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Fate of Inducer during Induction of Aryl Hydrocarbon Hydroxylase Activity in Mammalian Cell Culture

II. Levels of Intracellular Polycyclic Hydrocarbon during Enzyme Induction and Decay

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SUMMARY

The induction and degradation of aryl hydrocarbon hydroxylase activity in hamster fetal cell cultures was studied in the presence and absence of actinomycin D and/or cycloheximide; the concentration of polycyclic hydrocarbon in these cells was examined concomitantly. The level of intracellular polycyclic hydrocarbon diminishes after about 30 min of exposure of the cells to the inducer benz[*a*]anthracene. This decrease is presumably related to the appearance of the induced microsomal oxygenase.

We have estimated the minimal number of molecules of benz[*a*]anthracene per cell that are sufficient to stimulate the initial rate of hydroxylase induction maximally. In cells exposed to inducer plus cycloheximide and then grown in fresh control medium, no correlation exists between the amount of intracellular polycyclic hydrocarbon physically or covalently bound and the kinetics of hydroxylase induction. Exposure of the cells to benz[*a*]anthracene for 20 min or less does not stimulate hydroxylase activity if RNA synthesis is prevented thereafter. Inhibition of RNA synthesis at any time after 30 min of exposure of the cells to inducer does not prevent the initial, maximal rate of microsomal oxidase induction. The amount of intracellular polycyclic hydrocarbon that is sufficient to stimulate hydroxylase activity maximally in cells during the first several hours of exposure to inducer does not stimulate enzyme activity in cells exposed to benz[*a*]anthracene for 12 hr or more.

In cells previously treated with benz[*a*]anthracene and then grown in fresh control medium, actinomycin D produces a stimulation of hydroxylase activity for 7–10 hr. There is no correlation between the concentration of intracellular polycyclic hydrocarbon and this stimulatory effect. The rate of disappearance of induced hydroxylase activity is not affected by the concentration of intracellular polycyclic hydrocarbon in the presence of cycloheximide or actinomycin D plus cycloheximide.

INTRODUCTION

In the preceding paper (1), we examined the fate of the inducer benz[*a*]anthracene during aryl hydrocarbon hydroxylase[1] induction in hamster fetal cells grown in culture. The entrance rate of benz[*a*]anthracene into

[1] This enzyme system is also called *benzpyrene hydroxylase* and *aryl hydroxylase*. The nomenclature *aryl hydrocarbon hydroxylase* is preferred, since the enzyme from rat liver microsomes, or from hamster fetal cells grown in culture, converts a variety of polycyclic hydrocarbons to phenolic derivatives and is not specific for benzo[*a*]pyrene.

the cell, the amount of physical and covalent binding, and the rate of intracellular metabolism of inducer have now been determined. With this information (1), we estimate in this paper the minimal concentration of intracellular polycyclic hydrocarbon required for the initial maximal rate of enzyme induction. Also, we compare this estimated minimal requirement of intracellular inducer with the concentration of polycyclic hydrocarbon found in the cell during various experiments on hydroxylase induction and degradation in the presence and absence of inhibitors of RNA and protein synthesis.

MATERIALS AND METHODS

The chemicals and cell culture materials were obtained and prepared as described previously (1, 2). Generally labeled ^{3}H-uridine (4.25 Ci/mole) was purchased from Nuclear-Chicago. Actinomycin D and cycloheximide were generous gifts from the Cancer Chemotherapy National Service Center, National Institutes of Health. The preparation of cell cultures and cell samples, the determination of aryl hydrocarbon hydroxylase specific activity, and the measurements of polycyclic hydrocarbon bound physically and covalently to cellular material were carried out as described previously (1–3). The determinations of ^{3}H-uridine and ^{14}C-protein hydrolysate incorporation into perchloric acid-precipitable and trichloracetic acid-precipitable cellular material were used as parameters of RNA and protein synthesis, respectively, as previously described (4).

RESULTS

Intracellular concentration of polycyclic hydrocarbon required to produce a maximal initial rate of hydroxylase induction. We have found (5) that the initial rate of aryl hydrocarbon hydroxylase induction is the same over a 20-fold concentration range of BA[2] in the medium (0.65–13 μM). The fact that a maximal rate of enzyme induction can be obtained indicates that the process of hydroxylase induction involves a rate-limiting step, which may be the saturation of "inducer-binding" sites in the cell, or a metabolic process such as transcription or translation. Presumably, at some concentration of BA in the medium below 0.65 μM, the initial rate of enzyme induction would be less than maximal. Therefore, in the experiment depicted in Fig. 1, it is possible to estimate the minimal amount of intracellular polycyclic hydrocarbon necessary to produce the maximal initial rate of stimulation of microsomal oxygenase activity. We define the initial rate of hydroxylase induction as the rate of increase in enzyme activity between 30 and 60 min of exposure of the cells to BA.

Figure 1A shows that BA in the medium at concentrations of 0.43 μM or higher stimulated aryl hydrocarbon hydroxylase activity at the maximal rate for the first 3 hr, whereas 0.043 μM BA produced an initial rate of enzyme induction that was less than maximal. Thus, a starting concentration of BA in the growth medium that is 10 times greater than 0.043 μM BA approximately doubled the initial rate of hydroxylase induction.

