ron. Since the specific activity of the uridine triphosphate in the cell was not measured, it is not possible to choose between these alternatives. However, the high uridine concentration in the incubation medium renders the last interpretation unlikely.

Since it has been shown that a neuron can still initiate and conduct impulses for up to 24 hours during inhibition of RNA synthesis by actinomycin D (*4*), it was necessary to establish the viability of this preparation over the duration of the experiment. Evidence for this consists of the facts that (i) although the stimulus voltage necessary to fire R2 increased during the experimental period, the neuron was still capable of being synaptically activated after 9 hours in all cases, and (ii), as seen in Table 1, the incorporation ratio is linear through 9 hours for all three tissue fractions.

Table 1 also shows that when R2 was stimulated, the ratio increased significantly. There was no change in the rate of uptake of radiouridine from the medium, that is, no change in the mean activity of the acid-soluble fraction. Therefore, the increased ratio actually represents an increase in the rate of appearance of labeled RNA.

Unstimulated R2's produced about 20 spikes per hour. Stimulated preparations were driven at 200 to 500 spikes per hour, depending on the ability of the neuron to follow the stimulus. There was a definite trend toward greater incorporation in those cells which fired more impulses. This is seen in Fig. 2, where the incorporation ratio for R2 is plotted against spike output. The relationship is clearly linear and shows a highly significant rank-order correlation.

The close agreement between the ratios for the groups of cells from the right and left sides of the ganglion, shown in Table 1, indicates the reproducibility of the extraction method, especially since the mass of cells taken from each side of each ganglion varied. The rate of RNA synthesis in these neurons did not increase significantly with stimulation, although the mean value was raised. This is to be expected, since stimulation of the left connective nerve excites many of the neurons in the ganglion (*5*), but is unlikely to fire each of them.

These data point out the disadvantages of studies on even small populations of neurons, when the electrical activity cannot be accurately specified. The demonstration of electrical-metabolic coupling reported here is a direct result of the precision attainable in single neuron analysis. The same factors contributing to that precision should make this preparation useful for investigating the mechanism by which the two processes are linked.

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Alpha-Naphthoflavone: An Inhibitor of Hydrocarbon Cytotoxicity and Microsomal Hydroxylase

Abstract. *Alpha-naphthoflavone inhibits the metabolism of 3,4-benzopyrene and 7,12-dimethylbenz*(a)*anthracene in hamster embryo cell cultures and protects the cells against the inhibition of cell multiplication by these carcinogens. Alpha-naphthoflavone also inhibits the aryl hydrocarbon hydroxylase activity in homogenates of induced hamster embryo cells and in liver microsomes from rats previously treated with polycyclic aromatic hydrocarbons, but not in microsomes from control rats.*

The microsomal hydroxylases are important enzymes in the metabolism of aromatic hydrocarbons and are involved in the conversion of many drugs, steroids, and carcinogens (*1*). Some of the polycyclic aromatic hydrocarbons which are carcinogenic in animals are present in the environment and are ingested by man and thus may be factors in human carcinogenesis. Studies in vitro have shown that carcinogenic polycyclic hydrocarbons are toxic for normal embryonic rodent cells and inhibit their multiplication in monolayer cell cultures. Transformed rodent cells and both normal and transformed primate cells are relatively resistant to this effect (*2*). Cells that are sensitive to the cytotoxic effect metabolize the polycyclic hydrocarbons to water-soluble derivatives (*3*) and contain the NADPH-requiring (nicotinamide adenine dinucleotide phosphate, reduced) microsomal enzyme system, aryl hydrocarbon hydroxylase (AHH) (*4, 5*); in these cells the enzyme is also readily inducible (*5*). In cells that are resistant to the cytotoxicity, both the metabolic conversion and the enzyme system are markedly reduced or absent (*3, 6*). We have found that α-naphthoflavone (ANF) inhibits both AHH activity and the cytotoxicity induced by carcinogenic polycyclic hydrocarbons.

