpyruvic acid*	0.14	0.02	0.02	0.03	0.62	0.11	0.10	0.06
3-phenylacrylic acid	0.00	0.00	0.00	0.01	–	–	–	–
3-phenoxypropionic	0.05	0.00	0.00	0.01	–	–	–	–
phenoxyacetic acid	0.03	0.00	0.00	0.01	–	–	–	–
2-OH-phenylacetic*	0.03	0.00	0.00	0.01	–	0.01	–	0.01
3-OH-phenylacetic*	0.02	0.00	0.00	0.01	0.03	0.00	0.00	0.01
4-OH-phenylacetic*	0.03	0.00	0.00	0.01	0.00	0.00	0.00	0.01
3.4 diOHphenylacetic	0.05	0.00	0.00	0.01	–	–	–	–
2-phenylpropanal	1.20	0.34	0.67	0.13	–	–	–	–
3-phenylpropanal	–	–	–	–	–	–	–	–
2-Cl-benzaldehyde	–	–	–	–	–	–	–	–
3-OH-benzaldehyde	–	–	–	–	–	–	–	–
4-nitrobenzaldehyde	–	–	–	–	–	–	–	–
4-(dimethylamino) benzaldehyde	2.07	0.98	2.23	0.18	–	–	–	–
4-hydroxy-3-methoxy benzaldehyde*	0.07	0.01	0.01	0.01	0.26	0.02	0.03	0.03
3-hydroxy-4-methoxy benzaldehyde	0.04	0.00	0.00	0.01	–	–	–	–
2-phenylpropanol	0.01	0.00	0.00	0.01	–	–	–	–
3-phenyl-2-propenol	0.21	0.07	0.08	0.04	–	–	–	–
2-phenylethanol	0.08	0.01	0.01	0.01	–	–	–	–
phenylmethanol	0.31	0.05	0.08	0.04	–	–	–	–
epoxysterol	0.37	0.05	0.14	0.05	–	–	–	–
4-isopropyl toluene	0.01	0.00	0.00	0.01	–	–	–	–
phenylalanine*	0.08	0.00	0.00	002	0.20	0.02	0.00	0.06
tyrosine*	0.02	0.00	0.00	0.02		0.00	0.00	0.04
valine*	0.02	0.00	0.00	0.02	0.00	0.00	0.00	0.01
tartaric acid	0.01	0.00	0.00	0.01	–	–	–	–

SA (U/mg protein) of tropic acid enzymes in bacterial extracts, cultivated with 0.025% of the compound + 0.1% succinic acid or 0.005% succinic acid. * sole source of carbon – = no growth after 40 hours
2-OH-phenylacetic = 2-hydroxyphenylacetic acid etc

The compounds that can be used as carbon source were investigated once more for their ability to induce in synthetic medium with 0.025% of the compound and 0.005% succinic acid (table 10.4). In almost all cases, a stronger induction was observed under these conditions, especially in the case of benzaldehyde. Now, phenylpyruvic acid caused some induction as well. The other compounds which in the previous experiment did not induce, appeared in this experiment to induce the tropic acid enzymes neither.

Induction of the enzymes has been investigated in a synthetic medium with 0.2% atropine, tropic acid or benzoic acid (table 10.5). This leads in the cultures with atropine and tropic acid to a higher enzyme content as compared with the induction at lower concentrations of these compounds. Due to the induction, the specific activity of AtrE, TDH and PDC is 150-400 times higher compared with the enzyme levels in non-induced bacteria. The induction results in only 20 times higher enzymatic dehydrogenation of phenylacetaldehyde.

Table 10.5
Induction of the tropic acid enzymes inPMBL-1

	SPECIFIC ACTIVITY			
Carbon source	AtrE	TDH	PDC	PDH
Atropine	3.40	1.10	1.80	0.355
Tropic acid	2.85	1.10	1.50	0.345
Benzoic acid	0.03	0.004	0.005	0.020
Tropine	0.01	0.004	0.003	0.019

Specific activity of the tropic acid enzymes (U/mg protein) in the extract of PMBL-1 cultivated in synthetic medium with 0.2% carbon source

10.4 GRATUITOUS INDUCTION BY TROPIC ACID AND PHENYLACETALDEHYDE

Induction of the tropic acid enzymes seems less specific as initially expected. Compounds of very diverse chemical character are able to command the cell to produce the tropic acid enzymes. It is even difficult to indicate a structural similarity in these compounds that can make understandable that the inducing compounds in table 10.3 can induce, whereas many other compounds with structural similarity to tropic acid cannot.

This lack in similarity between compounds able to induce could indicate that some of the inducing compounds do not induce as such, but have to be converted

in another compound that serves as the real inducer. In that way, the number of actual inducing agents would be much smaller and the mutual resemblance much greater than data from table 10.3 suggest.

Gratuitous induction of the tropic acid enzymes has been observed for tropic acid, the first metabolite of atropine. The presence of tropic acid in the medium results in the induction of AtrE, as shown in table 10.3. This induction is gratuitous since AtrE is not required for the metabolism of tropic acid. (for data on Pseudomonas mutants supporting the conclusion of gratuitous induction see 10.6).

An even more remarkable fact is that phenylacetaldehyde induces the synthesis of AtrE, TDH and PDC, whereas none of these enzymes is required for the breakdown of this compound. Only PDH is required for the conversion of phenylacetaldehyde into phenylacetic acid. Phenylacetaldehyde is the real inducer of PDH. The next product in the pathway, phenylacetic acid, is not an inducer, because PMBL-1 cultivated in the presence of phenylacetic acid does not contain any of the tropic acid enzymes. These results could indicate that the induction in the culture with atropine or tropic acid as carbon source is not effectuated by these compounds themselves, but by phenylacetaldehyde, the breakdown product of tropic acid.

The fact that phenylacetaldehyde is able to induce the three enzymes gratuitously leads to conclusions with regard to the pattern of induction. The induction cannot be the result of sequential induction when under influence of a gratuitous inducer more than one enzyme is induced and the breakdown product(s) of the gratuitous inducer do not induce.

The induction by phenylacetaldehyde occurs therefore in a parallel or coordinated fashion.

10.5 GRATUITOUS INDUCTION BY PHENYLGLYOXYLIC ACID AND BENZALDEHYDE

Phenylglyoxylic acid and benzaldehyde satisfy both as carbon source for Pseudomonas PMBL-1 and moreover induce the tropic acid enzymes. The question arises here whether the tropic acid enzymes are induced to serve in the breakdown of these compounds or that this induction is also gratuitous. This could be gratuitous induction as well. The following arguments support the mechanism of gratuitous induction:

1. The enzymes PDC and PDH are not able to convert phenylglyoxylic acid and benzaldehyde respectively. This was shown in the investigation of the substrate specificity of these enzymes (7.5, 8.4).
2. Extracts of PMBL-1 cultivated with phenylglyoxylic acid as carbon source

contain a phenylglyoxylic acid decarboxylase and a $NADP^+$ dependent benzaldehyde dehydrogenase (9.5). These enzymes are absent in Pseudomonas PMBL-1 cultivated with tropic acid.

The data show that phenylglyoxylic acid and benzaldehyde cannot be converted by the tropic acid enzymes but that this breakdown is accomplished by other enzymes induced specifically for this purpose in PMBL-1. On basis of these observations, it is reasonable to assume that induction of the tropic enzymes in this case is gratuitous as well. This supports the conclusion that this synthesis is not regulated by sequential induction.

10.6 INDUCTION IN MUTANTS OF PMBL-1

As discussed in 10.4, it does not seem impossible that atropine and tropic acid as such are not capable of inducing the tropic acid enzymes, but that the actual induction is accomplished by a conversion product of tropic acid. Therefore, it has been tried to collect more information about the nature of the real inducer by an investigation of the induction process in mutants. Mutants, not any longer able to breakdown atropine into phenylacetic acid, were isolated. information was obtained for what reason that conversion was disturbed.