Figure 1B shows the kinetics of entry of inducer and the maximal level of intracellular polycyclic hydrocarbon during the experiment illustrated in Fig. 1A. With each of the three concentrations of BA in the medium, the highest intracellular concentration of polycyclic hydrocarbon was reached in 30 min, and a substantial decrease was seen after this time. This decrease after about 30 min may be caused directly or indirectly by the appearance of the induced hydroxylase. In cells exposed to BA, there is about a 35-min lag period until the induced enzyme system appears (4). In the 3½-hr period between 30 min and 4 hr, the intracellular polycyclic hydrocarbon diminished approximately 0.6×10^{-16}, 0.6×10^{-16}, and 0.04×10^{-16} mole/cell when the cells had been exposed to 13 μM, 0.43 μM, and 0.043 μM BA, respectively, in the growth medium. Between 30 min and 4 hr in cells exposed to either 13 μM or 0.43 μM BA in the medium,

However, the substrate specificity of either the constitutive or the induced hydroxylase system from the various mammalian tissues has not been determined. For example, endogenous substrates such as steroids may be hydroxylated by this same enzyme system.

[2] The abbreviation used is: BA, benz[*a*]anthracene (1,2-benzanthracene), as recommended by the American Chemical Society (6).

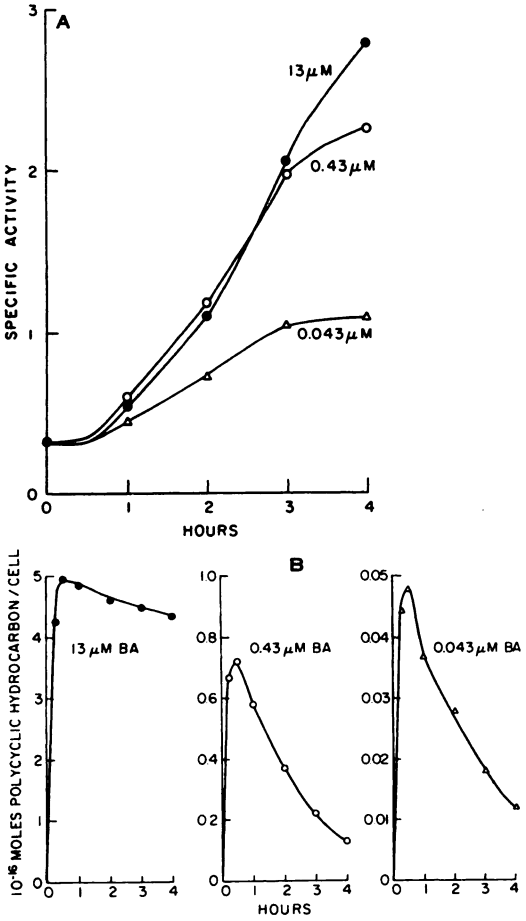

FIG. 1. *Concentration of BA in growth medium required to produce maximal initial rate of aryl hydrocarbon hydroxylase induction in hamster fetal cell culture.*

A. Kinetics of enzyme induction in response to different levels of BA. In all figures depicting enzymatic activity, the ordinate is expressed in specific activity, units per milligram of cellular protein. Each point represents duplicate determinations of both enzyme activity and protein content on a cellular homogenate. B. Intracellular content of polycyclic hydrocarbon during the 4-hr period as a function of the BA concentration in the growth medium. At 30 min, the extracellular to intracellular ratios of polycyclic hydrocarbon were 25, 5.2, and 2.3 in the experiments in which the starting levels of BA in the growth medium had been 13 μM, 0.43 μM, and 0.043 μM, respectively.

there are identical decreases in the intracellular polycyclic hydrocarbon concentration and similar rates of increase in the induced hydroxylase activity. In cells exposed to 0.043 μM BA, where the enzyme induction process was less than maximal, however, the decrease in intracellular polycyclic hydrocarbon levels between 30 min and 4 hr was 1/15 of that observed for cells treated with either 13 μM or 0.43 μM BA.

Therefore, an intracellular polycyclic hydrocarbon concentration of about 0.7×10^{-16} mole/cell during the first 30 min is capable of stimulating the microsomal enzyme activity maximally, while a level of less than 0.1×10^{-16} mole/cell is not adequate. These data thus indicate that, during the first 30 min of exposure, between 0.6×10^7 and 4×10^7 molecules of polycyclic hydrocarbon per cell are required to produce the maximal rate of hydroxylase induction in hamster fetal cell culture. This observation of such a large number of molecules per cell is in agreement with our recent findings that a large amount of polycyclic hydrocarbon is nonspecifically bound to cellular macromolecules.[3] In the subsequent figures and tables, we compare this estimated minimal requirement of intracellular inducer with the intracellular polycyclic hydrocarbon observed during the various phases of enzyme induction and degradation, in the presence and absence of RNA or protein synthesis.

