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Penicillinamidohydrolase in *Escherichia coli*

II. Synthesis of the Enzyme, Kinetics and Specificity of Its Induction and the Effect of O_2

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ABSTRACT. The differential rate of synthesis of penicillinamidohydrolase (penicillin acylase — EC 3.5.1.11) was studied in *Escherichia coli* growing in some chemically defined media and in a complex medium. The enzyme is synthesized at a constant rate only during the exponential phase of growth of cells. Its synthesis is induced most effectively (with respect to quantity) by phenylacetic acid. The induction lag of the enzyme synthesis in a medium with acetate corresponds to two generation times. The highest rate of the enzyme synthesis is reached in a medium containing phenylacetic acid as the only source of carbon and energy. The enzyme synthesis is fully repressed by an increased concentration of dissolved oxygen in the medium, even when *Escherichia coli* is cultivated in the medium with phenylacetic acid as the only carbon and energy source.

Penicillinamidohydrolase in *Escherichia coli*, namely the stimulation and induction of the enzyme by phenylacetic acid were studied by Kaufmann and Bauer (1964), Szentirmai (1964) and Levitov and coworkers (1967). The above-mentioned authors presented also optimal conditions for production of this enzyme by *Escherichia coli*, during aerobic growth of this microorganism in an aerated medium with inorganic salts, containing a mixture of amino acids and peptides (usually corn-steep liquor) serving as carbon and energy sources, together with phenylacetic acid. The presence of vitamins and growth factors in the form of corn-steep stimulated the enzyme synthesis. The enzyme production decreases with increasing temperature. The enzyme is not synthesized at all at temperatures higher than 37°C. The authors also studied specificity of the enzyme induction in *Escherichia coli*; however, certain aspects of this problem remained unclear. Thus Levitov and coworkers (1967) claimed that the later the addition of phenylacetic acid to the complex medium during growth, the lower the degree of induction. Kleyner and Lopatnev (1972) showed that synthesis of this enzyme is influenced by a different transfer of O_2 during growth of *Escherichia coli* in a medium containing corn-steep and phenylacetic acid. The aeration efficiency was changed by changing content of the medium in the flasks. The production of the enzyme was strongly dependent on the material of the stopper used to close the flasks. The above-mentioned authors suggested a limited range of the oxygen transfer required for the maximal production of the enzyme. The rate of the enzyme decreases at values below or above this range. The mechanism of induction, specificity of induction and the effect of different factors on the synthesis of the enzyme in *Escherichia coli* still remain unclear. In addition, complex, chemically undefined media were used in the papers referred to here. In such media the

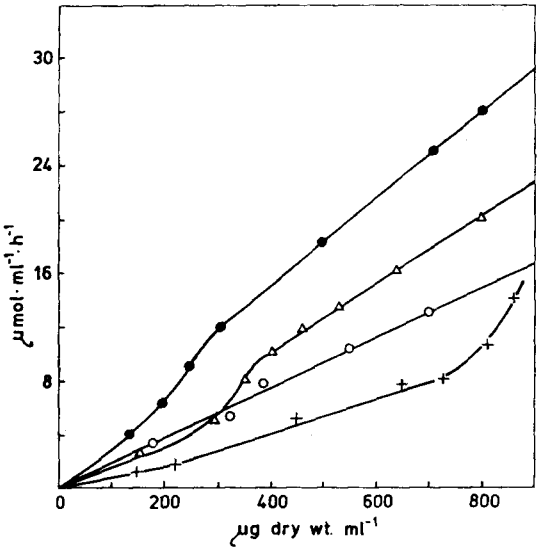

FIG. 1. Differential rate of enzyme synthesis in cultures (ordinate) of *Escherichia coli* exponentially growing in different media. +, Complex medium with phenylacetic acid; ○, acetate with phenylacetic acid; △, lactate with phenylacetic acid; ●, phenylacetic acid as the only carbon and energy source. Unwashed preinduced cells grown in the same media and taken from the exponential phase of growth (5×10^7 cells/ml) were used for the inoculation.

enzyme is not synthesized at a constant rate, its specific activity increasing during growth of the cells. Therefore, it appeared useful to study these problems in more detail.

