Induction and Repression of *Pseudomonas aeruginosa* Amidase

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SUMMARY

The synthesis of an inducible amidase by *Pseudomonas aeruginosa* 8602/A was studied in cultures growing exponentially in succinate medium. Induction by both the substrate inducer acetamide, and the non-substrate inducer *N*-acetylacetamide, was repressed by cyanoacetamide. Induction by 10^{-2} M-*N*-acetylacetamide was significantly repressed by 10^{-4} M-cyanoacetamide, but repression of induction by 10^{-3} M-acetamide required a tenfold excess of cyanoacetamide. Amidase synthesis in a medium in which acetamide was the sole carbon + nitrogen source was also repressed by cyanoacetamide, which under these conditions inhibited the growth of non-induced bacteria. Several tricarboxylic acid cycle intermediates, and related compounds, repressed amidase synthesis in exponentially growing organisms. Catabolite repression by propionate in succinate medium was decreased by increasing the concentration of acetamide. These findings are discussed in relation to general theories of regulation of microbial enzyme synthesis.

INTRODUCTION

Kelly & Clarke (1962) showed that *Pseudomonas aeruginosa* 8602/A produced an aliphatic amidase which hydrolysed acetamide and propionamide. They showed that the enzyme could be induced by either of these amides, and also by certain non-substrate amides, including *N*-methylacetamide and *N*-acetylacetamide. Amides were tested for inducer activity by measuring the amount of enzyme produced after overnight growth of the organism in a medium containing succinate and ammonium chloride, to which the test amide had been added. Under these conditions amidase induction by *N*-methylacetamide was repressed by the addition of cyanoacetamide or thioacetamide to the growth medium. Estimations of enzyme activity after overnight growth of cultures can give only limited information about the course of enzyme synthesis. We have followed amidase induction in cultures of *P. aeruginosa* 8602/A in the exponential phase of growth, with succinate as carbon source. This proved a convenient technique for comparing induction by substrate and non-substrate inducers. Under these conditions it was possible to investigate the nature of amidase repression by amide analogues such as cyanoacetamide, and also repression by general cell metabolites. A brief report of part of this work has appeared previously (Brammar & Clarke, 1963).

METHODS

Organism. The strain used was *Pseudomonas aeruginosa* 8602/A, described by Kelly & Clarke (1962). The culture was maintained on slopes of Lemco agar and subcultured weekly. Stock cultures were stored at 4°.

Growth. The cultures were grown in conical flasks, of capacity 10 times that of the medium volume and shaken vigorously at 37°. To obtain exponentially growing organisms 5 ml. of an overnight culture was used to inoculate 100 ml. of the test medium. Samples (4 ml.) were removed at intervals for determinations of amount of bacteria; they were then stored at 0° and enzyme activities determined at the end of the experiment. The inducers and other test compounds were added at suitable times after the culture had reached the exponential phase of growth, and the experiments were continued over 3–4 generations.

Media. Lemco agar was prepared as described by Clarke & Meadow (1959). The minimal salt medium contained (per litre): K_2HPO_4 , 12.5 g.; KH_2PO_4 , 3.8 g.; $(NH_4)_2SO_4$, 1.0 g.; $MgSO_4 \cdot 7H_2O$, 0.1 g.; trace element solution, 5 ml. (Kelly & Clarke, 1962).

Acetamide was sterilized by Seitz filtration and added aseptically to the minimal salt medium to a final concentration of 0.08 M. Most of the experiments with non-substrate inducers were done in minimal salt medium containing 0.05 M-succinate. A few experiments were made with other compounds as carbon source, and these were also added to a final concentration of 0.05 M.

Dry weight of bacteria. The optical extinctions of the cultures were measured at 670 m μ with a Unicam SP 600 spectrophotometer. A standard curve was used to convert the readings to dry weights.