Intracellular content of polycyclic hydrocarbon and hydroxylase induction in the presence and absence of cycloheximide. Figure 2A illustrates aryl hydrocarbon hydroxylase activity in cells grown in control medium alone, after a previous 12-hr exposure to BA, or to BA plus cycloheximide. In the presence of inducer but with a block in protein synthesis, there is no rise in hydroxylase activity. During the 10-hr period following the replacement of the medium containing BA or BA plus cycloheximide with fresh control medium, however, enzyme induction occurs in those cells previously treated with inducer plus cycloheximide; the hydroxylase activity declines logarithmically, after about a 2-hr lag period, in those cells previously exposed to BA only. These observations are consistent with our previous report (5), in which

[3] D. W. Nebert and L. L. Bausserman, in preparation.

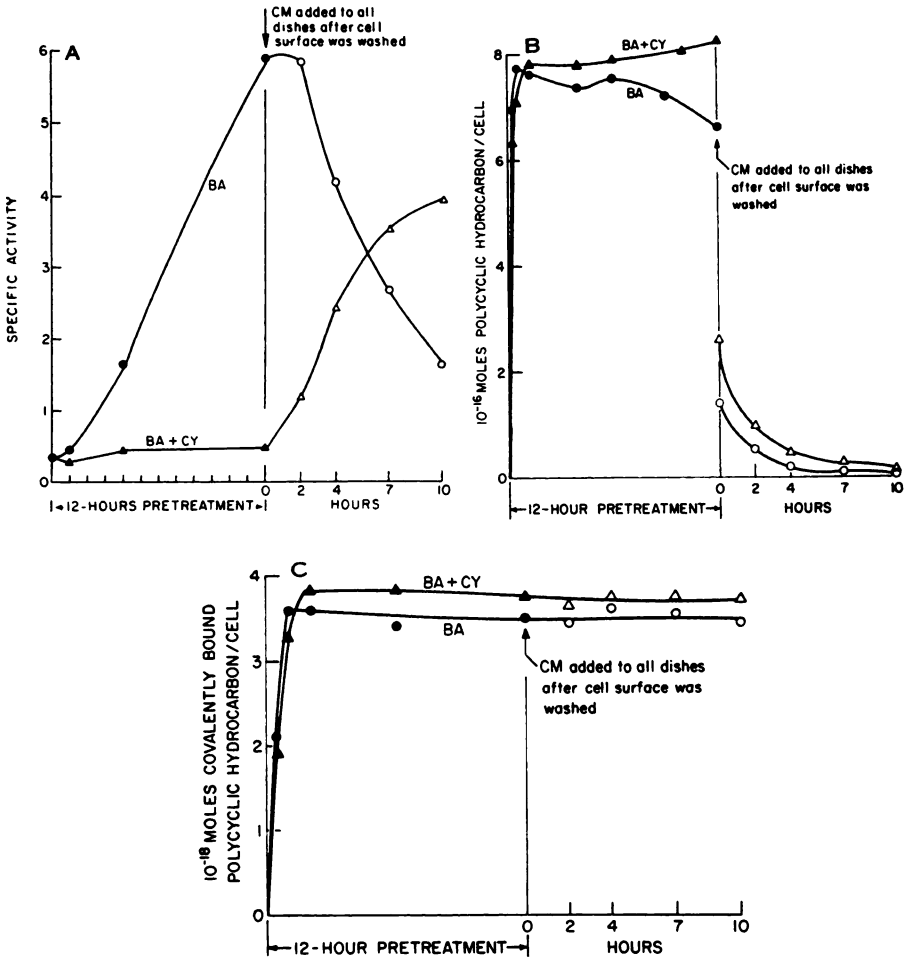

FIG. 2. *Aryl hydrocarbon hydroxylase activity (A), total intracellular polycyclic hydrocarbon concentration (B), and amount of polycyclic hydrocarbon bound to trichloracetic acid-precipitable cellular material (C) in cells grown in control medium alone (CM) following previous treatment with either 13 μM inducer (BA) or 13 μM inducer plus 3.5 μM cycloheximide (BA + CY)*

we suggested that there may be an accumulation of an induction-specific RNA species during the inhibition of protein synthesis by cycloheximide.

Figure 2B shows that the total polycyclic hydrocarbon taken up by cells exposed to inducer alone reached a maximum at 30 min and declined thereafter, from about 7.7 × 10^{-16} mole/cell at 30 min to about 6.6 × 10^{-16} mole/cell after 12 hr of treatment with BA. Similar decreases in intracellular polycyclic hydrocarbon were seen in each of more than 20 experiments. The presence of cycloheximide did not significantly alter the initial rate of entry of inducer into the cell, although the equilibrium between intracellular and extracellular polycyclic hydrocarbon was not reached until about 60 min, and remained relatively unchanged, or increased slightly, between 1 hr and 12 hr of exposure of the cells to BA plus cycloheximide. This latter curve is identical with that seen with hepatoma tissue culture cells,[4] which contain no constitutive or inducible aryl hydrocarbon hydroxylase activity. Also, we have found in another cell line that the lag time before increases in the hydroxylase activity

[4] D. W. Nebert and E. B. Thompson, unpublished data.

are found is about 60 min, and the maximal level of polycyclic hydrocarbon in these cells is always reached at about 60 min and declines thereafter.[3] Thus, the results of Fig. 2 further support the concept that the decline in intracellular polycyclic hydrocarbon concentration after 30 min in hamster fetal cells is caused by the appearance of the induced microsomal hydroxylase system.