MATERIALS AND METHODS

The bacterial strain and the basic medium were those previously described (Vojtíšek and Slezák, 1975). When required, sterile solutions of the following compounds were added to the basic medium: 1.0% (w/v) Na-lactate (Erba, Italy), 1.0% K-acetate, 0.1 or 0.2% phenylacetic acid (sodium salt, Loba Chemie, Wien, Austria) and 2.0% sucrose. Bacto Nutrient Broth (Difco, 8 g/litre) with 0.1% phenylacetic acid served as the complex medium; pH of the medium was adjusted with NaOH to 7.2. When phenylacetic acid served as the only carbon and energy source, its concentration was 0.2%. Its concentration was 0.1% when used as the inducer. In certain experiments phenylacetic acid was applied as 0.1% doses during cultivation of bacteria, due to the fact that this compound is toxic and inhibits growth of bacteria when applied at higher concentrations. Derivatives of phenylacetic and phenoxyacetic acid were supplied by Fluka A. G., Buchs, Switzerland.

The bacteria were cultivated either in a rotating shaker in flasks or in twenty-litre laboratory fermentors, in which an oxygen electrode (Development Workshop of the Czechoslovak Academy of Sciences) was inserted when required. Cultivation conditions, as well as parameters of the cultivation apparatus were the same as described previously (Vojtíšek and Slezák, 1975). Occasional changes of these parameters are mentioned in the text.

Bacteria were taken during the exponential phase of growth, centrifuged for 10 min at 18 000 ***g***, washed twice with 0.05M phosphate buffer and optical density of the culture was measured in a Spekol spectrophotometer at 600 nm using 1 cm glass cuvettes. Dry weight of cells was calculated from a relationship relating optical density to the dry weight. Washed suspensions of bacteria were killed by toluene and the enzyme activity was determined by means of the Berthelot photocolorimetric method modified according to Weatherburn (1967) as described in detail earlier

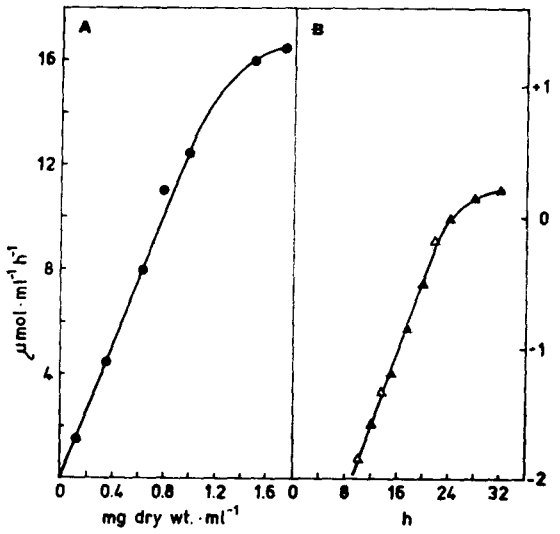

FIG. 2. Differential rate of enzyme synthesis (A) and growth (B) of *Escherichia coli* in a medium with acetate and in the presence of the inducer. ●, Activity of the enzyme; △, growth of the culture; ▲, samplings from the exponential and non-exponential phases of growth, equivalent to the enzyme activities (●). Unwashed preinduced cells grown in the same medium and taken from the exponential phase of growth (5×10^7 cells/ml) were used for the inoculation.

(Vojtíšek and Slezák, 1975). 5×10^{-3}M phenylacetamide dissolved in 0.1M phosphate buffer pH 7.6 was used as substrate. The enzyme activity is expressed as the amount of NH_4^+ ions released by hydrolysis of phenylacetamide in μmoles/ml/h at 42°C and pH 7.6 within zero order kinetics. The specific activity is expressed as μmol h^{-1} (mg dry wt.)$^{-1}$.

RESULTS

In addition to the complex medium, synthetic, chemically defined media containing ammonium sulphate as the nitrogen source and different carbon and energy sources, were used to follow the differential rate of the enzyme synthesis in *Escherichia coli*. The inducer, phenylacetic acid, was added to all media just before the inoculation. A synthetic medium with phenylacetic acid serving as the only carbon and energy source was also used. The media were inoculated with a needle from a meat-peptone agar slant (inoculation flasks). When the cultures reached the end of the exponential phase of growth, the unwashed bacteria were transferred to the same media in the presence of the inducer and the differential rate of the enzyme synthesis and growth of bacteria were followed. The obtained results are presented in Fig. 1.