Enzyme assays. The hydrolytic activity of the amidase was measured by Conway's microdiffusion technique (Conway, 1957) as used by Kelly & Clarke (1962). The transferase activity was measured by estimating the amount of acylhydroxamate formed from hydroxylamine and the amide substrate in a standard time. The transferase reaction became the routine method for the enzyme assay and was carried out as follows. Bacterial suspension (0.25 ml.) was added to 0.75 ml. of the substrate mixture, which was prepared by mixing equal volumes of 0.4 M-acetamide solution, 2.0 M-hydroxylamine hydrochloride freshly neutralized, and 0.1 M-tris buffer (pH 7.2). After incubation at 37° for 15 min. the reaction was stopped by adding 2 ml. of a solution of $FeCl_3$ (6%, w/v) in HCl (2%, w/v). The extinction was read at 500 m μ with a Unicam SP 600 spectrophotometer and related to the amount of acethydroxamate formed in the reaction by comparison with a standard curve. Under these conditions the enzyme activity was linear with time over at least 30 min. The substrate concentrations were such as to give complete saturation of the enzyme system. Disruption of the bacteria with acetone, toluene, ultrasonic treatment or with the French press did not increase the specific activity.

Enzyme units. The units of enzyme activity used in this paper are defined as follows. One unit of hydrolase activity corresponds to that amount of enzyme which catalyses the formation of 1 μ mole ammonia/min. under the conditions used for the hydrolase assay. One unit of transferase activity corresponds to that amount of enzyme which catalyses the formation of 1 μ mole acethydroxamate/min. under the conditions used for the transferase assay.

Amides. Acetamide was obtained from Hopkins & Williams Ltd. and purified by recrystallizing twice from ethanol. *N*-Acetylacetamide was prepared by refluxing acetamide with excess acetic anhydride, removing the residual acetic anhydride by distillation, and twice recrystallizing the product from methylethylketone. Cyanoacetamide was obtained from Hopkins & Williams Ltd. and purified by recrystallizing twice from methanol. The purity of these preparations was tested by melting-point determinations; occasional preparations were also examined by paper chromatography. *N*-Methylacetamide was obtained from Hopkins & Williams Ltd. and was not further purified.

Paper chromatography. Amides were analysed by ascending chromatography on Whatman No. 1 paper in toluene + ethanol (80 + 20, by vol.) solvent. Hydroxamates were produced by spraying with a saturated solution of hydroxylamine hydrochloride in methanol (80 %, v/v) in water and containing NaOH 3 % (w/v). After heating at 100° for 10 min. the papers were sprayed with a solution of FeCl₃ (10 %, w/v) + HCl (0.1 %, w/v) in aqueous methanol (95 %, v/v, methanol in water). The hydroxamates appeared as the red ferric complexes.

RESULTS

Comparison of hydrolase and transferase activities

During the earlier investigation of the *Pseudomonas aeruginosa* 8602/Δ amidase the enzyme activity was measured by estimating the ammonia produced under standard conditions, with propionamide as substrate (Kelly & Clarke, 1962). It had been shown that propionamide was hydrolysed more than twice as rapidly as acetamide, and propionamide was therefore chosen as the more sensitive substrate for enzyme assays. Some studies done with partially purified enzyme preparations suggested that the enzyme also had acyl transferase activity, and could transfer the acyl group of the substrate amides to hydroxylamine to form acylhydroxamates (Kelly, 1961). The transferase showed different substrate affinities, acetamide being more than twice as active as propionamide as a substrate in this reaction.

The two reactions can be represented as follows, with acetamide as the substrate in both reactions:

The transferase assay offered many advantages in sensitivity and speed but, before it could be adopted as a routine assay for the enzyme, it was necessary to show that both activities were due to a single enzyme protein. This was investigated in two ways. First, the relative transferase and hydrolase activities of cultures induced by either a substrate or by a non-substrate inducer, over a wide range of enzyme activities, were compared. Figure 1 shows the results obtained. Transferase activity with acetamide as substrate is plotted against hydrolase activity with propionamide as substrate. Each point represents the enzyme activity of a separate overnight culture. It can be seen that over a wide range of activity there is parallel induction of transferase and hydrolase activities. This result would be expected both for the induction of a single enzyme and also for the co-ordinate induction of two different enzymes whose synthesis was controlled by a single operon. This has been reported

for several systems, the best known being β -galactosidase and β -galactoside transacetylase (Jacob & Monod, 1961).

Secondly, we tried to separate the two activities by the usual methods for protein separation. After ammonium sulphate precipitation followed by chromatography on a column of diethylaminoethyl-cellulose, both activities came from the column with the same protein peak. When such purified preparations were subjected to starch gel electrophoresis, only one protein band was obtained, and both transferase and hydrolase activities were associated with it. Kelly & Kornberg (1962*b*, 1964) reported a constant ratio of propionamide hydrolase to propionamide trans-

Fig. 1

Fig. 2

Fig. 3

Fig. 1. Correlation between hydrolase and acyl transferase activities of induced cultures of *Pseudomonas aeruginosa* 8602/A.