After replacement of the medium containing inducer or BA plus cycloheximide with fresh control medium, more than two-thirds of the intracellular polycylic hydrocarbon present after the 12-hr initial treatment period was instantly removed. In those cells previously treated with BA alone for 12 hr, the intracellular level of polycyclic hydrocarbon was about 1.3×10^{-16} mole/cell after the cells had been washed, and about 0.5×10^{-16} mole/cell after the cells had been grown in control medium for 2 hr. This latter concentration is of the same magnitude as that which we estimated from the data in Fig. 1 to be necessary to stimulate hydroxylase activity at a maximal rate. Thus, our data of Fig. 2B are consistent with the fact that the induced enzyme activity decreases after about a 2-hr lag period. In those cells previously treated with BA plus cycloheximide for 12 hr, the intracellular level of polycyclic hydrocarbon was about 2.6×10^{-16} mole/cell after the cells had been washed, and about 0.4×10^{-16} mole/cell following growth of the cells in control medium for 4 hr; however, the hydroxylase activity in these cells continued to increase for about 8–10 hr in the control medium. Therefore, in cells previously exposed to BA plus cycloheximide for 12 hr, there is no correlation between the total amount of intracellular polycyclic hydrocarbon and the processes of induction and degradation of aryl hydrocarbon hydroxylase activity. Hence, the data in Fig. 2B are in agreement with our previous hypothesis (5) that exposure of cells in culture to BA in the absence of protein synthesis may result in the accumulation of an induction-specific RNA.

Figure 2C shows the intracellular polycyclic hydrocarbon covalently bound to cellular material during the same experiment. The covalently bound compounds represent between 0.5% and 1.0% of the total intracellular polycyclic hydrocarbon (1). A plateau in the curve depicting covalently bound polycyclic hydrocarbon occurred after about 1 hr in cells exposed to inducer alone, and within about 2 hr in cells treated with BA plus cycloheximide. The concentration between 3×10^{-18} and 4×10^{-18} mole of chemically bound intracellular polycyclic hydrocarbon per cell remained unchanged during the last 20 hr of the 22-hr experiment. Thus, these results show that cycloheximide does not prevent the covalent binding of polycyclic hydrocarbon to cellular macromolecules. Furthermore, actinomycin D does not inhibit the covalent binding of some polycyclic hydrocarbon to cellular material.[4] These findings are therefore consistent with our suggestion (1) that the control hydroxylase system alone is sufficient to metabolize BA to covalently bound metabolites in the cell. Also, we interpret the results depicted in Fig. 2C to mean that the intracellular sites for the chemical binding of metabolites are filled during the first 1–2 hr of exposure of the cells to BA. Furthermore, while most of the physically bound inducer leaves the cell during the time the cells are washed and then grown in control medium alone for 10 hr, it is not surprising that covalently bound polycyclic hydrocarbon remains in the cell. Therefore, the data in Fig. 2C demonstrate that no obvious correlation exists between the concentration of intracellular, covalently bound polycyclic hydrocarbon and the stimulation and disappearance of hydroxylase activity.

Intracellular concentration of polycyclic hydrocarbon and hydroxylase during short exposures of cells to BA. Figure 3 shows that, after short exposures of the cells to inducer, the kinetics of aryl hydrocarbon hydroxylase induction is identical whether the treatment with BA was carried out for 30 min or 4 hr. The peak in the curve of enzyme induction was reached in about 8, 10, and 13 hr in cells previously treated with inducer for 4, 2, and ½ hr. respectively. Table 1 lists the level of total intracellular polycyclic hydrocarbon at several points in time during this experiment. When the washed cells were grown in fresh control medium, approximately a first-order rate of loss of polycyclic hydrocarbon from the cell was observed over

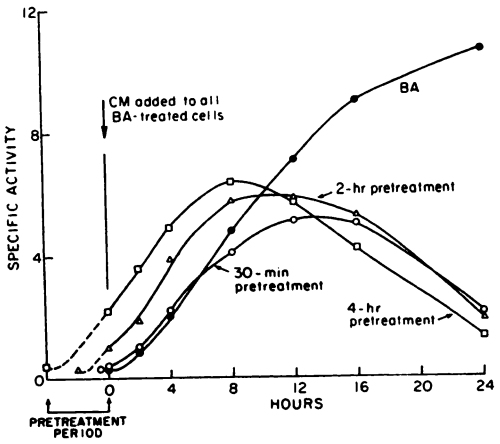

FIG. 3. *Kinetics of aryl hydrocarbon hydroxylase induction after short exposures of cells to 13 μM inducer (BA)*

During a prior treatment period, cells were exposed to the polycyclic hydrocarbon for 30 min, 2 hr, or 4 hr. At zero time, all BA-containing medium was replaced with fresh control medium (CM) for the next 24 hr. Other cells were treated with BA for 24 hr starting at zero time, producing the usual curve of enzyme induction.