It follows from the results presented in Fig. 1 that the highest rate of the enzyme synthesis was reached in the medium, in which phenylacetic acid served as the only carbon and energy source (specific activity of 35.0 μmol/mg dry wt.), followed by the media with lactate (25.0 μmol/mg dry wt.) and acetate (15.0 μmol/mg dry wt.). The differential rate of the enzyme synthesis in cells growing in the complex medium is not constant during the exponential phase of growth for the specific activity increases; average values of 20.0 μmol/mg dry wt. were reached. The differential rate of the enzyme synthesis during the initial phase of the exponential growth of cells slightly changes, both in the medium with lactate and in the medium with phenylacetic acid. However, this rate is constant in the medium with acetate, from the beginning to the end of the exponential growth of bacteria.

In further experiments we wanted to find out whether the enzyme is synthesized

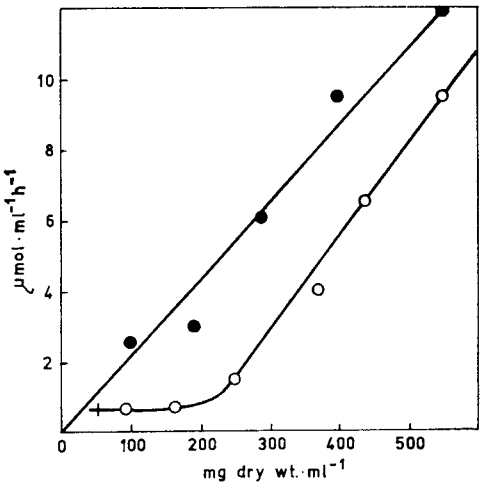

FIG. 3. Kinetics of the induction of enzyme synthesis by phenylacetic acid in a culture of *Escherichia coli* growing exponentially in a medium with acetate. +, Basal enzyme activity in exponentially grown bacteria (inoculum) in a medium with acetate and without the inducer (10^8 cells/ml); ○, differential rate of enzyme synthesis in the exponentially growing subculture 1 (acetate with the inducer) inoculated with the non-preinduced bacteria; ●, differential rate of enzyme synthesis in the exponentially growing subculture 2 (acetate with the inducer) inoculated with the preinduced bacteria taken from the exponential phase of growth of the subculture 1.

even in non-logarithmically growing, or occasionally even in non-growing, bacteria. The results are presented in Fig. 2.

It follows from the results presented in Fig. 2 that the enzyme is synthesized at a constant rate only during the exponential phase of growth, the change from the exponential phase to the phase of decelerated growth being related with a substantial decrease of the rate of the enzyme synthesis. In the non-growing cells the enzyme is not synthesized. This fact was demonstrated in experiments in which the non-preinduced bacteria, grown in the medium with acetate or lactate, were taken at the end of the exponential phase of growth, washed, and suspended in a phosphate buffer. The suspension of washed bacteria was then transferred to a flask and placed in a shaker. The cell suspension was temperature stabilized, phenylacetic acid was added and the enzyme activity was followed at time intervals. The enzyme activity did not increase above the basal level values, even after an 8 h incubation.

As no experimental data concerning kinetics of synthesis of the studied enzyme could be found in the literature and as the only demonstrated fact had been that the rate of the enzyme synthesis is not constant, being lower, the later phenylacetic acid is added to the medium, we followed the kinetics of induction of the enzyme synthesis. The acetate medium was chosen for this purpose. Non-preinduced cells grown in the same medium were employed here. The results are presented in Fig. 3.

It follows from the results presented in Fig. 3 that the induction lag of the enzyme synthesis in *Escherichia coli* equals roughly two generation times in the used medium and under the given experimental conditions.

The effect of various derivatives of phenylacetic acid and phenoxyacetic acid on the enzyme synthesis was further studied, with the aim of finding out whether these compounds, chemically and structurally related to phenylacetic acid, also induce the enzyme synthesis in *Escherichia coli*. These experiments were arranged as follows: (i) Non-preinduced cells of *Escherichia coli* (basal activity), grown in the same medium and taken from the exponential phase of growth, were used for the inoculation of the first subculture in the medium with acetate or lactate containing a given compound in the form of its sodium salt. (ii) Due to the length of the observed induction lag (Fig. 3), the bacteria grown in the first subculture were transferred to the second subculture, *i.e.* to the same medium containing the given compound.