Mutants were obtained using the method described in 2.5. Selection was applied for the phenotype $Atr^-Tro^+Pac^+$ or $Atr^-Tro^-Tpn^+Pac^+$. These mutants were studied as follows.

The mutants with the phenotype Atr^- Tro^+ Pac^+ were cultivated in synthetic medium with tropic acid as carbon source. In extracts of these bacteria, the AtrE was absent, whereas the presence of TDH, PDC and PDH could be demonstrated. This is in agreement with the phenotype.

Mutants with phenotype Atr^- Tro^- Tpn^+ Pac^+ were cultivated in synthetic medium with 0.2% succinic acid (as carbon source) and 0.1% tropic acid. The level of tropic acid enzymes in all 33 mutants of this type being analyzed was less than 10% as compared with the level of the enzymes in PMBL-1 cultivated under the same conditions. This could be a consequence of a disturbance of the induction mechanism, but also of the loss of genetic information for any of the tropic acid enzymes in each of these mutants. The option that this observation is caused by a disturbance in the capacity to take up tropic acid from the medium, seems unlikely since this compound is not taken up actively. Induction in these mutants was studied using the gratuitous character of the induction by phenylglyoxylic acid. In this case, the inductor was the carbon source at the same time. In this experiment, the 33 mutants were grown with 0.2% phenylglyoxylic acid. The experiment had a surprising result. In experiments with tropic acid, the induction of the four enzymes

nearly failed. In the experiments with phenylglyoxylic acid, the induction of three of the four tropic acid enzymes occurred in more than half of the mutants as good as in the wild type. In these mutants only TDH or PDC was absent, no mutants were found with only PDH missing.

Induction by atropine, tropic acid, phenylacetaldehyde, phenylglyoxylic acid and benzaldehyde has been compared in a number of mutants with the induction by these compounds in the wild type under comparable conditions. In this way, the possible differences in induction were investigated more in detail. The bacteria were cultivated in a synthetic medium with 0.05% succinic acid and 0.025% of the compound of interest. The induction in these cultures is reported in table 10.6.

In the wild type, all compounds mentioned are able to induce four enzymes. In a mutant PMBL-42 that lacks AtrE, only atropine is no longer able to induce; in the presence of tropic acid or phenylacetaldehyde, the other enzymes were induced normally. A similar result was obtained with mutant PMBL-33, which having lost the ability to produce AtrE – due to a mutation – can grow normally on tropic acid.

Table 10.6
Induction in PMBL-1 and mutants

	PMBL-1 (wild type)				PMBL-42 (AtrE$^-$)			
	AtrE xxxx	TDH xxxx	PDC xxxx	PDH xxxx	AtrE xxxx	TDH xxxx	PDC xxxx	PDH xxxx
atropine	0.89	1.38	1.28	0.38	0.00	0.09	0.21	0.03
tropic acid	0.88	1.59	1.55	0.41	0.00	1.02	1.91	0.06
phenylacetaldehyde	0.45	0.44	0.42	0.13	0.00	0.57	1.27	0.05
phenylglyoxylic acid	1.59	0.85	0.69	0.23	–	–	–	–
benzaldehyde	0.51	1.00	1.18	0.32	–	–	–	–
	PMBL-174 (TDH$^-$)				PMBL-194 (PDC$^-$)			
atropine	0.00	0.00	0.00	0.01	0.12	0.05	0.00	0.04
tropic acid	0.00	0.00	0.00	0.01	0.21	0.08	0.00	0.05
phenylacetaldehyde	0.30	0.00	0.22	0.11	0.71	1.28	0.00	0.37
phenylglyoxylic acid	0.37	0.00	0.37	0.10	1.75	0.44	0.00	0.24
benzaldehyde	0.74	0.00	0.48	0.17	1.17	0.97	0.00	0.25

Specific activities of the tropic acid enzymes (U/mg protein) in the extract of PMBL-1 and PMBL-mutants, cultivated in synthetic medium with 0.05% succinic acid and 0.025% of the compound under investigation.
AtrE$^-$ = mutant lacking the AtrE etc.

This leads to the conclusion that not atropine is the real inducer, but tropic acid or one of the metabolites of tropic acid. Neither atropine nor tropic acid appears to be able to induce the tropic acid enzymes in mutants that lack the TDH or the PDC. On the contrary, with phenylacetaldehyde the enzymes were induced normally, with the exception of the enzyme which is absent in the pertaining mutant. Comparable data were obtained in a number of other mutants of this type. Moreover, the difference in induction by tropic acid and that by phenylglyoxylic acid was already observed in the investigation of Tro^- mutants described above. As suggested before, the induction by atropine or tropic acid is apparently dependent of an intact metabolic pathway to phenylacetaldehyde. Because phenylacetaldehyde in PMBL-1 is converted in phenylacetic acid that is known not to induce the tropic acid enzymes (10.3), one can conclude that phenylacetaldehyde is the real inducer of the tropic acid enzymes and atropine, tropic acid and pma only induce if these compounds can be broken down to phenylacetaldehyde. Although pma could not be tested as inducer, this conclusion is justified also with regard to the pma, in view of the fact that tropic acid is not able to induce in a mutant that lacks PDC.

Research on the gratuitous induction by phenylglyoxylic acid and benzaldehyde brought to light that exclusively benzaldehyde can act as inducer of the tropic acid enzymes and that phenylglyoxylic acid only induces upon conversion into benzaldehyde. This was demonstrated in an investigation on the induction of the tropic acid enzymes by phenylglyoxylic acid and benzaldehyde. Enzyme levels in PMBL-1 were compared with those in mutant PMBL-132, a mutant unable to grow with phenylglyoxylic acid as carbon source. But it can grow with benzaldehyde or benzoic acid. In enzymological research, it was demonstrated that PMBL-132 lacks the phenylglyoxylic acid decarboxylase. The results of this experiment are shown

Table 10.7

Induction in PMBL-1 and PMBL-132

	PMBL-1				PMBL-132			
	AtrE	TDH	PDC	PDH	AtrE	TDH	PDC	PDH
phenylglyoxylic acid	1.09	0.34	0.39	0.16	0.00	0.01	0.00	0.01
benzaldehyde	0.30	0.16	0.18	0.05	0.19	0.08	0.05	0.03

Specific activity of the tropic acid enzymes (U/mg protein) in the extract of PMBL-1 and PMBL-132, cultivated in synthetic medium with 0.1% succinic acid and 0.025% phenylglyoxylic acid or benzaldehyde. In PMBL-132, phenylglyoxylic acid decarboxylase is absent.

in table 10.7. Tropic acid enzymes are induced in the wild type by both phenylglyoxylic acid and benzaldehyde; in the mutant, only benzaldehyde acts as inducer. This confirms that phenylglyoxylic acid itself is not able to induce. In this case, benzaldehyde is the real inducer of the tropic acid enzymes since benzoic acid – the metabolic conversion product of benzaldehyde – does not induce either.

The results of these investigations show that only two compounds which can be used by PMBL-1 as carbon source and cause induction of these enzymes, can be identified as inducer with certainty: phenylacetaldehyde and benzaldehyde. Four other compounds atropine, tropic acid, pma and phenylglyoxylic acid have been shown not to be inducers themselves, but only give rise to induction after conversion into phenylacetaldehyde and benzaldehyde respectively. It might not be impossible that the low level of induction observed in cultures with phenylpyruvic acid should not be ascribed to this carbon source itself, but is related to its conversion in phenylacetaldehyde. Since mutants disturbed in the metabolism of phenylpyruvic acid not have been found, this could not be confirmed.