TABLE 1

Concentration of intracellular polycyclic hydrocarbon following short exposures of cells to BA

The cells were obtained during the experiment depicted in Fig. 3. After short exposures to 13 μM ^{14}C-BA, the cell surface was washed with isotonic phosphate buffer at zero time, and fresh control medium was added.

Growth period in fresh control medium	Intracellular polycyclic hydrocarbon after treatment with BA for		
	½ hr	2 hr	4 hr
hr	*(moles/cell)* × 10^{16}		
0, not washed	6.1	5.6	5.1
0, washed	1.0	1.6	1.2
½	0.25	0.46	0.65
1	0.24	0.28	0.46
2	0.22	0.26	0.39
4	0.14	0.15	0.28
6	0.080	0.12	0.20
12	0.047	0.048	0.11
24	0.032	0.033	0.035

the 24-hr period. If the minimal concentration of intracellular polycyclic hydrocarbon required for maximal stimulation of hydroxylase activity is between 0.1 × 10^{-16} and 0.7 × 10^{-16} mole/cell (Fig. 1), the results in Table 1 indicate that one might expect a maximal rate of enzyme induction during at least the first 6 hr in which the cells are grown in fresh control medium, with less than a maximal rate of stimulation during the second 6-hr period. This is approximately what was observed.

With respect to the level of intracellular polycyclic hydrocarbon and the resultant hydroxylase induction, however, a discrepancy exists between cells treated with BA for ½ hr (Fig. 3) and cells exposed to inducer for 12 hr (Fig. 2). In cells exposed to BA for ½ hr and then grown in control medium for 2 hr, there was about 0.22 × 10^{-16} mole of polycyclic hydrocarbon per cell and hydroxylase induction persisted for about 11 hr more. Yet, in cells treated with BA for 12 hr and then grown in control medium alone for 2 hr, there was about 0.5 × 10^{-16} mole of polycyclic hydrocarbon per cell and the induced enzyme activity began to decline. One possible explanation is that the radioactivity in cells previously exposed to BA for 12 hr represents mainly inactive metabolites of BA, while the polycyclic hydrocarbon in cells previously treated with BA for ½ hr represents mostly active inducer. In any event, the cells appear *more sensitive* to the amount of intracellular polycyclic hydrocarbon during the first several hours compared to their response after 12 hr or more.

Induction of hydroxylase activity is initially inhibited by actinomycin D, but after 20 hr or more of exposure of the cells to BA, the process of enzyme induction is insensitive to actinomycin D inhibition (4, 5). How soon does this insensitivity develop? From the data in Fig. 3, one might speculate that development of the insensitivity may occur early, perhaps in the first 30 min. Since a lag time of about 35 min is necessary before a significant increase in hydroxylase activity is detectable (4), RNA synthesis presumably takes place during this lag period.

Figure 4 and Table 2 show the hydroxylase activity and polycyclic hydrocarbon concentrations in cells exposed to BA for short periods of time, after which high levels of actinomycin D were added. Figure 4 illustrates that the inhibition of RNA synthesis,

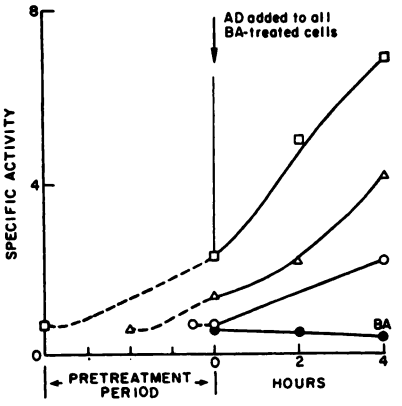

FIG. 4. *Kinetics of hydroxylase induction in the presence of 0.40 μM actinomycin D (AD) following short exposures of cells to 13 μM inducer (BA) during prior treatment period*

The concentration necessary for the immediate inhibition of more than 90% of RNA synthesis was 0.40 μM actinomycin D; in cells exposed to this level of inhibitor for more than 4 hr, toxic and usually irreversible effects on cellular metabolism caused by the antibiotic were encountered.

TABLE 2

Concentration of intracellular polycyclic hydrocarbon in the presence of actinomycin D, following short exposures of cells to BA

Dishes of cells were obtained during the experiment shown in Fig. 4. After short exposures to 13 μM ^{14}C-BA, 0.40 μM actinomycin D (AD) was added to the washed cells.

Treatment of cells	Intracellular polycyclic hydrocarbon
	(*moles/cell*) × 10^{16}
BA, 10 min	4.4
BA, 10 min; AD, 20 min	0.14
BA, 20 min	4.9
BA, 20 min; AD, 10 min	0.42
BA, ½ hr	5.5
BA, ½ hr; AD, ½ hr	0.23
BA, 2 hr	4.8
BA, 2 hr; AD, ½ hr	0.47
BA, 4 hr	4.4
BA, 4 hr; AD, ½ hr	0.57
BA, 4 hr; AD, 4 hr	0.26

in cells exposed to BA for as short a time as 30 min, did not prevent the hydroxylase activity from rising normally for the next 4 hr. In fact, in cells previously treated with inducer for 4 hr, we consistently found that the rate of the enzyme induction in the presence of actinomycin D was greater than the rise in hydroxylase activity in cells grown in control medium alone. This observation probably represents the stimulation by actinomycin D that we previously noted (4, 5) in fetal cell cultures which had previously been treated with BA for 20 or 24 hr. Numerous other earlier reports (7–13) also have described a stimulatory effect of actinomycin D on enzymatic activity.