Primary monolayer cultures of Syrian hamster embryo cells were initiated by treating 11- to 14-day-old embryos with trypsin and were grown in Eagle's basal medium supplemented with fetal calf or calf serum (10 percent). All experiments were done with 8- to 48-hour-old secondary or tertiary subcultures in which the cell densities were 4×10^4 to 8×10^4 cell/cm^2. The ANF (Eastman Kodak, or Aldrich) (*7*) was dissolved in dimethyl sulfoxide, and the polycyclic hydrocarbons (Eastman Kodak) were dissolved in dimethyl sulfoxide or acetone; these solutions were further diluted with culture medium. The final concentrations of dimethyl sulfoxide and acetone in the cell cultures never exceeded 0.5 and 0.1 percent, respectively.

The metabolism of 3,4-benzopyrene to water-soluble derivatives was measured by adding tritium-labeled benzopyrene (Amersham/Searle; specific activity, 4 c/mmole) to hamster embryo cultures and extracting the medium, at specified times, with four volumes of water and 20 volumes of a mixture of

chloroform and methanol (2:1 by volume) (*3*). The radioactivity in the aqueous and organic solvent phases was measured in a Packard Tri-Carb scintillation spectrometer with Bray's mixture (*8*). Corrections were made for the counting efficiencies of the two phases. The extent of binding of benzopyrene to cell components was determined by adding H^3-benzopyrene to cover-slip cultures of hamster embryo cells (*2*). At specified times, the cover slips were washed twice with fresh medium and twice with Dulbecco's phosphate-buffered saline. The cells were fixed in Carnoy's solution (60 percent absolute ethanol, 30 percent chloroform, 10 percent glacial acetic acid) and rinsed thoroughly in 70 percent ethanol. After drying at 100°C for 2 hours, the cover slips were assayed for radioactivity.

Homogenates of hamster embryo cells (*5*) and liver microsomes from rats treated with methylcholanthrene and from control rats (*9*) were assayed for AHH activity (*5*), with $10^{-4}M$ benzopyrene as substrate. By definition, one unit of activity catalyzes the production, during a 30-minute incubation period, of an amount of fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo(*a*)pyrene per milliliter (*10*). Protein was determined by modification of the Lowry method described by Sutherland *et al.* (*11*).

Table 1. The effect of α-naphthoflavone (ANF) on the aryl hydrocarbon hydroxylase (AHH) activity of whole hamster embryo cells. Specific activity is expressed as the number of enzyme units per milligram of protein (*10*). Hamster embryo cultures were treated with control medium (CM) or with medium containing 1,2-benzanthracene (BA), ANF, or a combination. Eight hours later, homogenates of these cells were prepared and assayed for AHH activity. When used, the concentration of BA in the medium was 3 μg/ml and that of ANF, 1 μg/ml. Cultures were preinduced by treatment with BA (3 μg/ml) for 18 hours. At the end of this time, the AHH specific activity was 298, and the cultures were fed with the indicated medium. Five hours later, cell homogenates were prepared and assayed for AHH activity. The concentration of BA and of ANF in the medium was 3 μg/ml.

Treatment	AHH specific activity
Hamster embryo cells	
CM	16
BA	271
ANF	20
BA + ANF	160
Preinduced hamster embryo cells	
BA	247
BA + ANF	126
CM	198
ANF	58