Fig. 2. Amidase induction in *Pseudomonas aeruginosa* 8602/A by *N*-acetylacetamide in succinate medium. Inducer added at the time indicated by the arrow to exponentially growing cultures. ○—○, 10⁻²M; △—△, 3 × 10⁻³M; ●—●, 10⁻³M; □—□, 10⁻⁴M.

Fig. 3. Amidase induction in *Pseudomonas aeruginosa* 8602/A by 10⁻³M-acetamide in succinate medium.

ferase activities at several stages of enzyme purification. We therefore concluded that we were dealing with a single enzyme capable of catalysing both reactions. The term 'amidase' in this paper refers to an enzyme protein with both hydrolase and transferase activities. The transferase assay was used to measure amidase activity in all subsequent work.

Amidase induction by N-acetylacetamide

N-Acetylacetamide was chosen as the non-substrate inducer for studies of the kinetics of amidase induction under conditions of gratuity (Monod & Cohn, 1952). It could be readily prepared in a crystalline form and its purity checked. Succinate was used as the carbon source for growth. This was an arbitrary choice, but it was already known that the organism grew well under these conditions and that several non-substrate amides were effective inducers in this medium (Kelly & Clarke, 1962). Figure 2 shows the results obtained when four different concentrations of *N*-acetylacetamide were added to exponentially growing cultures. Total enzyme activity was plotted against dry weight of bacteria to give the differential rate of enzyme synthesis. Little induction occurred at 10⁻⁴M-*N*-acetylacetamide. As the concentra-

tion of inducer was increased the differential rate of enzyme synthesis increased. The highest concentration of inducer shown on the graph is 10^{-2}M , and there was no increase in the differential rate of amidase synthesis when the concentration was increased beyond this value.

It was assumed that the system was saturated at 10^{-2}M -*N*-acetylamine and this was used as the standard concentration for investigating the effects of repressors on amidase synthesis. At the saturating concentration there was a lag of about one generation before enzyme synthesis started. Amidase synthesis then continued at a constant differential rate during the experimental period. There was a similar lag of about one generation before enzyme synthesis started when *N*-methylacetamide was used as the inducer under these conditions. The saturating inducer concentration for *N*-methylacetamide was also approximately 10^{-2}M .

The long lag before enzyme synthesis started might be a function of the carbon source used. The induction kinetics when the organism was growing on other carbon compounds was then examined. 10^{-2}M -*N*-acetylacetamide was added to cultures growing exponentially in minimal media containing malate, citrate, pyruvate or glycerol as carbon source for growth. The mean generation times in these media varied from 42 min. with succinate or citrate to 220 min. with glycerol. In all cases a lag occurred after the inducer had been added and before amidase synthesis started. The shortest lag was 0.25 of a generation time with pyruvate as carbon source. With all the other compounds the lag was about one generation, as with succinate.

Amidase induction by acetamide in succinate medium

Acetamide was a very effective inducer in succinate medium. Amidase synthesis could be detected in response to the addition of as little as 10^{-5}M -acetamide, although this small amount was soon metabolized. Figure 3 shows the results obtained with 10^{-3}M -acetamide as inducer. There was again a lag of about a generation, followed by a period of constant differential rate of amidase synthesis. This rate was not maintained during the experimental period, and it can be seen from Fig. 3 that it rapidly decreased. The change of rate corresponded to the time at which the acetamide would have almost disappeared from the medium.

In these experiments we were attempting to compare substrate and non-substrate inducers under conditions as similar as possible. In succinate medium, although acetamidase is not necessary for the growth of the culture, enzyme synthesis is not strictly gratuitous, since the acetamide will be hydrolysed and metabolized as soon as some amidase has been synthesized. It is therefore not possible to keep the acetamide concentration constant over a long period of time. It was concluded that if measurements were made during the early stages of enzyme induction it would be possible to compare substrate and non-substrate inducers by following amidase synthesis in succinate medium.

Repression of amidase synthesis by amide analogues

Kelly & Clarke (1962) showed that cyanoacetamide and thioacetamide repressed amidase induction by *N*-methylacetamide during overnight growth of *Pseudomonas aeruginosa*. These amide analogues also repressed amidase synthesis in exponentially growing cultures; Fig. 4 gives the results of a typical experiment. 10^{-2}M -*N*-acetylacetamide was added to a culture growing exponentially in succinate medium.

