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EFFECTS OF 3-METHYLCHOLANTHRENE AND SOME RELATED COMPOUNDS UPON THE BENZPYRENE HYDROXYLASE ACTIVITY IN FETAL RAT LIVER EXPLANTS*

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Abstract—The addition of 3-methylcholanthrene (3-MC) or some of its derivatives to explants of fetal rat liver caused a reproducible "induction" of benzpyrene (BP) hydroxylase. In fresh explants, a lag of 6–12 hr was observed, whereas a more immediate increase in enzyme activity was noted when 3-MC was added to 40-hr preincubated cultures. The addition of 3-MC and 1-keto-3-MC (10^{-5} M in the medium) caused a 4-fold elevation in BP-hydroxylase, while the 1-hydroxy and the cis-11,12-dihydroxy-11, 12-dihydro derivatives produced a stimulatory activity of only 2·7- and 2-fold respectively. The most potent compound in this regard was the trans-1,2-dihydroxy-3-MC; its administration resulted in a 6·7-fold increase in enzyme activity. This study points out certain advantages of studying the induction phenomenon in this system *in vitro*.

THE ADMINISTRATION of 3-methylcholanthrene (3-MC) to young and adult rats is attended by a marked increase in the activity of several enzymes of biotransformation located in the endoplasmic reticulum of the liver cells. Benzpyrene (BP) hydroxylase is one of the mixed-function oxidases which responds in this manner to polycyclic hydrocarbons.[1–4] Since studies of the induction ‡ of this enzyme *in vivo* by pharmacologically active drugs may be complicated by factors such as absorption and distribution, systemic toxicity or hormonal changes, it was felt that utilization of an experimental system, such as organ culture, would prove more advantageous. Previous studies *in vitro* of the induction phenomena have been made by Wattenberg *et al.*[5] with rat lung explants, and by Alfred and Gelboin[3] and Nebert and Gelboin[6,7] with cell cultures derived from rodent fetuses.

The purpose of this paper is to report studies on the effect of 3-MC and some related compounds on the BP-hydroxylase enzyme system in explants of fetal rat liver tissue. The results demonstrate that in fetal rat liver tissue, the BP-hydroxylase system can be induced by these polycyclic hydrocarbons. This explant system may have great utility in studying the induction phenomenon *in vitro*.

MATERIALS AND METHODS

The standard complete medium consisted of Eagle's minimal essential medium (MEM) with Hanks' balanced salt solution (BSS), pH 7·4, 2 × glucose and 2 ×

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‡ The term "induction" is used to describe an elevation in enzyme activity without suggesting any genetic mechanism.

$NaHCO_3$, 0·05 M Tricine, penicillin (100 units/ml) and streptomycin (100 μg/ml). The medium, which was prepared fresh every 4 weeks, was sterilized by passing through a millipore filter. Plastic tissue culture dishes were obtained from Falcon Plastics; stainless steel grids (type 316, 20 mesh, 16 wire diameter) from Metal Goods, Houston, Tex. The derivatives of 3-MC were prepared as described by Sims.[8] We are grateful to Dr. Sims for providing authentic samples of many of these derivatives for comparative purposes. Pregnant Sprague–Dawley rats, estimated at 18–20 days of gestation, were received weekly from Holtzman Rat Company, Wisconsin, or from Texas Inbred Company, Houston.

The explant technique which was employed is a modification of the method of Wicks.[9] Pregnant rats (one to two rats per assay) were sacrificed by decapitation, the fetuses were removed aseptically, decapitated, and the livers were cut into cubes averaging approximately 1–2 mm with sterile scalpel blades. After washing twice with sterile complete medium and mixing for the purpose of randomization, eight cubes of liver tissue (approximately 2 g protein) were placed on sterile stainless steel grids in plastic tissue culture dishes. Sufficient complete medium (approximately 5·5 ml) was added to just wet the lower surface of the explants. The vapor-sealed dishes were placed in a humidified Napco incubator at 37° with a circulating gas phase of 95% air–5% CO_2. All operations were carried out under sterile conditions; about 2 hr elapsed between killing and incubation. Less than 30 per cent of the control BP-hydroxylase activity in fresh fetal liver tissue was lost during the preparation of the cultures. Occasional adjustment of pH at 7·4 was required; dilute sterile NaOH was used for this purpose. (The liver explant technique was learned in the laboratory of Dr. W. D. Wicks, and we are grateful to him for the opportunity.)

The additions of 3-MC or derivatives were made at varying times after the cultures were set up. The polycyclic hydrocarbon was first dissolved in dimethylsulfoxide (DMSO) and then added to the medium with the final DMSO concentration of 0·1% (v/v). Control dishes were also treated with DMSO in a final concentration of 0·1%. This concentration of DMSO has no demonstrable effect on the BP-hydroxylase activity of the liver tissue nor upon the morphology of the explant. Furthermore, the addition of up to 0·1 ml DMSO to the assay mixture had no effect upon BP-hydroxylase activity in the fetal liver homogenates, either from control or drug-treated systems. After an appropriate time, the liver pieces were washed twice in 0·9% NaCl and were homogenized in 1 ml of cold 0·25 M sucrose with the use of a microhomogenizer of the Potter–Elvehjem type.

