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 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.

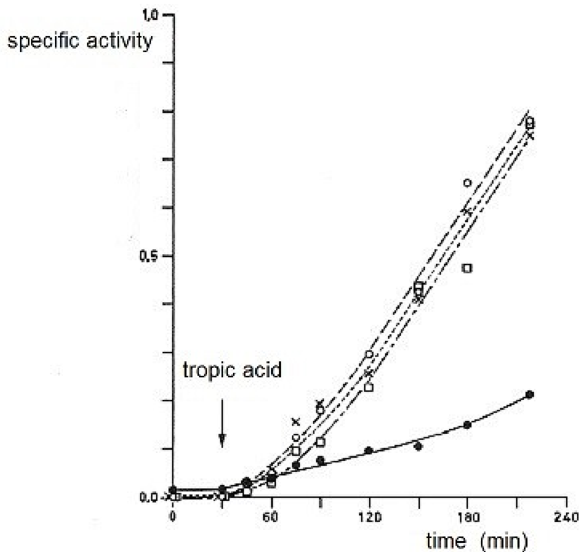
More experiments will be presented comparing the enzyme production in PMBL-1 and in a number of mutants of PMBL-1, leading to the conclusion that atropine and tropic acid are not recognized as such by the system that the regulates their production. The induction is of the gratuitous type where these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



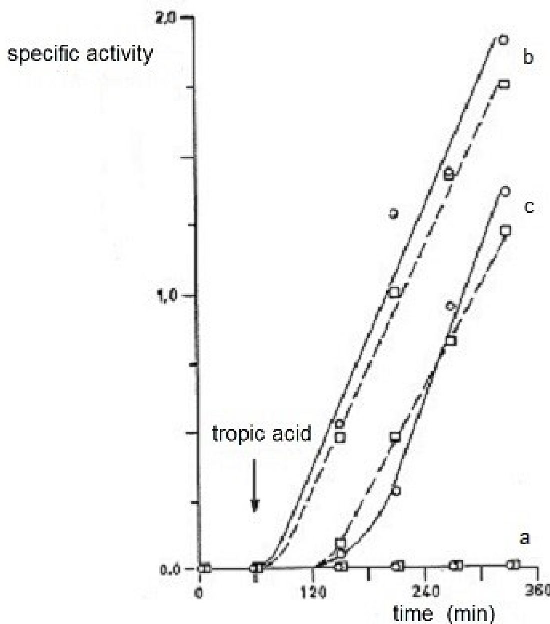
Tropic acid as added to a non-induced culture in the logarithmic growth phase on time t=30 min. Samples were taken at different times. Specific activity (U/mg protein) was measured of AtrE (x – x), TDH (o – o), PDC (□ – □) and PDH (• – •).

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

	Table 10.3				Table 10.4			
compound	0.1% succinic acid				0.005% succinic acid			
(0.025%)*	AtrE	TDH	PDC	PDH	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	TDH	PDC	PDH	AtrE	TDH	PDC	PDH
	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	xxxx	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 phenyl-acetaldehyde 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.