Soon after enzyme synthesis had started, the repressor amides were added to the same final concentrations as the inducer. Both cyanoacetamide and thioacetamide repressed amidase induction, and the rate of enzyme synthesis decreased almost at once; there was no effect on the growth rate. Figure 5 shows that cyanoacetamide produced a considerable repression of amidase induction by *N*-acetylacetamide even when it was present at only one hundredth of the concentration of the inducer.

Amidase induction by acetamide was also repressed by cyanoacetamide. In this case addition of cyanoacetamide to an exponentially growing culture at the same initial concentration as the inducer had no effect on the rate of amidase synthesis. It was necessary to increase the ratio of cyanoacetamide to acetamide at least tenfold to obtain significant repression. Figure 6 gives the results of an experiment in which the initial concentration of the inducer acetamide was 10^{-3} M and that of the repressor cyanoacetamide was 10^{-2} M. It can be seen that there was a marked repression of enzyme synthesis under these conditions.

Fig. 4

Fig. 5

Fig. 6

Fig. 4. Effect of cyanoacetamide and thioacetamide on amidase induction in *Pseudomonas aeruginosa* 8602/A. 10^{-2} M-*N*-acetylacetamide added to an exponentially growing culture at time indicated by the first arrow. At the time indicated by the second arrow, the culture was split into three parts, and the following additions made: ○—○, no addition; △—△, 10^{-2} M-cyanoacetamide; □—□, 10^{-2} M-thioacetamide.

Fig. 5. Cyanoacetamide repression of amidase induction in *Pseudomonas aeruginosa* 8602/A by *N*-acetylacetamide. At the time indicated by the first arrow, 10^{-2} M-*N*-acetylacetamide was added to an exponentially growing culture. At the time indicated by the second arrow, the culture was divided into 3 parts, and the following additions made: ○—○, no addition; △—△, 10^{-4} M-cyanoacetamide; □—□, 10^{-2} M-cyanoacetamide.

Fig. 6. Cyanoacetamide repression of amidase induction in *Pseudomonas aeruginosa* 8602/A by acetamide. At the time indicated by the first arrow 10^{-3} M-acetamide was added as inducer. At the time indicated by the second arrow, the culture was split into two parts: ○—○, control culture; △—△, 10^{-2} M-cyanoacetamide added.

These experiments showed that cyanoacetamide repressed amidase synthesis in a similar way, whether acetamide or *N*-acetylacetamide was used as inducer. Because of the structural similarity between these amides, mutual competition for an amide-binding site seemed possible. If this were the case, it should be possible to annul the repression by using a sufficient concentration of inducer analogue. The

results of an experiment to test this possibility are shown in Fig. 7. An exponentially growing culture was divided into three parts, to which *N*-acetylacetamide was added to give final concentrations of 10^{-3} M, 5×10^{-3} M and 2×10^{-2} M. At the points marked on Fig. 7, cyanoacetamide was added to a final concentration of 1.2×10^{-4} M in each case. The results (Fig. 7) showed that cyanoacetamide repression of induction was prevented by an approximately 200-fold excess of *N*-acetylacetamide.

The results shown in Figs. 5, 6 and 7 suggest that cyanoacetamide repressed amidase synthesis by competing with the inducer, acetamide or *N*-acetylacetamide for an inducer-recognizing site. The affinities of the amides for such a site would appear to be in the order acetamide > cyanoacetamide > *N*-acetylacetamide.

Fig. 7

Fig. 8

Fig. 7. Effect of concentration of *N*-acetylacetamide on repression of amidase synthesis in *Pseudomonas aeruginosa* 8602/A by cyanoacetamide. *N*-Acetylacetamide was added to 3 flasks early in exponential growth: ○—○, 10^{-3} M; △—△, 5×10^{-3} M; □—□, 2×10^{-2} M; at the times indicated by the arrows, 1.2×10^{-4} M-cyanoacetamide was added to each flask.

Fig. 8. Catabolic repression of amidase induction in *Pseudomonas aeruginosa* 8602/A. 10^{-3} M-*N*-acetylacetamide was added to an exponentially growing culture in succinate medium. At the time indicated by the second arrow, the culture was split into three parts, and the following additions made: ○—○, control culture, no additions; △—△, 10^{-3} M-acetate; □—□, 10^{-3} M-propionate.