Table 2 shows that the polycyclic hydrocarbon content in cells which had been exposed to inducer for 10 or 20 min ranged between 4×10^{-16} and 5×10^{-16} mole/cell; this concentration is at least 6 times greater than the minimal amount of intracellular polycyclic hydrocarbon required to stimulate hydroxylase induction maximally. However, enzyme induction is completely prevented in these cells upon addition of actinomycin D after 10 min of treatment with BA, while there are variable, small increases in hydroxylase activity if the antibiotic is added after 20 min of exposure to BA.[4] In cells previously treated with BA for 30 min and then with actinomycin D for 30 min, we found about 0.23×10^{-16} mole of intracellular polycyclic hydrocarbon per cell; yet hydroxylase activity increased at its maximal rate of induction in these cells during the 4-hr exposure to actinomycin D. Therefore, a sequence of events apparently takes place during the initial 35-min period of microsomal oxidase induction in which no detectable enzyme induction occurs: between 0.6×10^{7} and 4×10^{7} molecules of intracellular polycyclic hydrocarbon per cell produce a rise in hydroxylase activity only if an induction-specific RNA is allowed to be synthesized, and then only after the RNA is allowed to be translated.

Stimulation of hydroxylase activity by actinomycin D as indicator of induction-specific RNA. The stimulatory effect of actinomycin D has been interpreted (7–10, 12, 14) as due to the inhibition of synthesis of a labile cytoplasmic translational repressor by relatively high concentrations of the antibiotic. Reel and Kenney (13) have proposed that actinomycin D inhibits the

degradation of induced tyrosine transaminase activity. Another interpretation (5) of this phenomenon is that induction-specific RNA molecules bound to DNA may be displaced by the binding of actinomycin D to DNA, thereby leading to a sudden increase in the induction-specific RNA available in the cell for translation. In any event, perhaps one can use the stimulatory effect of this inhibitor as an indication of the relative amount of intracellular induction-specific RNA.

Figure 5 shows the effect of actinomycin D on aryl hydrocarbon hydroxylase activity in cells previously exposed to inducer for 16 hr and then grown in fresh growth medium. In five experiments the stimulatory phenomenon produced by actinomycin D was observed for 7–10 hr after the BA-containing medium had been replaced with control medium. The response to actinomycin D diminished with time. This response is strikingly similar to that reported recently

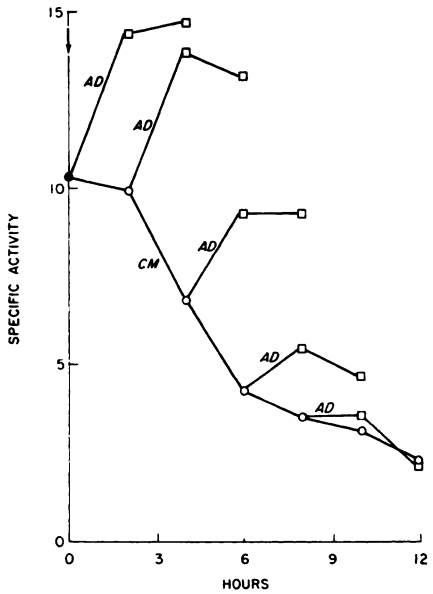

FIG. 5. *Stimulation of aryl hydrocarbon hydroxylase activity by 0.40 μM actinomycin D (AD) inhibition in cells previously treated with 13 μM BA for 16 hr*

Following the initial treatment period with BA, fresh control medium (CM) was added to all dishes, and the control medium was replaced by actinomycin D in two dishes every 2 hr.

(14) with tyrosine transaminase induction in cell culture. From the hydroxylase activities observed at different times in cells treated with the inhibitor for 2 hr, we estimated in five experiments that the average half-life of the "enzyme activity able to be stimulated by actinomycin D" was 3.2 hr. The half-life of induced aryl hydrocarbon hydroxylase activity is estimated to be 3.3 hr (4). Thus, this finding of a similar half-life indicates that the decay of induced hydroxylase activity in hamster fetal cell culture may reflect the stability of an induction-specific RNA.

The same response of the hydroxylase activity to actinomycin D in Fig. 5 was observed whether or not BA had been added with the antibiotic. Therefore, the stimulation produced by actinomycin D has no obvious relationship to a high, or low, level of polycyclic hydrocarbon in the cell.