TABLE I. Specificity of enzyme induction in exponentially growing cultures of *Escherichia coli* in a medium with acetate and the compound shown (0.1% v/v), and growth of the strain on these compounds as the only carbon sources.

Compounds and derivatives		Growth	μmol/h per mg dry wt.	Induction ratio[a]
Phenylacetic acid		+	14.0	280
Substitution in the benzene ring	2-OH	−	0.05	1
	4-OH	+	0.05	1
	4-NH_2	−	0.05	1
	4-NO_2	−	0.05	1
	3,4-OCH_3	−	0.05	1
Substitution at the α-carbon	−OH	−	0.05	1
	$-CH_3$	−	0.05	1
	$-CH_2CH_3$	−	0.05	1
	$-CH_2OH$	−	0.1	2.0
	$-CH_3$, −OH	−	0.05	1
Elongation of the side chain	Phenylpropionic acid	+	0.15	3.0
	Phenylacrylic acid	−	0.05	1
	Phenylbutyric acid	−	0.05	1
Phenoxyacetic acid		−	3.5	70
Its derivatives	2,4-Cl	−	0.05	1
	2,4-OCH_3	−	0.05	1
	4-NO_2	−	0.05	1
Basal activity			0.05	

[a] $\frac{\text{differential rate of enzyme synthesis with the compound shown}}{\text{differential rate of enzyme synthesis without the compound}}$

The enzyme activity during the exponential phase of growth was quantitatively evaluated only in these second subcultures. The ability of *Escherichia coli* to grow on these compounds as the only carbon and energy sources was determined at the same time. In cases, when the bacteria were able to metabolize a derivative of phenylacetic acid or other acid, the enzyme activity during growth of the cells in the second subculture, was also quantitatively determined. Specificity of the induction was expressed by the so-called induction ratio (I.R.). The results are summarized in Table I.

It follows from the results presented in Table I that none of the derivatives of phenylacetic or other acid exhibited such effect on the degree of induction of the enzyme synthesis in *Escherichia coli* as phenylacetic acid itself. Also 4-hydroxyphenylacetic acid and beta-phenylpropionic acid, the only derivatives chemically related to phenylacetic acid that were metabolized by cells of *Escherichia coli* as the only carbon and energy sources in the medium, did not influence the enzyme synthesis. It also follows from the results presented in Table I that substitution of the OH group in the para position on the benzene ring, or substitution of the OH or other group at the α-carbon, or extension of the side chain of phenylacetic acid by further carbon atoms, completely remove the induction effect. Only the insertion of oxygen between the benzene ring and side chain (phenoxyacetic acid) facilitates a significant effect on the enzyme induction. However, the effect is about 4 times lower as compared with that obtained when using phenylacetic acid. It was also of