Catabolite repression of amidase synthesis

The synthesis of many induced enzymes has been shown to be repressed by glucose or other cell metabolites. This repression was known for a long time as 'the glucose effect', but is more correctly described as catabolite repression (Magasanik, 1961). In a few cases it has been shown that the repression is specific, and that the catabolite repressor is a common intermediate in the metabolic pathways of both glucose and the normal substrate of the induced enzyme (McFall & Mandelstam, 1963). We

examined the effect of various cell metabolites on amidase induction, using either acetamide or *N*-acetylacetamide to induce enzyme synthesis.

With cultures of *Pseudomonas aeruginosa* 8602/Δ growing exponentially in succinate medium, and induced with 10^{-2} M-*N*-acetylacetamide, there was marked repression of amidase synthesis by tricarboxylic acid cycle intermediates and metabolically related compounds. Figure 8 shows that the most effective repressor under these conditions was propionate, which at 10^{-2} M caused 70 % repression of amidase synthesis. The same concentration of citrate, pyruvate or malate resulted in 60 % repression, and acetate was slightly less effective. These effects were not transient, but persisted through the experimental period, even though the concentration of the repressor compounds would be decreased by cell metabolism. Enzyme induction by acetamide was also repressed by adding these compounds shortly after amidase synthesis had been induced.

We attempted to annul catabolite repression of amidase synthesis by increasing the concentration of substrate and non-substrate inducers. With *N*-acetylacetamide we were unable to find any effect. There were technical difficulties in carrying out this experiment, since the saturating concentration of *N*-acetylacetamide required for induction was so high. Propionate was metabolized rapidly under these conditions, and concentrations lower than 10^{-2} M could not be used. When the propionate concentration was kept constant at about 10^{-2} M and the inducer concentration increased, it would have been necessary to take the *N*-acetylacetamide concentration to unreasonably high values to reach a significant excess. It was thought that acetamide might be a more suitable inducer for this purpose, since the results with amide-analogue repression had indicated that it had a much higher affinity for an inducer-binding site than had *N*-acetylacetamide. Cultures growing exponentially in succinate medium were induced with various concentrations of acetamide. When a constant differential rate of amidase synthesis had been reached, propionate was added to some of the flasks. Table 1 summarizes the results obtained. Amidase induction by 10^{-3} M and 3×10^{-3} M-acetamide was more than 90 % repressed by 3×10^{-2} M-propionate; with 10^{-2} M-acetamide as inducer, repression by this concentration of propionate was only 65 %. Under these conditions there would be a high concentration of metabolites in the cell, and complete de-repression by additional inducer would be unlikely. Preliminary experiments with carbon-starved bacteria showed that catabolite repression was annulled by increasing the inducer concentration.

Amidase synthesis required for growth of the culture

In all the previous experiments, amidase induction by acetamide was studied under conditions in which the enzyme was not necessary for the growth of *Pseudomonas aeruginosa*, since the medium already contained amounts of carbon and nitrogen compounds adequate to maintain growth. We also examined the kinetics of amidase synthesis under conditions in which the organism could grow only if it could synthesize this enzyme.

A culture of *Pseudomonas aeruginosa* 8602/Δ grown overnight in succinate medium has a very low basal amidase activity. When succinate-grown organisms are inoculated into a medium containing acetamide as the sole carbon and nitrogen source, there is an appreciable growth lag. During this time, amidase synthesis occurs and some of the acetamide is hydrolysed. When the amidase content of the organisms

has reached a significant value, the culture begins to grow and, soon after multiplication has become established, the acetamide originally added is completely hydrolysed. The amidase is then no longer synthesized and becomes diluted out, and the culture continues to grow on the acetate and ammonia.

Table 1. *Effect of acetamide concentration on catabolite repression of amidase synthesis by Pseudomonas aeruginosa 8602/A*

Acetamide concentration (mM)	Propionate concentration (mM)	Differential rate of amidase synthesis (units/ml./mg. dry wt.)	Repression (%)
1	0	17.5	—
1	30	1.2	93
3	0	17.5	—
3	30	1.5	91
10	0	17.0	—
10	30	5.8	65

The pattern of amidase synthesis in acetamide medium is, however, more complex than this, since at the end of the exponential growth phase, and long after acetamide has disappeared from the growth medium, there is a second stage of amidase synthesis. We have observed this second phase of amidase synthesis, at the same growth stage, with cultures growing with acetate as carbon source, but with no other carbon compounds. Figure 9 shows the course of amidase synthesis, growth and acetamide disappearance. Kelly & Kornberg (1962*a*) reported this phenomenon, and were able to show cyanoacetamide repression of the second phase, but not of the first phase of amidase synthesis. They showed that the enzymes produced at the two growth phases had identical properties (Kelly & Kornberg, 1964).