10.7 OVERVIEW OF MUTANTS OF PMBL-1 USED

Mutants of Pseudomonas PMBL-1 lacking only one of the tropic acid enzymes as the result of mutagenic treatment have been very useful in the investigation of the metabolic pathway and the elucidation of the regulation by product induction. Several of these mutants had been isolated at the start of this research but only recognized as such following the discovery of by phenylglyoxylic acid as gratuitous inducer.

Table 10.8
Induction of tropic acid enzymes in PMBL-1 and mutants

PMBL	type	AtrE	TDH	PDC	PDH
1	wild	1.10	0.360	0.535	0.200
33	$AtrE^-$	0.00	0.535	0.260	0.155
42	$AtrE^-$	0.00	0.785	1.47	0.060
174	TDH^-	1.60	0.000	0.610	0.255
176	TDH^-	1.05	0.000	0.390	0.085
192	PDC^-	0.55	0.355	0.001	0.150
194	PDC^-	1.05	1.19	0.000	0.285

The specific activity of the tropic acid enzymes (U/mg protein) in extracts of PMBL-1 and mutants cultivated in synthetic medium with 0.2% phenylglyoxylic acid as carbon source and harvested as described in 10.2. $AtrE^-$ = mutant that lacks AtrE activity etc.

Phenylglyoxylic acid is particular useful for the study of the induction pattern in mutants because this induction is not hindered by a possible block in the atropine-metabolism. Moreover, growth in the presence of the gratuitous inducer phenylglyoxylic acid gives clear information how the genotypic changes in the mutants exert their effect on the expression on enzyme levels resulting in the disturbance of the conversion of atropine into phenylacetic acid.

Table 10.8 lists the specific enzyme activities in the mutants, used in this study. The mutants were isolated as described in 2.5; the bacteria were cultivated according to 2.10. Due to the relative high frequency of back mutation (± 1 : 10^7) for some mutants, cultures were used grown from one single colony.

10.8 DISCUSSION

From the induction pattern of mutants of Pseudomonas, one can deduce that atropine, tropic acid and phenylglyoxylic acid are not recognized as inducer by the regulatory system, but only give rise to induction if those compounds can be metabolized into phenylacetaldehyde and benzaldehyde respectively. This implies a specificity of the regulatory mechanism of the tropic acid enzymes much higher than expected on basis of the data in table 10.3. This solves the problem that a number of structurally very different compounds can induce the enzymes.

Out of the carbon sources studied in this investigation, the aromatic aldehydes phenylacetaldehyde and benzaldehyde appear able to induce; in addition, induction is observed for the non-metabolizable compounds 2-phenylpropanal and p-dimethylaminobenzaldehyde. None of the other derivatives of benzaldehyde used in this study was able to induce.

As argued in section 10.4, the gratuitous induction of the AtrE, TDH and PDC by phenylacetaldehyde cannot be the result of sequential induction. Induction of these enzymes is therefore coordinated induction, parallel induction or a combination of both induction patterns. The available data do not allow to distinguish any further.

The induction by the third product of the metabolic pathway throws some light upon the odd mechanism, Pseudomonas is using to regulate its enzyme synthesis. One would not expect exactly that phenylacetaldehyde is the inducer of the AtrE indeed, and that neither atropine nor tropic acid are able to do so. Yet, there are more cases of product induction elucidated in micro-organisms.

It has been shown that in Escherichia coli lactose as such is not able to induce the lactose enzymes, but for this purpose first has to be converted in another not yet identified β-galactoside (Müller-Hill et al 1964). The four enzymes, involved in the breakdown of L-histidine in Aerobacter aerogenes are induced by the product of the first enzyme (Schlesinger 1965 a and b). In Pseudomonas putida, induction of the catechol-oxygenase is mediated by the product of this enzyme, while the three enzymes involved in the breakdown of β-carboxy-muconic acid are induced by the end product β-keto-adipinic acid (Ornston 1966).

In a discussion on product-induction in bacteria, Ornston (1966) compared this form of induction with the induction by substrate. In case of induction of an enzyme by its substrate, the specificity of induction depends on the accuracy of recognition between the repressor and the inductor molecule. It cannot be excluded that a substrate analogue, that cannot be converted by the enzyme in question, can form a complex with the repressor and in that way gives rise to a meaningless induction of the enzyme. In the case of induction of an enzyme by its product, the specificity of induction is controlled not only by the specificity of the repressor but also by the specificity of the enzyme to be induced because the product has to be formed through the basal level of this enzyme. The substrate analogue that cannot be converted by the enzyme will not give rise to induction (even when the product that would be formed if the substrate analogue could be converted, would be recognized by the repressor). Ornston concluded that induction by the product is a more specific process compared to induction by substrate.

In case of product induction of the tropic acid enzymes, an atropine analogue will only act as inducer, if that analogue by AtrE, TDH and PDC can be converted into phenylacetaldehyde or in a compound recognized by the repressor as phenylacetaldehyde. However, all specificity requirements of the repressor and those of the three enzymes have to be met. The odd regulatory mechanism now gets a meaning: it is an extra precaution for economic housekeeping in which the substrate is checked firstly by the enzymes. This in order to make sure that after induction the substrate can be broken down by the induced enzymes. However, the product-inducer should belong specifically to the metabolic pathway in question, because otherwise the enzymes will be induced as well if the product is formed by another metabolic route.

Induction by product can be also more economical as induction by substrate substrate for other reasons. The basal dehydrogenation of phenylacetaldehyde is clearly greater than the basal activity of the enzymes AtrE, TDH and PDC (table 10.5). If this reflects the situation in vivo as well, another advantage of product induction above substrate induction can be argued.

A fully induced level of the four tropic enzymes is meaningless if, due to external conditions like insufficient NAD^+ supply, the conversion of atropine in phenylacetaldehyde can proceed only slowly. If a relative high concentration of atropine would be added to a non-induced culture, this would result - in the case of substrate induction - to a high concentration of enzyme. But due to the shortage of NAD^+ the induction would be useless because the extra supply of enzyme cannot function. In the case of induction by the product there will be in this situation no induction of the enzymes. Now, only a small amount of phenylacetaldehyde will be produced by the basal level of the AtrE, TDH and PDC. Due to the relative high basal conversion of phenylacetaldehyde in phenylacetic acid, not sufficient phenylacetaldehyde will accumulate to initiate induction.

This is an attractive mechanism. Under conditions that the four tropic acid enzymes cannot function, even a large amount of atropine does not induce. In case of product induction, not only the substrate and its metabolites are checked in advance by the enzymes in the metabolic pathway, but also whether the speed of the conversion of the substrate induction makes sense. Independent of the amount of substrate offered, the crucial factor for induction is the rate of conversion of the substrate into the inducer.

As the result of induction, the enzyme activities of AtrE, TDH and PDH increase much more than that of PDH with respect of the basal levels of activity (table 10.5). If this would be representative for the ratio of induced and non-induced levels in vivo, induction could proceed under non-limiting conditions as follows: atropine is metabolized in non-induced bacteria into phenylacetaldehyde by the action of the basal enzyme level of the three enzymes. The PDH with its relative high basal dehydrogenation probably is localized on certain spots in the cell. Elsewhere in the cell the concentration of phenylacetaldehyde will increase and the induction of the tropic enzymes will get going.

It is due to the ratios between the induced and non-induced levels of the tropic acid enzymes that the production of phenylacetaldehyde as result of the induction will increase more than compared with the conversion of phenylacetaldehyde in phenylacetic acid. Therefore, the phenylacetaldehyde concentration will increase. This leads to even more induction. This process will proceed auto-catalytically as long as other factors do not limit the induction or the conversion of atropine in phenylacetaldehyde. Maybe the autocatalytic effect is the explanation of the shape of curve of the induction curve (fig. 10.1). The curve is somewhat concave, in spite of special precautions for a logarithmically growing culture. An intracellular increasing phenylacetaldehyde concentration could be responsible.