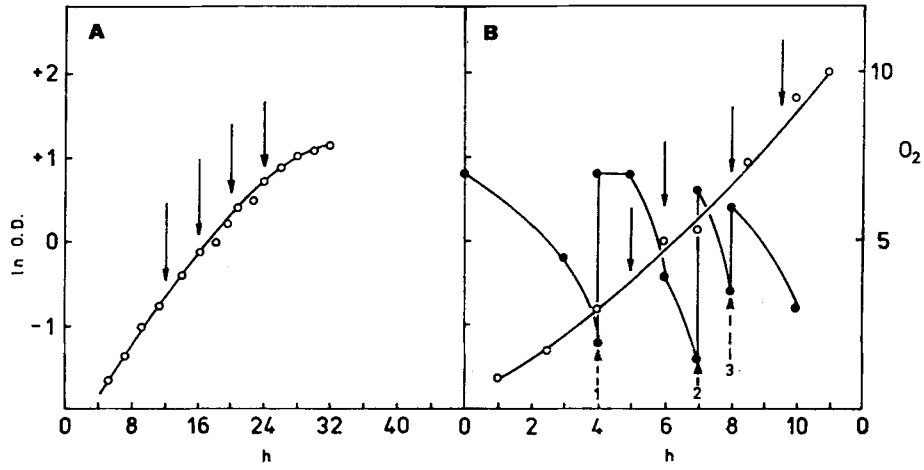

FIG. 4. Submerged cultivation of *Escherichia coli* in a medium with 2% sucrose and dosed phenylacetic acid in a 20-litre fermentor. A, Control Specific enzyme activity high (24.0 μmol/mg dry weight). Arrows indicate doses of 0.1% phenylacetic acid. Inoculation was with a 10% (v/v) inoculum grown in a medium with 2% corn-steep and phenylacetic acid. Cultivation conditions: 300 r.p.m. 1/2 volume of air/min, 4 baffles, overpressure 0.2 atm, content of medium 10 litre. B, Raised concentration of dissolved oxygen. Specific enzyme activity zero. Solid arrows indicate doses of 0.1% phenylacetic acid. Cultivation conditions: 1/2 volume of air/min, 4 baffles, content of medium 10 litre. Inoculation was with a 10% (v/v) inoculum grown in a medium with corn-steep and phenylacetic acid. 1, R.p.m. raised from 300 to 700; 2, overpressure increased from 0.2 to 0.8 atm; 3, overpressure increased from 0.8 to 1.0 atm. ○, ln O.D. of bacteria; ●, concentration of dissolved oxygen in μg O_2/ml.

interest to investigate whether some amides of the used acids are comparable or even better inducers than phenylacetic acid itself. The effect of phenylacetamide, phenoxyacetamide, D(−)mandelamide, L(+)mandelamide, 4-hydroxyphenylacetamide and beta-phenylpropionamide on the degree of induction of synthesis of the studied enzyme was also investigated. The above-mentioned amides, with the exception of phenylacetamide, also did not exhibit any effect on the enzyme induction. The induction effect of phenylacetamide was roughly 4—5 times lower than that of phenylacetic acid. The obtained value is probably an artefact as under conditions when phenylacetamide is present in the medium during growth of cells in two subcultures (see the previously described experimental arrangement), it is hydrolyzed due to the basal activity of the growing cells. Thus, phenylacetic acid, originating by its hydrolysis, is released to the medium, although at concentrations lower than fully saturating ones. It can hence be concluded that also the origin of the covalent CONH bond in the molecule of phenylacetic acid or its derivatives, completely removes the enzyme induction in *Escherichia coli*.

As during growth of *Escherichia coli* in all complex media the differential rate of the enzyme synthesis is not constant, the specific activity of the enzyme increasing during the growth, and as also in some chemically defined media this rate changes although only at the beginning of the exponential phase of growth, this phenomenon was considered as being due to suppression of the enzyme synthesis by oxygen.

To disprove or confirm the repressive effect of oxygen on the enzyme synthesis, 20 litre fermentors with an introduced oxygen electrode were used. By increasing r.p.m. and air pressure, the concentration of dissolved oxygen in the medium during the submerged cultivation of *Escherichia coli* varied around 7 μg O_2/ml, this value

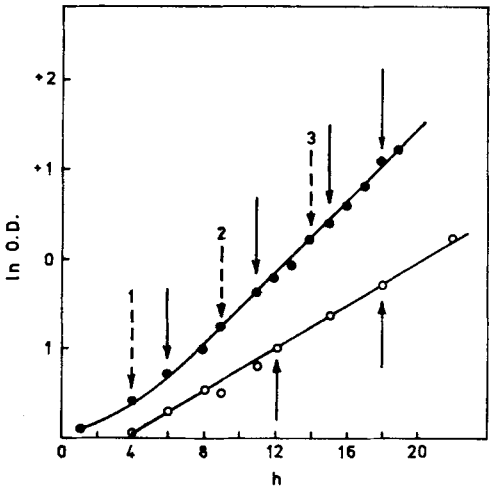

FIG. 5. Effect of a raised concentration of dissolved oxygen in the medium on growth and synthesis of the enzyme during a submerged cultivation of *Escherichia coli* in a medium with phenylacetic acid as the only carbon and energy source in a 20-litre fermentor; comparison with the control experiment. ○, specific enzyme activity high (35.0 μmol/mg dry weight); ●, specific enzyme activity zero. ○, ln O.D. of bacteria growing at a normal concentration of dissolved oxygen in the medium; ●, ln O.D. of bacteria growing at a raised concentration of oxygen in the medium; solid arrows indicate doses of 0.2% phenylacetic acid. Cultivation conditions: 1/2 volume of air/min, 4 baffles, content of medium 10 litre. Inoculation was with a 10% (v/v) inoculum grown in a medium with phenylacetic acid as the only carbon and energy source. In the control experiment (○) mixing was 300 r.p.m., overpressure 0.2 atm. 1, R.p.m. raised from 300 to 700; 2, overpressure increased from 0.2 to 0.8 atm.; 3, overpressure increased from 0.8 to 1.0 atm.