It had become clear from our results on amidase induction by acetamide in succinate medium that the relative concentrations of the inducer and repressor were extremely important in determining whether or not enzyme synthesis would take place. We therefore tried to repress the first phase of induction by using a higher concentration of cyanoacetamide. Figure 10 shows that amidase synthesis was completely repressed when 0.5 M-cyanoacetamide was added to a medium which contained 0.02 M-acetamide as carbon source. The concentration of cyanoacetamide required to repress the first phase of amidase induction in acetamide medium was very high. It therefore seemed possible that this was a non-specific effect, and that cyanoacetamide was a general inhibitor of cell metabolism. This possibility was examined by comparing the behaviour of pre-induced and non-induced bacteria. *Pseudomonas aeruginosa* 8602/A was grown overnight in acetamide medium, to give pre-induced bacteria, and in succinate medium to give non-induced bacteria. The pre-induced bacteria were inoculated into medium containing 0.02 M-acetamide + 0.5 M-cyanoacetamide. The bacteria already contained enough amidase to hydrolyse the acetamide in the medium and, after a short lag, normal growth occurred.

When non-induced bacteria were inoculated into this medium, growth was completely inhibited. In the absence of cyanoacetamide, the bacteria grew normally after a short lag, during which time amidase was synthesized. From these results (see Fig. 11) it is clear that cyanoacetamide is not a general growth inhibitor, even at

0.5 M, and that the inhibition of growth of the non-induced bacteria in acetamide medium was due to specific repression of amidase synthesis.

Fig. 9

Fig. 10

Fig. 11

Fig. 9. Growth and amidase synthesis of *Pseudomonas aeruginosa* 8602/A in acetamide medium. Succinate-grown organisms were inoculated into a medium containing 0.08 M-acetamide. ●—●, Growth as dry wt. bacteria/ml.; ○—○, amidase as total enzyme units/ml.; △—△, acetamide concentration.

Fig. 10. Effect of cyanoacetamide on amidase synthesis in *Pseudomonas aeruginosa* 8602/A in acetamide medium. Succinate-grown organisms were inoculated into acetamide medium: △—△, control culture 0.02 M-acetamide; ○—○, 0.02 M-acetamide + 0.5 M-cyanoacetamide.

Fig. 11. Effect of cyanoacetamide on growth of pre-induced and non-induced *Pseudomonas aeruginosa* 8602/A in acetamide medium: ○—○, pre-induced organisms + 0.02 M-acetamide + 0.5 M-cyanoacetamide; △—△, non-induced organisms + 0.02 M-acetamide; □—□, non-induced organisms + 0.02 M-acetamide + 0.5 M-cyanoacetamide.

DISCUSSION

The results obtained in this study of amidase synthesis by *Pseudomonas aeruginosa* 8602/A confirm the view that enzyme induction by substrate and non-substrate inducers follows essentially the same pattern. Acetamide and *N*-acetylacetamide induced amidase synthesis in cultures growing exponentially in succinate medium, and this induction by both amides was repressed by cyanoacetamide. The main difference between the two inducers is that acetamide is an effective inducer at a much lower concentration than is *N*-acetylacetamide. Induction of amidase by acetamide is repressed by a tenfold excess of cyanoacetamide, whereas induction by *N*-acetylacetamide is repressed by one-hundredth of its concentration of cyanoacetamide. Amidase synthesis in acetamide medium was also repressed by cyanoacetamide at high concentrations. Kelly & Kornberg (1962a) found that the initial phase of amidase synthesis in medium containing 5×10^{-2} M-acetamide was not affected by 5×10^{-3} M-cyanoacetamide, and we have found that it was necessary to increase the concentration to at least 20-fold excess of cyanoacetamide to show any repression. These results with acetamide and the two amide analogues suggest that they have different affinities for the same site, and that repression by cyanoacetamide is due to competition with the inducers for a site concerned with the binding of inducer and the initiation of enzyme synthesis.