Both the hydroxylase activity and protein content of the homogenate were determined in duplicate. The activity of BP-hydroxylase was estimated using an incubation mixture essentially as described by Nebert *et al.*[10] The reaction mixture contained, in a total volume of 1·0 ml: 50 μmoles of Tris chloride buffer, pH 7·5; 0·54 μmole TPNH; 3 μmoles $MgCl_2$; 0·32 μmole glucose 6-phosphate; 0·6 unit glucose 6-phosphate dehydrogenase; 0·4 ml of fetal liver homogenate (approximately 1 mg protein); 80 mμmoles benzpyrene in 50 μl DMSO. The mixture was incubated with gentle shaking in the dark at 37° for 10 min in air. The reaction was stopped by the addition of 1·0 ml of cold acetone and 3·25 ml hexane. The hydroxylated BP was extracted into the alkali as described by Nebert and Gelboin[6] and the fluorescence was measured in an Aminco spectrophotofluorometer set at activation and fluorescence wavelengths of 396 and 522 nm respectively. Linearity of hydroxylated BP formation during the

10-min period of incubation was observed at each level of enzyme activity tested. One unit of hydroxylase activity was arbitrarily defined as the amount of enzyme catalyzing the formation, during a 1-min incubation at 37°, of a hydroxylated product with a fluorescence equivalent to 1 pmole of 3-hydroxybenzo(a)pyrene. (We are grateful to Dr. H. V. Gelboin who supplied authentic 3-hydroxybenzpyrene for calibration of our fluorescence assay.)

The protein concentration of aliquots of the homogenate was determined by the method of Lowry *et al.*,[11] with bovine serum albumin as the reference standard.

RESULTS

Figure 1 illustrates the effects of 3-MC administration on BP-hydroxylase activity in fresh explants and in explants of fetal liver which were preincubated. The control level of hydroxylase activity in fresh fetal liver explants was very low. The addition

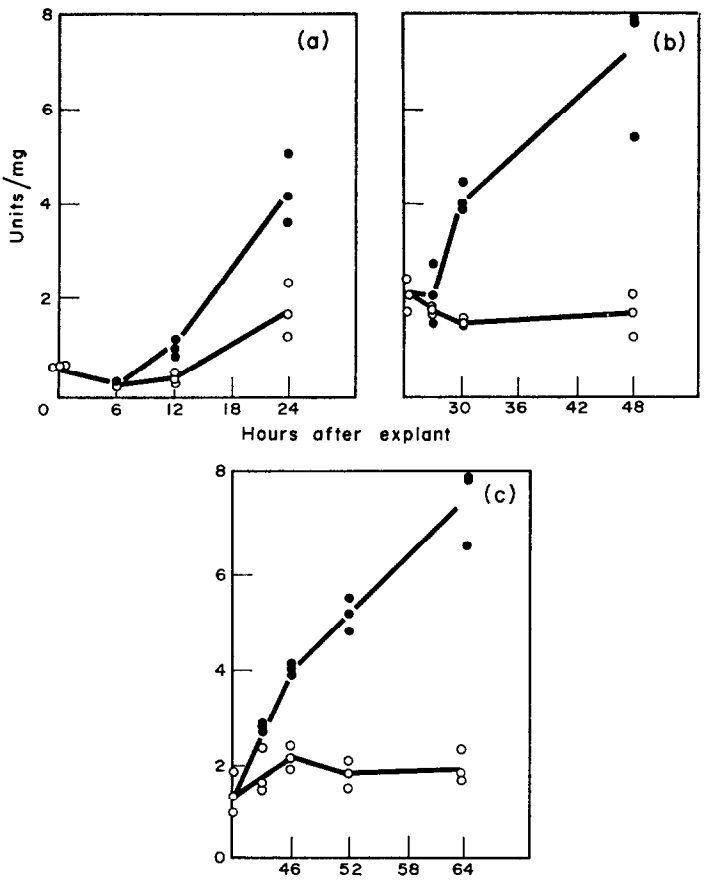

FIG. 1. Kinetics of induction of BP-hydroxylase by 3-MC. 3-MC, 10^{-5} M (●—●), or DMSO (○—○) was added to fresh explants (a) of liver from 18-day fetuses or to explants which had been maintained in culture for 24 (b) or 40 hr (c) previously. Periodically, after addition of the drug, explants were homogenized and assayed for enzyme activity. Each point represents a single culture dish except the 0 and 6 hr points in (a); there was virtually no variation among the three dishes at these times. BP-hydroxylase activity is given on the ordinate as units/mg of protein.