corresponding approximately to the concentration of dissolved oxygen immediately after the inoculation. Phenylacetic acid had to be added to the medium in doses, because it is toxic and strongly inhibits the growth of bacteria at concentrations above 0.2%. The results are presented in Figs. 4a, b and 5. It follows from the results presented in these figures that: (i) At the elevated concentration of dissolved oxygen in the medium the enzyme is not synthesized at all, even in the medium with sucrose and dosed phenylacetic acid and in the medium with phenylacetic acid as the only carbon and energy source, in spite of the fact that high specific enzyme activities were reached in both control experiments. This finding is surprising, especially in the case when the inducer, phenylacetic acid, serves as the only carbon and energy source, *i.e.* under conditions when the differential rate of the enzyme synthesis was the highest (Fig. 1 and 5). (ii) In the medium with sucrose, during growth of bacteria in the control experiment, the growth stopped after about 30 h of cultivation at a yield of 9.3 mg dry weight of cells/ml (Fig. 4a). At the increased concentration of dissolved oxygen in the medium a three-fold yield of dry weight of cells was reached already after 11 h of cultivation (Fig. 4b). After 18 hours of cultivation in the medium with phenylacetic acid as the only carbon source, about a 4-fold yield of dry weight of cells was obtained in the presence of a higher concentration of dissolved oxygen, as compared with the control experiment (Fig. 5).

Both described findings are interesting both from the theoretical and practical point of view and show clearly that the enzyme synthesis is suppressed by oxygen. Thus, a certain value of O_2 concentration exist, which is optimal for the production of the enzyme in *Escherichia coli*. In order to find this value, experiments were performed in such a way that the aeration effect was determined (by means of the sulphite method) in 500 ml flasks containing 40, 60, 100 and 200 ml of the medium on a rotary shaker. The optimal value of transfer of O_2 for the production of the enzyme was determined, using two types of media, *viz.* acetate and lactate with the inducer. However, in spite of the fact that a number of experiments were performed the results were not reproducible, the values were scattered and subject to considerable error, even with the use of chemically defined media, in which the differential rate of the enzyme synthesis is constant during a predominating phase of

the exponential growth under normal conditions of cultivation. The main problem was the quality of the stopper used to close the flasks. When the flasks were not stoppered at all, the enzyme was not synthesized in any of the differently filled flasks. Preparation of a stopper of standard type has remained unresolved so far.

Discussion

Szentirmai (1964) claimed that phenoxyacetic acid is as good an inducer of the enzyme synthesis in *Escherichia coli* as phenylacetic acid. The results obtained in the present work are not in agreement with the above-mentioned finding. It was found that phenoxyacetic acid also partially influences synthesis of this enzyme, but that the induction ratio with phenoxyacetic acid is 4 times lower as compared with that obtained when using phenylacetic acid. None of other 17 studied compounds, structurally and chemically related to phenoxyacetic acid, exhibited a significant effect on the degree of the induction of the enzyme synthesis under the given experimental conditions. This fact was also confirmed when using amides of corresponding derivatives of phenylacetic and phenylacetic acid. The interpretation of the results presented in the paper of Levitov and coworkers (1967) and concerning specificity of the induction of the enzyme synthesis in *Escherichia coli* by different N-substituted derivatives of phenylacetic acid or some other derivatives, is problematic for the reasons mentioned in the experimental part of this paper. It rather follows from the studies on specificity of the induction of the studied enzyme that a product of a regulatory gene facilitating derepression of this enzyme might be highly specific. Phenylacetic acid is thus the quantitatively most effective inducer of the synthesis of this enzyme in *Escherichia coli*. Levitov and coworkers also showed (1967) that the later phenylacetic acid added during growth of cells to the medium, the lower the degree of the induction of the enzyme synthesis by this compound. A relatively long induction lag, approximately two generations, can explain the quantitative differences obtained by the authors referred to above. In addition, it was not mentioned in the above paper whether preinduced cells of *Escherichia coli* were used for the inoculation. It was also not presented, from which phase of growth the cells were taken. In the paper referred to above a complex medium with corn-steep was used. In this medium the specific enzyme activity increases during growth of *Escherichia coli*, so that the results can only with difficulty be quantitatively compared. It follows from the results presented in Fig. 1 that the differential rate of the enzyme synthesis in *Escherichia coli* is typical under defined conditions and graded quantitatively for a given type of a chemically defined medium. The highest rate of its synthesis was reached in the medium in which phenylacetic acid served as the only carbon and energy source. This fact can be explained by two alternatives: (i) During growth of *Escherichia coli* on phenylacetic acid as the only carbon and energy source, the intracellular pool of the compound is high, thus completely saturating a product of a regulatory gene, resulting in a high rate of expression of a structural gene governing the studied enzyme. (ii) Phenylacetic acid is not as quantitatively effective an inducer as an intermediate "X" originating by its degradation. This intermediate would then be a more effective inducer, or, perhaps, the inducer proper of this enzyme.