The behavior of $Tro^{-}Tpn^{+}$ mutants which have an intact tropine metabolism and which contain AtrE after growth in the presence of phenylglyoxylic acid, is interesting. Nevertheless, these are unable to grow on the tropine moiety with atropine as sole source of carbon. With the knowledge on the mechanism of product induction, this can be understood. In these mutants the induction of AtrE is disturbed. These mutants are therefore phenotypically Atr^{-}.

In summary, induction by the product explains induction pattern of mutants of PMBL-1 with a disturbance in the atropine-metabolism and the observations discussed above. On theoretical grounds, product induction offers significant advantages over induction by substrate. Product induction is a more specific process and offers a check on beforehand whether induction under the actual circumstances will lead to a faster rate of conversion. In this way, induction by the product offers the possibility to a fine tuning which is not possible with induction by substrate. The mutual ratio between the enzyme activities of enzymes induced by the same inducer might also have an important meaning; it would not be surprising it might in general play a role in an efficient regulation of protein synthesis.

SAMENVATTING EN NABESCHOUWING

In dit proefschrift is een onderzoek beschreven naar de stofwisselingsketen van atropine in Pseudomonas PMBL-1, naar de enzymen die hierbij betrokken zijn en naar de wijze waarop de synthese van deze enzymen wordt gereguleerd. Atropine wordt in de bacteria afgebroken volgens het schema weergegeven in bijlage 1. Na hydrolyse van atropine in tropazuur en tropine wordt tropazuur via 2-fenylmalonzuur semi-aldehyde (pma) en fenylaceetaldehyde tot fenylazjnzuur afgebroken.

De rol van fenylazijnzuur in dit metabolisme blijkt ondermeer uit het feit dat PMBL-1 gekweekt met atropine of tropazuur geadapteerd is aan fenylazijnzuur. Bovendien blijken mutanten met een storing in het metabolisme van fenylazijnzuur ook gestoord te zijn in de groei met tropazuur als enige koolstofbron. Sommige mutanten zijn nog wel in staat tropazuur gedeeltelijk af te breken en een intermediaire verbinding in het medium uit te scheiden, die als fenylazijnzuur is geïdentificeerd. Bij de afbraak blijken de volgende enzymen betrokken te zijn: atropine esterase (AtrE), tropazuur dehydrogenase (TDH) 2-fenylmalonzuur semi-aldehyde decarboxylase (PDC) en fenylaceetaldehyde dehydrogenase (PDH); deze enzymen worden de tropazuur-enzymen genoemd.

De hydrolyse van atropine wordt gekatalyseerd door het atropine-esterase; de werking van het esterase is bewezen door het aantonen van de reactieproducten tropine en tropazuur. Er is een methode uitgewerkt waarmee het enzym vrijwel volledig wordt gezuiverd (>90%). Het enzym blijkt specifiek te zijn voor (-)atropine en enkele hiermee verwante verbindingen en wordt geremd door organofosfaten bv diïsopropyl-fosforofluoridaat (DFP).

De afbraak van tropazuur verloopt o.i.v. een NAD^+-afhankelijk dehydrogenase. Het dehydrogenase is zeer specifiek voor tropazuur. Tijdens de zuivering van het enzym zijn aanwijzingen verkregen dat de door het enzym gekatalyseerde omzetting in 2-fenylmalonzuur-semi-aldehyde (pma) een evenwichtsreactie is:

$$\text{tropazuur} + NAD^+ \rightleftharpoons \text{pma} + NADH + H^+$$

De instelling van het evenwicht is zowel door een NAD^+ afhankelijke dehydrogenering van tropazuur als door een NADH-afhankelijke hydrogenering van pma aangetoond. De evenwichtsconstante is bepaald op $\pm\ 0.30 \times 10^{-12}$ M; het evenwicht ligt sterk aan de kant van het tropazuur. Na de instelling van het evenwicht wordt een langzame NADH productie waargenomen, die veroorzaakt wordt door een spontane ontleding van pma.

Terwijl het effect van de tropazuur-, NAD^+- en NADH-concentratie op de enzymatische dehydrogenering hiermee kwantitatief kon worden verklaard, was het effect van pma op de instelling van het evenwicht veel geringer dan op grond van de toegevoegde hoeveelheid werd verwacht.

Dit was de aanleiding de keto-enol tautomerie van pma nader te onderzoeken. Hierbij kon door een combinatie van infrarood-spectroscopie en enzymologisch onderzoek worden aangetoond, dat het tropazuur-dehydrogenase specifiek is voor de keto-vorm van pma. Bij de enzymatische hydrogenering van een mengsel van keto- en enol-pma wordt in de initiële fase van de reactie de keto-vorm gehydrogeneerd, waarna de snelheid van de hydrogenering wordt bepaald door de tautomere omlegging van enol-pma in keto-pma.

De verklaring dat de langzame NADH-productie na instelling van het evenwicht o.i.v. het tropazuur-dehydrogenase wordt veroorzaakt door de snelheid, waarmee het gevormde pma ontleedt, is geverifieerd door de bepaling van de stabiliteit van keto- en enol-pma onder reactie-condities. Hierbij bleek dat de snelheid van de ontleding van keto-pma – in tegenstelling tot die van enol-pma – overeenstemt met de snelheidsconstante van de reactie die aan de NADH productie ten grondslag ligt.

Met een niet-gezuiverd TDH-preparaat werd bij neutrale pH de evenwichts-instelling niet waargenomen. Dit wordt veroorzaakt door de aanwezigheid van het pma-decarboxylase (PDC) dat een groot effect heeft op de stabiliteit van keto-pma. Bij de enzymatische dehydrogenering van tropazuur in de aanwezigheid van dit enzym wordt pma zo snel gedecarboxyleerd dat het evenwicht niet kan worden ingesteld. Dit heeft een sterke toename in de productie van NADH, CO_2 en fenylaceetaldehyde tot gevolg.

Fenylaceetaldehyde wordt o.i.v. een NAD^+-afhankelijk dehydrogenase omgezet in fenylazijnzuur. Ook dit enzym is zeer specifiek. Terwijl de drie eerder genoemde enzymen stabiel zijn, blijkt het PDH te worden geïnactiveerd tijdens dialyse en bij bewaren van minder geconcentreerde oplossingen. Deze inactivering kan worden voorkomen door een hoge concentratie kalium-ionen en door het toevoegen van mercapto-ethanol, EDTA, natrium laurylsulfaat en fenylaceetaldehyde.

De tropazuur enzymen blijken alle in PMBL-1 te worden geïnduceerd, wanneer de bacterie wordt gekweekt in een medium met atropine, tropazuur, fenylaceetaldehyde, fenylglyoxylzuur of benzaldehyde. Tropine en fenylazijnzuur induceren deze enzymen niet. In het onderzoek van mutanten van PMBL-1 , waarin de genetische informatie van één of meer van de tropazuur-enzymen verloren is gegaan, is geconcludeerd dat atropine en tropazuur niet als zodanig tot inductie aanleiding geven, maar eerst o.i.v het basale niveau van de tropazuur-enzymen moeten worden omgezet in fenylaceetaldehyde. Hetzelfde geldt voor fenylglyoxylzuur; slechts wanneer deze verbinding kan worden gedecarboxyleerd

tot benzaldehyde wordt inductie waargenomen. Blijkbaar zijn fenylaceetaldehyde en benzaldehyde de werkelijk inducerende verbindingen.