We are unable to define the site at which cyanoacetamide competes with the amide inducers from these experiments, but there are at least two possibilities

which have been suggested from the results of work with other systems. Experiments on β -galactosidase synthesis in *Escherichia coli* (Cohn & Monod, 1953) and *Staphylococcus aureus* (McClatchy & Rosenblum, 1963) have shown competition between inducer-analogues and inducers. Similar competition has been shown for the induction of the galactose enzymes of *E. coli*, where methyl- β -D-thiogalactoside represses the induction of the galactose group of enzymes by competing with the non-substrate inducer fucose (Buttin, 1963).

One possible site of competition would be a specific permease. This appears to be the case in the *Escherichia coli* β -galactosidase system, when arylthiogalactosides are added to cultures induced by methyl- β -D-thiogalactoside. We think it unlikely that cyanoacetamide represses induction of the amidase of *Pseudomonas aeruginosa* 8602/A by competing with an amide permease. Cyanoacetamide has no effect on the growth of pre-induced organisms in acetamide medium, and preliminary experiments have shown that it has no effect on the entry of radioactively labelled *N*-acetylacetamide.

Current theories of enzyme induction suggest that the inducer combines with a cytoplasmic repressor molecule, thereby releasing the structural genes from repression and allowing enzyme synthesis to occur (Jacob & Monod, 1961). Another possible site of competition of inducer analogues with inducers would be the inducer-binding site of the cytoplasmic repressor. Buttin (1963) found that the thiogalactosides had no effect on galactose permease, and concluded that the site of competition was within the cell. It would appear that amide analogue competition with amidase inducers also occurs at a site within the cell, which may be the cytoplasmic repressor.

The catabolite repressor could act at the site of entry of the inducer into the cell. Kessler & Rickenberg (1963) suggested that several metabolites may compete in this way with α -glucosides. Any effect of this sort would be overcome by increasing the inducer concentration. Alternatively, the catabolite repressor could act at a site on the pathway between the structural gene and the completed enzyme protein. We are rather surprised that amidase synthesis in *Pseudomonas aeruginosa* 8602/A can be so readily induced in a medium containing succinate, which would be expected to be a good precursor of catabolite repressors. With both substrate and non-substrate inducers there is a lag of about a generation before amidase synthesis can be detected. It seems possible that this amidase is normally under repression involving a metabolite closely related to the tricarboxylic acid cycle intermediates. Part of the explanation for the second-phase amidase induction in acetate medium may be that the culture is being released from catabolite repression. This cannot be the whole story, however, since the second-phase enzyme synthesis does not occur with any other carbon source we have tested.

In discussions of the regulation of enzyme synthesis, the process of enzyme induction is usually described as being diametrically opposed to that of end-product repression. Jacob & Monod (1961) postulated that, in an inducible system such as β -galactosidase, the regulator gene produces a cytoplasmic repressor molecule which prevents gene transcription until it is removed from the operator by combination with the low molecular weight inducer. In a typically repressible system, such as the enzymes of the histidine biosynthetic pathway, they postulated that the regulator gene produces an inactive cytoplasmic repressor, which requires to be combined with the low molecular weight co-repressor before it can combine with the

operator and prevent gene transcription. McFall & Mandelstam (1963) have demonstrated the specificity of catabolite repression. Gorini (1963) has shown that some of the enzymes of the arginine biosynthetic pathway are induced by glutamic acid and repressed by arginine. We think it likely that the synthesis of amidase is controlled by induction by amide inducers, and repression by certain compounds in the metabolic pool. We would predict that all catabolite repression would be overcome by inducer, if the inducer concentration could be raised to a sufficiently high value. This does not require a particular model for inducer and metabolite repressor sites of action, but, not wishing to multiply regulatory sites unnecessarily, we see no reason why they should not both act at the level of the cytoplasmic repressor. Recent work by Nakada & Magasanik (1964) and Attardi, Naono, Rouvière, Jacob & Gros (1963) supports the view that catabolite repression of β -galactosidase synthesis acts at the same stage as inducer, that is at the level of gene transcription to produce the specific messenger RNA. Loomis & Magasanik (1964) conclude that the catabolite in glucose repression of β -galactosidase does not react with the cytoplasmic repressor produced by the *i* gene, but that another regulator molecule is involved.

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