It should be added to the latter alternative (ii) that data exist in the literature concerning the metabolic degradation of phenylacetic acid and aromatic compounds closely related to it (4-hydroxyphenylacetic acid and beta-phenylpropionic acid) (Blakley *et al.*, 1967; Blakley, 1967); however, these studies were performed with some strains of the genus *Pseudomonas*. Assuming that phenylacetic acid is metab-

olized analogously also in *Escherichia coli*, *i.e.* that the initial stage of its degradation is hydroxylation of the benzene ring, the latter alternative (ii) appears unlikely, especially due to the fact that 4-hydroxyphenylacetic acid cannot serve at all as inducer of the studied enzyme, while supporting as the only carbon source in the medium the growth of *Escherichia coli* (Table I). For the above-mentioned reasons it appears likely that the first alternative (i) is correct. At the same time, it can be assumed that the induction of the studied enzyme in *Escherichia coli* is of gratuitous character. This assumption is also confirmed by some further data (Vojtíšek and Slezák, 1975). It should be pointed out here that the gratuitous induction of the enzyme synthesis is fully suppressed by oxygen. The mechanism of repression of the gratuitous induction of the enzyme synthesis by oxygen was not further studied. Kleyner and Lopatnev (1972) also showed that synthesis of this enzyme is influenced by transfer of oxygen in a medium with 2% corn-steep and phenylacetic acid. However, the enzyme production was highly dependent on quality of the used stopper. The same problem occurred also in the present communication (see the experimental part). The effect of oxygen on growth of microorganisms and synthesis of different enzymes was reviewed by Wimpenny (1969). However, it would be premature to postulate any hypotheses without a further experimental study of the mechanism of repression of this enzyme by oxygen.

REFERENCES

Blakley E. R., Kurz W., Halvorson H., Simpson F. J.: The metabolism of phenylacetic acid by a *Pseudomonas*. *Canad. J. Microbiol.* **13**, 147 (1967).

Blakley E. R.: The metabolism of aromatic compounds with different side chains by a *Pseudomonas*. *Canad. J. Microbiol.* **13**, 761 (1967).

Kaufmann W., Bauer K.: The production of penicillin amidase by *Escherichia coli* ATCC 9637. *J. Gen. Microbiol.* **35** (Soc. Gen. Microbiol. Proc. IV), p. iv (1964).

Kleyner G. I., Lopatnev S. V.: Role of aeration in the formation of benzylpenicillin acylase in a submerged culture of *Escherichia coli*. (In Russian) *Prikl. Biokhim. Mikrobiol. USSR*, **VIII**, 559 (1972).

Levitov M. M., Klapovskaya K. I., Kleyner G. I.: Induced biosynthesis of *Escherichia coli* acylase. *Mikrobiologiya* **36**, 912 (1967).

Szentirmai A.: Production of penicillin acylase. *Appl. Microbiol.* **12**, 185 (1964).

Vojtíšek V., Slezák J.: Penicillinamidohydrolase in *Escherichia coli*. I. Substrate specificity. *Folia Microbiol.* **20**, 224 (1975),

Weatherburn M. W.: Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* **39**, 971 (1967).

Wimpenny J. W. T.: Oxygen and carbon dioxide as regulators of microbial growth and metabolism, p. 161, in *Microbial Growth*, 19th Symp. Soc. Gen. Microbiol., Cambridge. University Press 1969.