Men kan zich afvragen of atropine in PMBL-1 uitsluitend o.i.v. de hier beschreven enzymen wordt afgebroken of dat de bacterie nog over een andere mogelijkheid beschikt om het alkaloïde te metaboliseren. Bij het onderzoek van mutanten, die gestoord waren in de afbraak van atropine en tropazuur, maar niet in het metabolisme van fenylazijnzuur, is echter gebleken dat in elk van deze mutanten één of meer van de tropazuur-enzymen afwezig was. Dit maakt het zeer onwaarschijnlijk dat PMBL-1 over een alternatieve mogelijkheid voor de afbraak van atropine en tropazuur beschikt. In dit verband kan worden opgemerkt dat het aantal moleculen substraat, dat door de tropazuur-enzymen in vitro per tijdseenheid kan worden omgezet, aanmerkelijk groter is dan vereist is voor de waargenomen groeisnelheid van PMBL-1 in een medium met tropazuur als enige koolstofbron, zodat de capaciteit van deze stofwisselingsketen geen belemmerende factor voor de groei behoeft te zijn.

Hiernaast kan men zich afvragen of de tropazuur-enzymen uitsluitend bij de afbraak van atropine zijn betrokken. Het lijkt op het eerste gezicht verwonderlijk dat Pseudomonas over een groep enzymen beschikt, die speciaal voor de afbraak van atropine en tropazuur dienen. Het ligt voor de hand te veronderstellen dat deze enzymen in de cel een andere, meer belangrijke functie bezitten. Hiertegen pleit echter de grote substraat specificiteit van deze enzymen en de specificiteit van het inductie mechanisme. Op grond hiervan kan een functie bij de stofwisseling van andere aromatische verbindingen zoals fenylalanine en fenylmelkzuur worden uitgesloten. Ook de waarneming dat Pseudomonas mutanten, waarin de informatie voor één of meer van de tropazuur-enzymen verloren is gegaan, ongestoord kunnen groeien met fenylazijnzuur als enige koolstofbron, duidt op een niet-essentiële functie van deze enzymen.

Indien men aannneemt, dat bij micro-organismen een zekere economie bestaat wat betreft het ontwikkelen van enzymsystemen, die de cel ter beschikking staan, zou de uitgesproken specificiteit van de tropazuur-enzymen er op kunnen duiden, dat atropine en tropazuur in de natuur een veel belangrijkere rol spelen dan op grond van de spaarzame gegevens over het metabolism van deze stoffen wordt verwacht. Het zou bijv. mogelijk zijn dat tropazuur in vivo wordt gevormd by de afbraak van 3-benzofuraan- en 3-indool-derivaten of dat tropazuur-derivaten ontstaan bij de afbraak van lignine, een polymeer van fenylpropeen-derivaten, dat in grote hoeveelheden in houtweefsel wordt aangetroffen. De afbraak van lignine zou kunnen verlopen via een afsplitsing van fenylisopropeen-derivaten, waaruit door oxidatie tropazuur derivaten kunnen ontstaan. Een andere mogelijkheid is dat

de synthese van atropine en tropazuur in de natuur veel meer algemeen voorkomt dan men zich realiseert. Hiervoor pleit de belangrijke plaats die de natuurlijke producenten van atropine, de Solanaceae, in de planten- wereld innemen.

Aan de bestudering van het werkingsmechanisme van het atropine esterase en het gebruik van dit enzym als beperkt model voor de atropine-gevoelige receptor (zie inleiding) is in dit onderzoek een bijdrage geleverd door de uitwerking van de zuiverings-procedure voor het esterase. Bij bestudering van de regulering van de tropazuur-enzymen bleek dat het atropine-esterase ook door benzaldehyde en benzaldehyde-derivaten wordt geïnduceerd. Hiermee is een goedkope inductor ter beschikking gekomen, die gebruikt kan worden bij de productie van grote hoeveelheden atropine-esterase, die nodig zijn voor het onderzoek van het katalytisch centrum van het enzym.

Onderzoek van de stofwisselingsketen heeft het mogelijk gemaakt bij mutanten, die gestoord zijn in het metabolisme van atropine, een onderscheid te maken tussen mutanten, waarin alleen de atropine esterase activiteit verlaagd is en mutanten waarin het niveau van alle vier enzymen is veranderd. Op deze wijze kunnen uit de $AtrE^{-}$ mutanten - voorafgaand aan het meer bewerkelijke enzymologisch onderzoek - die mutanten worden geselecteerd, waarin de kans op een gemodificeerd esterase het grootst is.

Voor een verder onderzoek naar de regulering van de eiwitsynthese en voor de bestudering van ioniserende en exciterende straling hierop, lijken de tropazuur-enzymen zeer geschikt. De enzymen kunnen snel en kwantitatief worden geëxtraheerd en bepaald en hebben een laag moleculair gewicht, zijn specifiek voor hun natuurlijke substraten en worden uitsluitend geïnduceerd door enkele aromatische aldehyden.

Het feit dat hun functie niet essentieel is voor het normale celmetabolisme maakt de tropazuur enzymen tot een geschikt object voor een dergelijke studie. Bij de bestudering van de de regulering van eiwit- en enzym-synthese maakt men nl. meestal gebruik van mutanten, waarin de informatie voor één of meer van de betreffende enzymen verloren is gegaan. Bij vele andere enzym-systemen leiden deze mutaties tot een verstoring van het normale cel-metabolisme. In PMBL-1 is het echter mogelijk het effect van mutaties op de regulering van de synthese van de tropazuur-enzymen te onderzoeken zonder dat deze mutaties het cel-metabolisme verstoren en daardoor het regulerings-proces beïnvloeden. Hierbij kan een nuttig gebruik worden gemaakt van de gratuïte inductie door fenylglyoxylzuur; in aanwezigheid van deze verbinding worden de tropazuur-enzymen geïnduceerd terwijl de groei geheel onafhankelijk verloopt van het intact zijn van de tropazuur-stofwisseling.

Bij het gebruik maken van mutanten voor het onderzoek van de regulering van eiwitsynthese is het vereist de aard van de aanwezige mutaties te kennen. Pseudomonas putida is in dit opzicht echter minder aantrekkelijk omdat de mogelijkheden voor genetisch onderzoek in deze bacterie tot op heden zeer beperkt zijn. Onlangs zijn door Holloway en van der Putte enkele bacteriofagen geïsoleerd (1968) waarmee in Pseudomonas putida PMBL-1 de overdracht van genetische kenmerken kan worden aangetoond. Hoewel deze overdracht tot nu toe alleen is gevonden voor enkele auxotrofe kenmerken, zal het wellicht spoedig ook voor het atropine-kenmerk mogelijk zijn. Een verdere bestudering van de regulering van eiwit- en enzymesynthese in Pseudomonas putida zal dan mogelijk worden door een genetische analyse van het atropine locus gecombineerd met biochemisch onderzoek van de gen-producten, de tropazuur enzymen.

Summary

From soil between the roots of Atropa belladonna L., Rörsch and Berends (1965) isolated a Pseudomonas putida (PMBL-1) which is able to grow with the alkaloid atropine as sole carbon source. It has been shown that the breakdown of atropine in this micro-organism proceeds according to the scheme facing the last page. Atropine is hydrolyzed, tropine and tropic acid are formed. The latter is broken down via 2-phenylmalonic semi-aldehyde (pma) and phenylacetaldehyde to phenylacetic acid, a common carbon source to many Pseudomonas strains.

The role of phenylacetic acid as an intermediate in the metabolism of atropine and tropic acid was deduced from the observations that PMBL-1 grown on atropine or tropic acid is adapted to phenylacetic acid (fig. 4.2) and that all mutants isolated for the inability to metabolize phenylacetic acid fail to grow with atropine or tropic acid as carbon source. Moreover, phenylacetic acid was isolated from the growth-medium of several of these mutants grown in the presence of tropic acid.

