Enzymology and regulation of the atropine metabolism in pseudomonas putida

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Citation

Stevens, W. F. (1969, June 18). *Enzymology and regulation of the atropine metabolism in pseudomonas putida*. Retrieved from https://hdl.handle.net/1887/77056

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Title: Enzymology and regulation of the atropine metabolism in pseudomonas putida
Issue Date: 1969-06-18

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

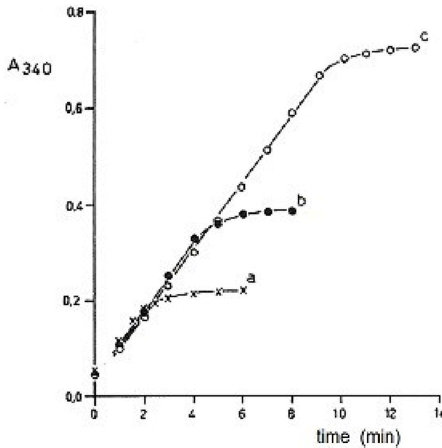
In this experiment, the amount of NADH was determined, which was formed at the complete conversion of a limited amount of the substrate. Since the reaction under the standard condition of the activity assay (2.9.3) proceeds quantitatively, there was no need for special precautions like the high pH in 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

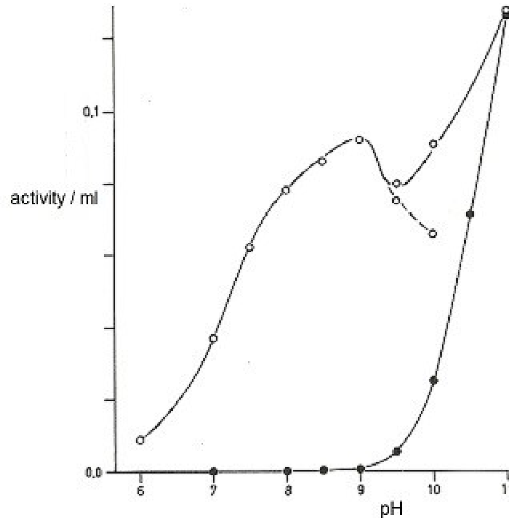
Substrate specificity

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.

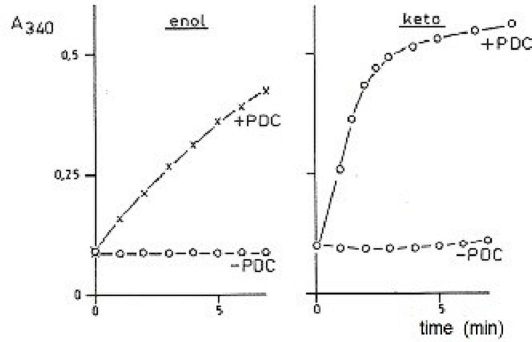
The pma formed from tropic acid by the TDH might be not a suitable substrate for PDH. However, a number of complications arise to prove this point. A priori this seems difficult to prove due to the limited stability of keto-pma; in aqueous environment, this compound is converted nearly completely in 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.

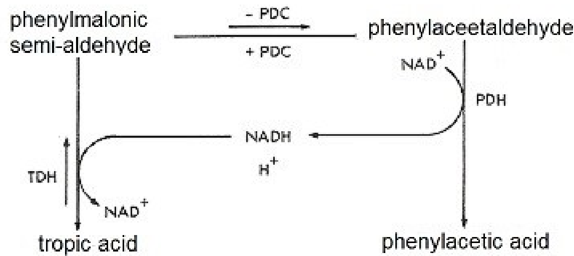
Fig 8.7

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.

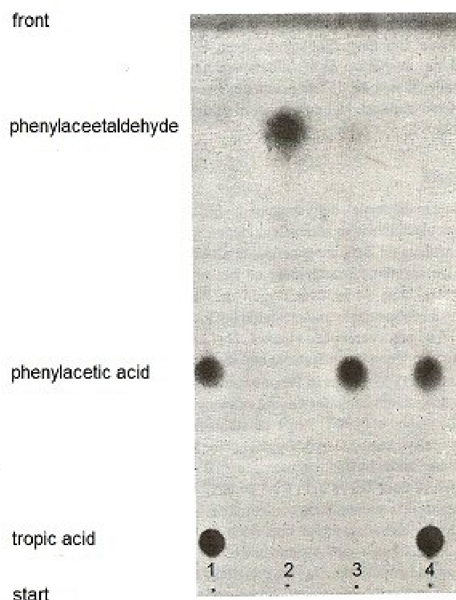
8.6 MISCELLANNEOUS

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.