Enzymology and regulation of the atropine metabolism in pseudomonas putida

Stevens, W.F.

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Author: Stevens, W.F.
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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° and 37°. After 3 days, growth was observed at 30°. 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°, 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 $H_2SO_{\underline{4}}$. 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 (± 1.6×10^5 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.

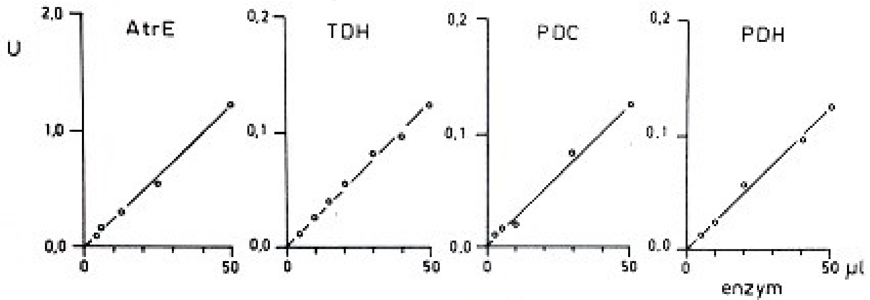
In the assay of PDH activity, 20 µl 20 mM phenylacetaldehyde (in acetone) was added to the NAD^+ incubation mixture in the cuvette. Next enzyme solution and water was added to a final volume of 3 ml. The A_{340} was registered during at least 4 min. Acetone in the amount used has no effect on the PDH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

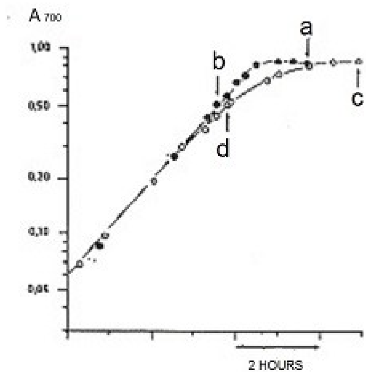
by pipette from the upper part of the centrifuge tube. The specific enzyme activities were calculated from these data. The assay of the enzyme activities was carried out as soon as possible following the extraction.

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 enzymes in duplicate
o — o without extra aeration

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.

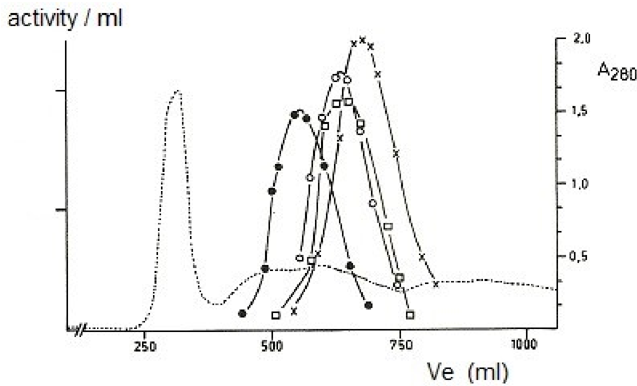
2.11.3 Estimation of the molecular weight of the tropic acid enzymes

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

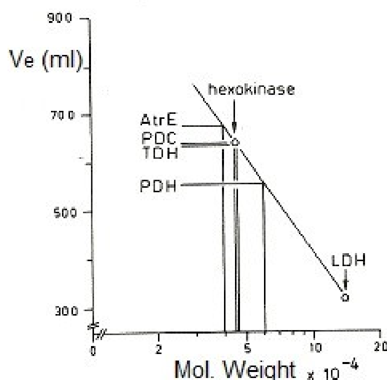
Ve = elution volume (ml)

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

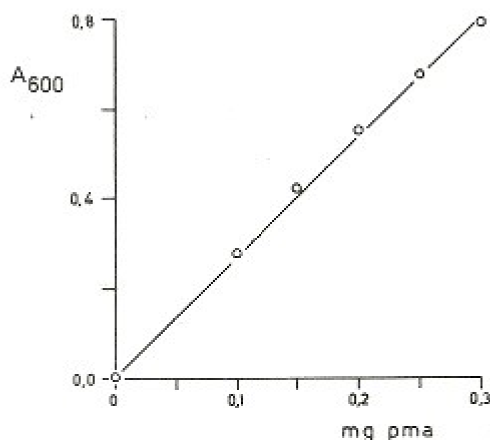
This assay is a variant of the Kaufmann and Richter method (1925) based on the colorimetric quantification of the complex of the enol compound with $FeCl_3$.

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.