The hydrolysis of atropine is catalyzed by the enzyme atropine-esterase (AtrE). The action of this esterase has been demonstrated by the identification of the reaction products tropine and tropic acid (fig. 5.2). This fairly stable enzyme has been purified extensively: estimated purity over 90% (table 5.1). The enzyme is specific for (-)atropine and closely related compounds (table 5.6) and is inhibited by organophosphates like diïsopropyl phosphorofluoridate (DFP).

The second enzyme playing a role in the metabolic pathway is the NAD^+ dependent tropic acid dehydrogenase TDH, the third is the 2-phenylmalonic semi-aldehyde decarboxylase (PDC). The combined action of the two enzymes on tropic acid in the presence of NAD^+ results in the formation of NADH (fig 6.3), phenylacetaldehyde (fig 7.4) and CO_2 (table 7.5). The last step is catalyzed by the phenylacetaldehyde dehydrogenase (PDH), also NAD^+ -dependent. It was shown that phenylacetic acid is the product of this reaction (fig. 8.4). The TDH, PDC and PDH have been purified partially (tables 6.2; 7.6; 8.3). For each enzyme, a reliable determination of enzyme activity has been developed (fig 2.2). The enzymes were found to be specific for the appropriate substrates (table 6.5.1 and 6.5.2).

When the dehydrogenation of tropic acid was studied in the presence of NAD^+ and purified TDH at neutral pH, a fast initial NADH-production was observed, followed by an abrupt slowing down (fig 6.6). This phenomenon was explained on the assumption that the enzyme catalyzes the equilibrium process:

$$\text{tropic acid} + NAD^+ \rightleftharpoons \text{pma} + NADH + H^+$$

The NADH concentration increases rapidly while the equilibrium is established;

the slow NADH-production thereafter is caused by spontaneous decomposition of pma, resulting in a shift of the equilibrium to the right. Variation of the concentrations of tropic acid and NAD^+, variation of the pH and the addition of NADH and pma all had the expected influence on the course of the reaction. The equilibrium was obtained either by dehydrogenation of tropic acid or by hydrogenation of pma (fig. 6.9). From the results, the equilibrium constant K_{eq} was calculated approximately to amount to 0.30×10^{-12} M (table 6.17); this means that the hydrogenation is favored.

The pma required for these experiments has been synthesized according to the only procedure described in literature (Strukov 1952). The melting point of the synthesized product was not the same as the one cited. The compound, however, was identified unquestionably as pma in its enol-form by means of chemical analysis and infrared spectroscopy.

The reaction of enol-pma with NADH in the presence of TDH also shows biphasic kinetics (fig. 6.11.a). Evidence is presented that the keto-pma is the proper substrate for the TDH; the initial rapid conversion of NADH into NAD^+ was found to correspond with the amount of keto-pma initially present. The slow phase of the reaction was attributed to a rate limiting tautomeric conversion of enol-pma into keto-pma.

The rate of decomposition of enol-pma and keto-pma was measured (fig 6. 15). These values were compared with the rate of reaction governing the NADH production after the establishment of the equilibrium. From these results can be concluded that keto-pma is the product formed during the enzymatic dehydrogenation of tropic acid.

When non-purified TDH preparations are used for the dehydrogenation of tropic acid, the equilibrium is not reached. This is shown to be the consequence of the pma-decarboxylase present in these preparations. The decarboxylase has a great effect on the stability of keto-pma. This enzyme can also be demonstrated by its great effect on the amounts of NADH (fig. 7.2), phenylacetaldehyde and CO2 formed after establishment of the equilibrium during enzymatic dehydrogenation of tropic acid at neutral pH. The effect on the NADH-production is used for a quantitative assay for the decarboxylase. During purification, the enzyme is separated from TDH (fig 7.1).

The phenylacetaldehyde dehydrogenase is less stable than the other enzymes. Much activity is lost during dialysis (table 8.1) and during storage of preparations with rather low protein content (table 8.2). In a phosphate buffer (0.05 M), containing mercapto-ethanol, EDTA, sodium-dodecylsulfate and phenylacetaldehyde, the enzyme can be handled without serious loss of activity.

The pathway from atropine to phenylacetic acid as given in the scheme is, owing to the substrate specificity of the four enzymes, the only possible route mediated by these enzymes. A quite different pathway seems unlikely since all mutants unable to grow with atropine as sole source of carbon lack one or more of these enzymes. Indications that PMBL-1 contains a permease or a racemase for atropine or tropic acid have not been found. The enzymes involved in the breakdown of atropine in PMBL-1 are quite different from the enzymes in Pseudomonas ATCC 12633 which are concerned with the metabolism of the structurally related mandelic acid (Hegeman 1966).

The AtrE, TDH, PDC and PDH are inducible enzymes. Growth in atropine and tropic acid containing media results in the induction of all four enzymes. Also phenylacetaldehyde, phenylglyoxylic acid and benzaldehyde act as inducers. (table 10.3 and 10.4). From studies of mutants of PMBL-1, with a mutation in only one of the genes for these enzymes, it was concluded that atropine and tropic acid themselves are unable to induce the “tropic acid enzymes” but have to be converted into the actual inducer phenylacetaldehyde (table 10.6). The same holds for the induction observed in PMBL-1 in phenylglyoxylic acid containing media; this compound is not a real inducer but must be converted into benzaldehyde by a decarboxylase present in PMBL-1 (table 10.7).

The advantages of induction of the enzymes by an intermediate product are discussed. This mode of induction is more specific than induction by the substrate (Ornston 1966): the compound has to be recognized by three specific enzymes before it can give rise to induction. Moreover, because of the relatively high basic activity of PDH, induction will only take place if atropine can be converted into phenylacetaldehyde in a really fast process. This enables the cell to check beforehand whether induction will be of advantage or not. This is very attractive from a regulatory point of view.

In the final discussion it is concluded that these enzymes present in PMBL-1 have the exclusive function to catalyze the breakdown of atropine into phenylacetic acid. These enzymes are therefore considered as a very promising object for a study of the regulation of protein synthesis in micro-organisms.

LITERATURE – REFERENCES

ADELBERG, E.A., Bact. Rev., 17 (1953) 253

AMMON, R. and W. SAVELSBERG, Z. Physiol. Chem., 284 (1949) 135

ASAKAWA, T., H. WADA and T. YAMANO, Biochem. Biophys. Acta, 170 (1968), 375

BÄCKLIN, K.I., Acta Chem. Scand., 12 (1958) 1279

BERENDS, F., personal communication (1965)

BERENDS, F., A. RÖRSCH, W.F. STEVENS : Proc. Conf. Struct. Reactions DFP-sensitive enzymes, ed. E. Heilbronn, Försvarets Forskninganstalt Stockholm 1967 p.45.

BERENDS, F., A.RÖRSCH, W.STEVENS, R.OOSTERBAAN, J.A.COHEN, 1969 in prep.

BERNHEIM, F., Interaction of Drugs and Cell Catalysts, Burgess, Minneapolis, 1948

BERNHEIM, F. and M.L.C. BERNHEIM, J. Pharmacol., 64 (1938) 209

CALLAHAN, J.W. and G. W. KOSICHI, Can. J. Biochem., 45 (1967) 839

CHAMBERLAIN, E.M. and S. DAGLEY, Biochem. J., 110 (1968) 755

CHAPMAN, P.J. and D.J. HOPPER, Biochem. J., 110 (1968) 491

CHILSON, O.P., G.B. KITTO, N.O. KAPLAN, Proc. Natl. Acad. Sci. U.S., 53 (1965) 1006

CIOTTI, M., N. KAPLAN, Meth.Enzymol. III, Colowick Kaplan, Acad. Press. NY 1957, 890

COHEN, J.A., R.A. OOSTERBAAN, H.S. JANSZ, F. BERENDS, J. Cell Comp. Physiol., 54 Suppl.1 (1959) 231

COHEN-BAZIRE, G., W. SISTROM, R.Y. STANIER, J. Cell. Comp. Physiol., 49 (1957) 25

COMMISSION OF ENZYMES. Rep. Intern.Union Biochem.,Pergamon, Oxford, 1961 p. 7

COZZARELLI, N., S. HAYAISHI and E.C.C. LIN, Federation Proc., 24 (1965) 417

DAGLEY, S. et al, Pseudomonas Workshop, Cold Spr. Harb. Quant. Biol., NY, 1964, p.7.