Intracellular polycyclic hydrocarbon level during decay of hydroxylase activity. The possibility that the relative amount of polycyclic hydrocarbon in the cell has some effect on the degradation of induced hydroxylase activity was examined. Figure 6 shows that cycloheximide, in the presence or absence of the inducer and/or actinomycin D, produced a first-order rate of disappearance of enzyme activity not different from that observed when the BA-containing medium had been replaced with fresh control medium. During these experiments we found that the levels of intracellular polycyclic hydrocarbon ranged between 4×10^{-16} and 12×10^{-16} mole/cell whether the cells had been exposed to BA for 30 min or 28 hr. The intracellular content was always somewhat higher in cells exposed to actinomycin D or cycloheximide for 4 hr or longer than in cells not treated with either antibiotic. After replacement of the BA-containing medium with inducer-free medium, the intracellular polycyclic hydrocarbon concentration was about 0.30×10^{-16} mole/cell after 8 hr of growth, and about 0.10×10^{-16} mole/cell after 12 hr of growth, in fresh control medium. Therefore, the data in Fig. 6 indicate that, at concentrations from 0.10×10^{-16} to 12×10^{-16} mole of intracellular polycyclic hydrocarbon per cell, the decay of the hydroxylase activity is

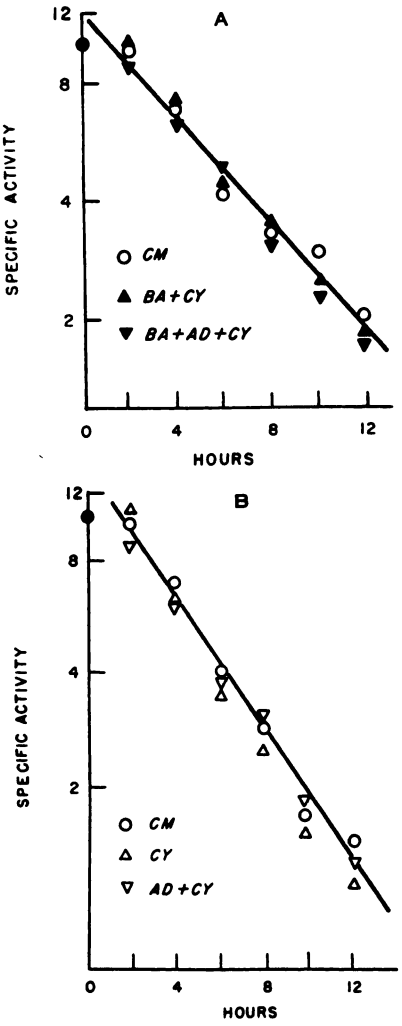

FIG. 6. *Semilogarithmic plot of disappearance of aryl hydrocarbon hydroxylase activity in the presence (A) and absence (B) of 13 μM inducer (BA)*

The cells were exposed to 13 μM BA for 16 hr, and the BA-containing medium was then replaced by control medium alone (CM), 3.5 μM cycloheximide (CY), and/or 0.40 μM actinomycin D (AD). At the concentrations employed, cycloheximide prevented more than 90% of protein synthesis and actinomycin D blocked more than 95% of RNA synthesis during the entire time the antibiotic was present. With 3.5 μM cycloheximide for 12 hr, the effects of the inhibitor could be reversed. With 0.40 μM actinomycin D for more than 4 hr, the toxicity of this antibiotic was usually not reversible; however, the decay of hydroxylase activity remained approximately first-order.

apparently unaffected. We interpret these results to mean that the inducer probably does not act primarily by preventing the degradation of microsomal oxygenase activity. These data also indicate that actinomycin D does not appreciably change the rate of decay of aryl hydrocarbon hydroxylase activity.

DISCUSSION

There is a decline in the concentration of intracellular polycyclic hydrocarbon concomitant with the appearance of the induced aryl hydrocarbon hydroxylase activity; the reason for this is not understood. After about 30 min, the amount of polycyclic hydrocarbon in the cell decreases, in spite of 5–25-fold excesses of extracellular BA. The formation of large amounts of polar metabolites of BA by the induced hydroxylase system (1) may somehow impede a portion of additional BA molecules from binding to sites in the cell or on the cell surface. The shape of the curves in Fig. 1B, with the maxima at about 30 min, is very similar to that in Fig. 2 of a paper by Andrianov and co-workers (15), who determined spectrophotofluorometrically the kinetics of uptake of 0.40 μM benzo[*a*]pyrene by fetal mouse cells in culture.

The possibility exists that a metabolite of BA, rather than the parent compound itself, is the active inducer. This hypothesis would explain why aryl hydrocarbon hydroxylase is not inducible in those established cell lines which have no constitutive levels of the hydroxylase (4). Also, evidence in the accompanying paper (1) indicates that the control microsomal enzyme begins to metabolize BA actively as soon as the compound enters the cells.

Our data indicate that there is a decreasing response of microsomal enzyme induction to the inducer as the length of exposure of the cells to BA is increased. In fact, between 32 and 40 hr of continuous exposure of hamster fetal cells to BA, the curve of hydroxylase induction reaches a maximum; replacement of the medium repeatedly with fresh BA-containing medium does not result in any further rise in enzyme activity (4). These findings are consistent with the hy-

pothesis that, during the initiation of microsomal hydroxylase induction, there exists a macromolecular process, such as the synthesis of an induction-specific RNA. In the twelfth hour of exposure of cells to BA, less induction-specific RNA may be synthesized than during the first hour. Also, if an induction-specific RNA is synthesized, there may be a difference between its expression in cells previously exposed to BA for only ½ hr as compared to cells previously treated with inducer for 12 hr; perhaps the induction-specific RNA is translated a greater number of times, or at a different rate in the two cases. Another possibility is that the half-life of the induction-specific RNA initially synthesized is longer than the half-life of RNA formed later. At these two different times during the process of microsomal oxygenase induction, therefore, we may be observing varying degrees of both derepression and repression of gene activity.