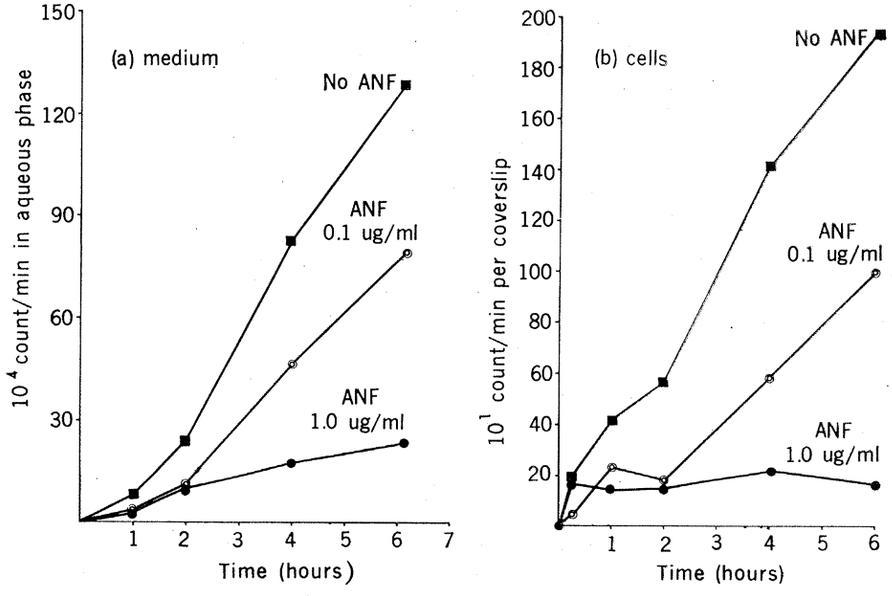

Fig. 1. The effect of α-naphthoflavone (*ANF*) on the metabolism of benzopyrene in hamster embryo cultures (a) and the binding of the carcinogen to cell components (b). Secondary hamster embryo cells were seeded in 50-mm petri plates containing three cover slips (9 by 2 mm). The cultures were treated with ANF for 18 hours, and H^3-benzopyrene was added at a final concentration of 0.06 μg/ml. At each interval, the medium was extracted with a mixture of chloroform and methanol, and the radioactivity in the aqueous phase was assayed (a). In the absence of cells, less than 1 percent of the input H^3-benzopyrene was degraded to water-soluble products. The cover-slip cultures were fixed in Carnoy's solution and assayed for radioactivity (b).

The inhibitory effect of ANF on the metabolism of benzopyrene and on the binding of the carcinogen or its derivatives to cell components is shown in Fig. 1. Six hours after H^3-benzopyrene was added to the hamster embryo cultures, half the input H^3-benzopyrene was metabolized to water-soluble derivatives, and H^3-benzopyrene became bound to cell components. Prior treatment of the cells with ANF (1 μg/ml) for 18 hours reduced by 70 percent the amount of metabolic conversion of H^3-benzopyrene (Fig. 1a) and reduced by more than 95 percent the amount of H^3-benzopyrene bound to cell components (Fig. 1b). In the presence of 0.1 μg of ANF per milliliter, conversion was inhibited by 30 percent, and binding was inhibited by 50 percent. The ANF also inhibited the metabolism of H^3-benzopyrene when it was added simultaneously with, or several hours after, the carcinogen. In similar experiments we found that ANF also inhibited the metabolism of the carcinogen, 7,12-dimethylbenz(*a*)anthracene (DMBA).

Fluorescence microscopy (*12*) showed that ANF did not prevent the uptake of benzopyrene by the cells. Cells were first treated with 5 μg of ANF per milliliter, then exposed to 1 μg of benzopyrene per milliliter and examined 2 hours later for the intracellular fluorescence produced by benzopyrene. No difference in the intensity of the fluorescence was observed in ANF-treated and untreated cells.

Figure 2 shows the inhibitory effect of ANF on the cytotoxicity induced by a carcinogenic polycyclic hydrocarbon. In the presence of ANF (1 μg/ml), there was almost complete protection against the inhibition of cell multiplication produced by DMBA. With 0.1 μg of ANF per milliliter, less protection against DMBA cytotoxicity was observed. Wheatley (*13*) has shown that SKF-525-A (β-diethylaminoethyl-diphenyl-*n*-propyl acetate), an inhibitor of microsomal drug metabolism (*14*), protects rats against DMBA-induced adrenal necrosis. We found that ANF also inhibited the cytotoxicity induced by benzopyrene in hamster embryo cultures. In the presence of 1 μg of ANF per milliliter, the growth inhibitory effect produced by 5 μg of benzopyrene per milliliter was reduced 40 to 50 percent.

In other studies, we examined the effect of ANF on the AHH activity of whole cells and of cell-free enzyme

Table 2. The effect of α-naphthoflavone (ANF) on the aryl hydrocarbon hydroxylase (AHH) activity of hamster cell homogenates and rat liver microsomes. Specific activity is expressed as the number of enzyme units per milligram of protein. The ANF was dissolved in methanol for the assay of cell homogenates and in dimethyl sulfoxide for the assay of microsomes. It was added to the reaction mixture of the enzyme assay so that the final concentration of solvent in each reaction flask was 1 percent. For induction, cultures were treated for 18 hours with 1,2-benzanthracene (3 μg/ml) and rats (50 to 70 g) were inoculated intraperitoneally with 3-methylcholanthrene (1 mg) 24 hours before isolation of the microsomes (*9*).