DAVIES, M. and W.J. ORVILLE-THOMAS, J. Chem Soc., (1951) 2858

FLEISCHMAN, P., Berl. Klin Wschr., 48 (1910) 135

FLODIN, P. and D.W. KUPLIE, Biochim. Biophys. Acta, 21 (1956) 368

FLUCK H., Pharm. Weekblad, 100 (1965) 1361

FRIEDMAN, C. A. and J.M.Z. GLADYCH, J. Chem. Soc., (1956) 310

GABOUREL, J.D. and R.E. GOSSELIN, Arch. Intern. Pharmacodyn., 115 (1958) 416

GIBSON, C.A. and H.W. YOUNGKEN, J. Pharm. Sci., 56 (1967) 854

GIBSON, D.T., Science, 161 (1968) 1093

GLICK, D., J. Biol. Chem., 134 (1940) 617

GUNSALUS, C.F., R.Y. STANIER and I.C. GUNSALUS, J. Bacteriol., 66 (1953 a) 548

GUNSALUS, I.C., C.F. GUNSALUS and R.Y. STANIER, J. Bacteriol., 66 (1953 b) 538

HEGEMAN, G.D., J. Bacteriol., 91 (1966) 1140

HOLLOWAY, B., P. VAN DE PUTTE, Nature, 217 (1968) 459

HOUBEN-WEIJL, Method. Organischen.Chem. Bd II Thieme, Stuttgart, (1953) p. 385

JANSON, J., J. Chromat., 28 (1967) 12

JINDRA, A., A. CIHAK, Abh. Deutch, Akad. Wiss., Chem. Geol. Biol, Berlin, 1963 nr 4

KACZKOWSKI, J., Acta Soc. Botan. Polon., 28 (1959) 677

KACZKOWSKI, J., Bull. Acad. Polon. Sci. Ser. Sci. Biol., 12 (1964) 375

KALSER, S.C., et al., J. Pharmacol. Exp. Therap., 121 (1957) 449

KAUFMAN, H.P. and E. RICHTER, Chem. Ber., 58 (1925) 222

KEDZIA, W., J. LEWON and T. WISNIEUWSKI, J. Pharm. Pharmacol., 13 (1961) 614

KENNEDY, S.I.T. and C.A. FEWSON, Biochem. J., 107 (1968) 497

KOHN, L.D., and W.B. JACOBY, J. Biol.Chem., 243 (1968) 2465

KRUPKA, R.M., Biochemistry, 2 (1963) 76

KUBOWITZ, F. and P. OTT, Biochem. Z., 314 (1943) 94

LAYNE, E., in: Meth. Enzymol. III, ed. Colowick Kaplan, Acad. Press, NY, (1957) p. 447

LEDERBERG, J. and E.M.LEDERBERG, J. Bacteriol., 63 (1952) 399

LINDEN, A.C, VAN DER and G.J.E.THIJSSE, Advances in Enzymology, 27 (1965) 469

LINEWEAVER, H. and D. BURK, J. Amer. Chem Soc., 56 (1934) 658

LINN, S. and I.R. LEHMAN, J. Biol. Chem., 240 (1965) 1287

LOEWUS, F.A., T.T. CHEN and B. VENNESLAND, J. Biol. Chem., 212 (1955) 787

LOWRY, O., N. ROSEBROUGH, A. FARR, R. RANDALL, J.Biol.Chem. 193 (1951) 265

MARGOLIS, F. and P. FEIGELSON, J. Biol. Chem., 238 (1963) 2620

MARGOLIS, F. and P. FEIGELSON, Biochem.Biophys. Acta, 90 (1964) 117

MATSUDA, K., Niigata Igakkai Zasshi, 80 (1966) 53
METZNER, R., Arch. Exp. Pathol. Pharmakol., 68 (1912) 110
MOORE, C.L., Arch. Biochem. Biophys., 128 (1968) 734
MÜLLER-HILL, B., H. RICKENBERG K. WALLENFELS, J. Mol. Biol., 10 (1964) 303
NEUMANN, D. and K. H. TSCHÖPE, Flora Jena, 156 (1966) 521
NIEMER, H., H. BUCHERER and A. KOHLER, Z. Physiol. Chem., 317 (1959) 238
NIEMER, H. and H. BUCHERER, Z. Physiol. Chem., 326 (1961) 9
ORNSTON, L.N., J. Biol Chem., 241 (1966) 3800
OTORII, T., Nippon Yakurigaku Zasshi, 61 (1965) 462
PARDEE, A.B., Science, 162 (1968) 632
QUINTON, R.M., J. Pharm. Pharmacol., 18 (1966) 579
RÖRSCH, A. and F. BERENDS, personal communication (1965)
RODD, E.H., Chem. Carbon Comp. Vol. III B, Elzevier, Amsterdam , 1956 p. 927
SAWIN, P.S. and D. GLICK, Proc. Natl. Acad. Sci. U.S., 29 (1943) 55
SCHACHMAN, H. K., Cold Spr. Harb. Symp. Quant. Biol., 28 (1963) 409
SCHLESINGER, S. and B. MAGASANIK, J. Biol. Chem., 249 (1965 a) 4325
SCHLESINGER, S., P. SCOTTO and B. MAGASANIK, J. Biol. Chem. 240 (1965 b) 4331
SCHROFF, Z., Z. Ges. Ärzte Wien, 3 (1852) 211
SEIDENBERG, M. et al, Arch. Biochem. Biophys., 97 (1962) 470
STACHOW, C.S., I.L. STEVENSON and D. DAY, J. Biol. Chem., 242 (1957) 5294
STAHL, E., Dünnschicht Chromatographie, Springer Verlag, Berlin, 1957.
STANIER, R.Y., Bact. Rev., 14 (1950) 179
STANIER, R.Y., I.C.GUNSALUS and C.F.GUNSALUS, J. Bacteriol. , 66 (1953) 543
STANIER, R.Y., N.J. PALLERONI, M. DOUDOROFF, J. Gen. Microbiol, 43 (1966) 159
STRUKOV, I.T., Zhur Obschei Khim., 22 (1952) 1025
SUBBA RAO, P.V., K. MOORE, G.TOWERS, Arch. Biochem. Biophys., 122 (1967) 466
UMBREIT, W.W. Manometric Techn.Tissue Metabol., Burgess, Minneapolis, 1951
VOGEL, A.I., Practical Organic Chemistry, Longmans, London, 1959, p. 781
WENSINCK, F., Appendix at Berends (1969)
WERNER, G. and G. BREHMER, Z. Phys. Chem. , 348 (1967) 1640
WERNER, G. and K.H. SCHMIDT, Z. Physiol Chem., 349 (1968) 741

OVERVIEW OF THE ACADEMIC CAREER OF WILLEM FRANS STEVENS

Presented here at the request of the Faculty of Science,
State University Leyden, The Netherlands.

After graduation in 1959 at the Grammar School β-division of the Twentsch Carmel Lyceum in Oldenzaal, The Netherlands, I was enrolled in the same year for a study in chemistry at the State University Leyden. In 1962, I passed the bachelors exam and in 1965 the master's exam with honors in the main discipline of biochemistry and in organic chemistry and pharmacology, under guidance of Prof. Dr. L. Bosch, Prof. Dr. E. Havinga and Prof. Dr. E. L. Noach.