The stimulation of hydroxylase activity by actinomycin D increases in intensity during the process of hydroxylase induction, and decreases several hours after the removal of polycyclic hydrocarbon from the medium. Therefore, it is not unreasonable to postulate that an induction-specific RNA species is responsible in some way for this stimulatory phenomenon. Understanding the mechanism by which actinomycin D enhances enzyme activity may determine whether enzyme induction in higher organisms is regulated by post-transcriptional or transcriptional control. The presence of a post-transcriptional repressor (14) is consistent with the concept that there exists a constant amount of cytoplasmic, induction-specific RNA which is not being translated at its maximal rate. On the other hand, the displacement of induction-specific RNA molecules from DNA by actinomycin D would produce a sudden increase in the level of cytoplasmic, induction-specific RNA; this effect is consistent with transcriptional control. It would be of interest to determine the concentration of extranuclear, induction-specific RNA during the stimulation by actinomycin D in an experimental system in in which the amount of newly synthesized RNA to be measured comprises a significant portion of the total cytoplasmic RNA.

With the knowledge of precise concentrations of extracellular and intracellular polycyclic hydrocarbon in fetal cell culture monolayers at various points in time, we can make certain stoichiometric estimations which would be impossible in pharmacological studies of the intact animal. After 12 hr of exposure of the cells to BA (Figs. 2A and 3), the specific activity of the induced aryl hydrocarbon hydroxylase activity is approximately 6 units/mg of cellular protein,[5] or the linear rate of accumulated enzyme activity is about 0.5 unit/hr. We have previously reported (2) that the induced hydroxylase system metabolizes both benzo[*a*]pyrene and BA to alkali-extractable products at similar rates, and that about one-fourth of the total alkali-extractable product is the 3-hydroxybenzo[*a*]pyrene which we have used to measure aryl hydrocarbon hydroxylase activity. Thus, in a single 100-mm tissue culture dish containing about 2 mg of cellular protein and about 6×10^6 cells, the induced enzyme system after 12 hr of exposure to BA is capable of metabolizing a maximum of about 5×10^{-11} mole of BA per minute to alkali-soluble derivatives. Hence, if the hydroxylase system, as it becomes induced in the cell, metabolizes BA at its maximal rate, we can estimate by integration that approximately 17 nmoles of alkali-extractable products should be formed during a 12-hr exposure of the cells to the inducer. The experimental data in the accompanying paper (1) show that about 460 pmoles of alkali-soluble radioactivity per milliliter are found in the 6 ml of growth medium after a 12-hr exposure of the cells to BA. This is approximately 2.8 nmoles of polar derivatives per dish of cells; i.e., about one-sixth of the estimated theoretical maximal value. Thus, these data indicate that the induced enzyme in cells saturated with BA metabolizes BA at about one-sixth the rate observed when the hydroxylase system is saturated with substrate *in vitro*.

In summary, upon the exposure of 6×10^6 hamster fetal cells to 7.8×10^{-8} mole of

[5] *One unit* of aryl hydrocarbon hydroxylase activity has been defined (1, 3) as that amount of enzyme catalyzing the formation, per minute at 37°, of hydroxylated metabolites causing fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo[*a*]pyrene.

BA (i.e., 6 ml of 13 μM BA in the growth medium), about 3×10^{-9} to 7×10^{-9} mole of inducer enters, or binds on the surface of, the cell during the first 30 min. The intracellular polycyclic hydrocarbon therefore represents between 4% and 9% of the total compound initially present in the medium. Within 1–2 hr, the cell becomes saturated with covalently bound polycyclic hydrocarbon: a total of approximately 2×10^{-11} to 3×10^{-11} mole of metabolites, which is between 0.5% and 1.0% of the total intracellular polycyclic hydrocarbon (1). More than 99% of the intracellular polycyclic hydrocarbon, therefore, is physically bound and presumably available for metabolism by the hydroxylase, which possesses a remarkably high affinity for its substrate. Using benzo[a]pyrene as substrate, we have previously estimated (2) a K_m value of 0.6 μM for the induced hydroxylase system. The physically bound polycyclic hydrocarbon is thus metabolized by both the control and the induced microsomal oxygenase, so that after 12 hr of exposure to 13 μM BA, 6×10^6 cells contain approximately 2×10^{-11} to 3×10^{-11} mole of intracellular covalently bound polycyclic hydrocarbon and have lost about 2.8×10^{-9} mole of polar metabolites into the growth medium. The physically bound intracellular polycyclic hydrocarbon is decreased slightly during the 12-hr exposure of the cells to BA. However, this decrease accounts for only approximatly one-tenth of the metabolized inducer excreted into the medium. Thus, we conclude that there must be an influx of BA into the cell concomitant with the metabolism of the polycyclic hydrocarbon and excretion of the polar products.

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