ANF (*M*)	AHH specific activity	
	Induced	Control
Homogenates of hamster embryo cells		
None	301	15
10^{-4}	151	14
10^{-5}	226	13
Rat liver microsomes		
None	11927	1163
10^{-4}	2606	1298
5×10^{-5}	3031	1265
10^{-5}	6021	1423
10^{-6}	11369	1455

preparations (Tables 1 and 2). Cells treated with ANF only showed no change in enzyme activity, but the simultaneous addition of ANF with benzanthracene inhibited enzyme induction by 40 percent (Table 1).

When cells that had been preinduced with benzanthracene were exposed to fresh inducer, there was little effect on enzyme activity (Table 1). However, when the cultures were exposed to fresh inducer and ANF, enzyme activity fell after 5 hours to about one-third the induced level. Exposure to fresh control medium without inducer caused a 30 percent decrease in enzyme activity, but the greatest decrease, to one-sixth the original level in the preinduced cells, occurred when the cultures were exposed to ANF with no inducer present. Thus, the presence of ANF caused a greater fall in activity than simply the change to inducer-free medium. These results may reflect competition between ANF and inducer for the appropriate receptor site for induction or competition for an active site on the enzyme.

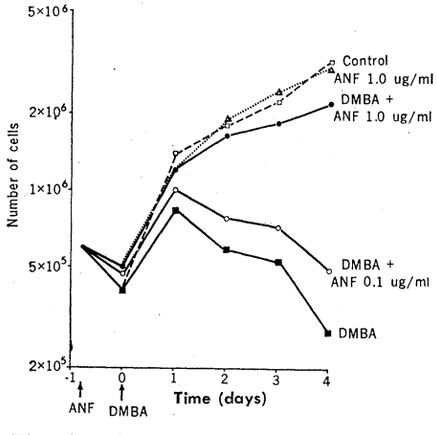

Fig. 2. The effect of α-naphthoflavone (*ANF*) on the inhibition of the growth of hamster embryo cells by 7,12-dimethylbenz(*a*)anthracene (*DMBA*). The cultures were treated with ANF for 18 hours before the addition of DMBA at a final concentration of 0.1 μg/ml. Control cultures received neither ANF nor DMBA.

Table 2 shows the effect on AHH activity when ANF was added to homogenates of benzanthracene-treated and control hamster embryo cells and to liver microsomes from rats treated with methylcholanthrene and from control rats. With $10^{-4}M$ ANF there was a 50 percent inhibition of the activity in homogenates of induced cells and an 80 percent inhibition of the activity in microsomes from induced rats. With $10^{-5}M$ ANF the activity in the induced cell homogenates and microsomes was inhibited 25 and 50 percent, respectively. On the other hand, concentrations of ANF ranging from 10^{-4} to $10^{-6}M$ failed to inhibit the AHH in liver microsomes from control rats. Thus, there seems to be a unique selectivity of ANF for the inhibition of AHH in induced, but not in normal, microsomes. This differential inhibition indicates that the microsomal hydroxylase complex of control rats differs from that of induced rats and that the enzymes can be selectively inhibited.

The relation between polycyclic aromatic hydrocarbon hydroxylation and carcinogenesis induced by these agents has not been adequately defined. Studies in vivo have demonstrated that the hydrocarbons become covalently bound to cellular DNA and protein (*15*); these findings are key facts in support of two of the major theories of chemical carcinogenesis—mutation and protein deletion. The formation of covalently bound benzopyrene-DNA complexes is catalyzed by microsomal preparations (*16*); presumably a microsomal enzyme is required to metabolize the hydrocarbon to a reactive derivative. In cells grown in culture, the ability to metabolize polycyclic aromatic hydrocarbons to water-soluble derivatives, the binding of hydrocarbon to cell macromolecules, and the sensitivity of the cells to the cytotoxic effects of carcinogenic hydrocarbons appear to be related to AHH activity. In the presence of ANF, an inhibitor of this enzyme system, cells which are normally sensitive to carcinogen-induced cytotoxicity behave like resistant cells. Their multiplication is not inhibited by the carcinogen, and the carcinogen is neither metabolized nor bound to cell components. These results suggest that metabolism of the polycyclic hydrocarbon is essential for its toxic effect on the cell. It may also be essential for the carcinogenic effect of these compounds.

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