To fulfill my duties in military service I followed a curtailed training for army officer. Thereafter, I was sent on secondment to the Medical Biological Laboratory (MBL) of the National Defense Organization TNO Rijswijk, The Netherlands. I got permission to make a start with the research presented in this thesis. After fulfillment of my military duties, I was engaged by the State University Leyden as co-worker of the Institute for Radiopathology and Irradiation Protection, but remained employed at the MBL in order to continue the research on the regulation of enzyme synthesis in Pseudomonas.

It is a deep honor to thank my PhD promoter Prof. Dr. A. Rörsch and Prof. Dr. J.A. Cohen, director of the MBL for their contributions to my academic education.

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ABBREVIATIONS

MBL	Medical Biological Laboratory
TNO	Dutch Organization for Applied Scientific Research
PMBL-1	Pseudomonas bacterium isolated from Atropa belladonna soil
Ps-atropine	PMBL-1 grown in the presence of atropine
Ps-tropic acid	PMBL-1 grown in the presence of tropic aid
AtrE	atropine esterase
TDH	tropic acid dehydrogenase
PDC	2-phenylmalonic semi-aldehyde decarboxylase
PDH	phenylacetaldehyde dehydrogenase
NAD+	nicotinamide adenine dinucleotide
NADH	reduced NAD+
NADP+	nicotinamide adenine dinucleotide phosphate
NADPH	reduced NADP+
pma	2-phenylmalonic semi-aldehyde
enol-pma	tautomeric enol-form of pma
keto-pma	tautomeric keto-form of pma
nm	nanometer
A_{340}	absorption at 340 nm
A_{700}	absorption at 700 nm
U	unit of enzyme activity
TA	total activity
SA	specific activity
HMP	10 mM K-phosphate buffer pH 7.0
EDTA	ethyleendiamino tetra-acetic acid
ME	mercapto ethanol
SDS	sodium lauryl sulfate
TRIS	tris hydroxymethyl aminomethane
LDH	lactic acid dehydrogenase

GENETIC MARKERS

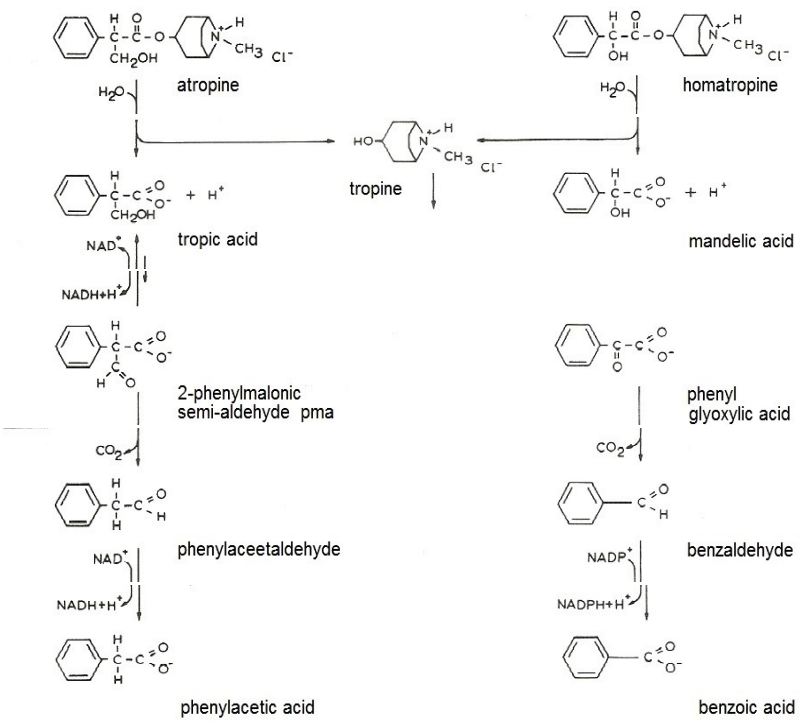
Atr	atropine
Tro	tropic acid
Tpn	tropine
Pac	phenylacetic acid
Php	p-hydroxylphenylacetic acid
Pgl	phenylglyoxylic acid
AtrE-	mutant lacking the AtrE

The relation between the amount or concentration and the absorption at 340 nm in a volume of 3 ml: 1 µmol NADH ≡ A_{340} 2.07 ; 1 mM NADH ≡ A_{340} 6.22

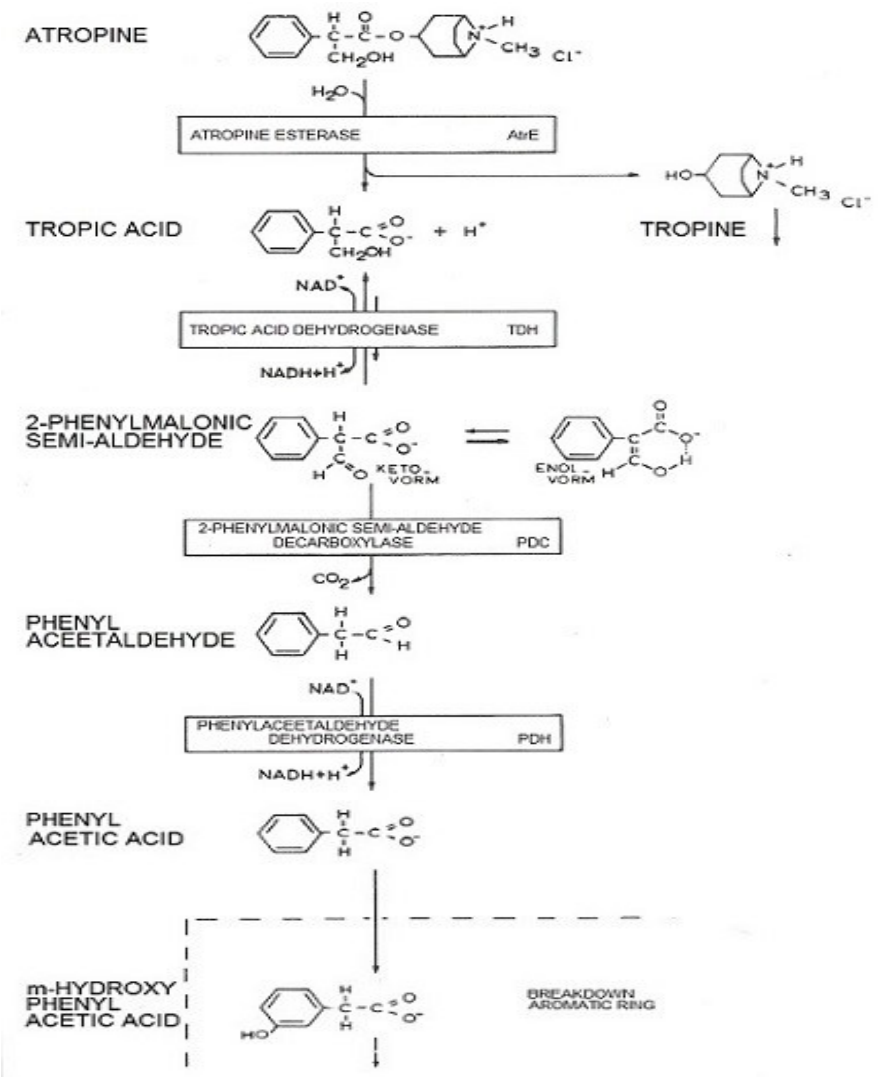
CONTENTS

Annex

PATHWAYS FOR ATROPINE AND PHENYLGLYOXYLIC ACID IN PSEUDOMONAS PMBL-1



TROPIC ACID ENZYMES :
INVOLVED IN THE METABOLISM OF
ATROPINE IN PSEUDOMONAS PUTIDA PMBL-1



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