of 3-MC in a final concentration of 10^{-5} M to fresh explants of fetal liver produced an increase in hydroxylase activity after a lag of 6–12 hr. If 3-MC was added to cultures after a preincubation period of 24 hr, the enzyme activity increased after a lag time of approximately 3 hr. However, the addition of the drug to 40-hr preincubated cultures caused a more immediate rise in enzyme activity. It may be noted that the rate of rise in activity was about the same in fresh explants and in preincubated cultures once the induction process started. In 40-hr cultures, a 3- to 4-fold increase over the control level was observed 24 hr after the addition of 3-MC. Similar observations have been reported by Wicks[9] for tyrosine α-ketoglutarate transaminase in fetal rat liver explants. He reported a lag in the hydrocortisone-mediated transaminase induction in fresh explants, whereas the addition of the inducer to 42-hr cultures caused an instant increase in enzyme activity. Although the reason for this phenomenon is not understood, the lag in inducibility may be caused either by the presence of inhibitors in the fetal liver tissue or by the stage of development and differentiation of the latter. Wicks[9] proposed that the synthesis of certain enzymes may be repressed *in utero* and that the postulated repressors of enzyme synthesis may be washed out or degraded during the early period in culture. The slow rise in basal enzyme level during the early period in culture is consistent with this idea. However, the possibility cannot be excluded from these studies that changes in membrane permeability occur in fetal liver with increased culture time.

Because of the lag in "inducibility" with fresh explants, further studies on the stimulatory effects of drugs upon the hydroxylase system were performed with explants which had been maintained in culture for 24 hr.

The effects of 3-MC and its derivatives, the 1-keto, 1- hydroxy, cis-11,12-dihydroxy-11,12-dihydro, and trans-1,2-dihydroxy-3-MC (at a final concentration of 10^{-5} M) upon the BP-hydroxylase system in 24-hr cultures of fetal liver are indicated in Fig. 2.

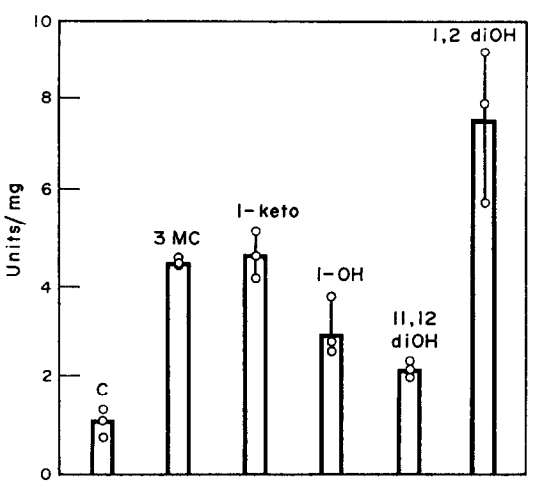

FIG. 2. Effect of derivatives of 3-MC upon BP-hydroxylase activity in 24-hr explants. DMSO (C), 3-MC, 1-hydroxy-3-MC (1-OH), 1-keto-3-MC (1 keto), cis-11,12-dihydroxy-11,12-dihydro-3-MC (11,12 diOH), or trans-1,2-dihydroxy-3-MC (1,2 diOH) was added to the liver explants in a final concentration of 10^{-5} M after 24 hr in culture. The explants were homogenized and 24 hr later BP-hydroxylase activity (units/mg of protein) was measured. Each point represents a single culture dish, and the average of three culture dishes is shown.

A 4-fold increase in enzyme activity over the control level was achieved 24 hr after addition of 3-MC. The addition of the 1-keto derivative was attended by a similar elevation in enzyme activity, whereas the administration of the 1-hydroxy derivative resulted in only a 2·7-fold elevation over the basal level. Occasionally in other experiments, the 1-keto and 1-hydroxy derivatives were equally effective. The 11,12-dihydroxy-11,12-dihydro-3-MC was less active in this regard; its administration produced a stimulatory activity of only 2-fold. The highest level of enzyme activity was observed after the addition of the trans-1,2-dihydroxy-3-MC; a 6·7-fold increase of BP-hydroxylase activity was noted after 24 hr.

DISCUSSION

The results in the present work demonstrate that the addition of 3-MC and some related compounds to explants of fetal rat liver effects a marked increase in the activity of the microsomal mixed-function oxygenase, BP-hydroxylase. This finding is not unexpected in view of the inducibility of BP-hydroxylase *in vivo* and *in vitro* by polycyclic hydrocarbons. This enzyme has been shown to be present and inducible in fetal cell cultures derived from whole animals and various tissues of different species[7] and in explants of rat lung in organ culture.[5]

The fetal liver explant system has several properties which are desirable in the study of the induction effect of polycyclic hydrocarbons. The fetuses are present in a sterile container which minimizes the bacterial contamination. The fetal liver is easily obtained, and the production of cubes is facile. The environment of the fetal liver explants can be carefully controlled and the explants are not subject to alterations in host nutrition or in circulating hormones. The culture of liver explants for the induction does not require expensive serum-containing media; the induction is easily seen in MEM–BSS containing buffer, glucose and bicarbonate.

Rat liver homogenates have been shown to catalyze the formation of a number of oxidized derivatives of 3-MC, including the ones which have been used in the present study.[8] From the studies *in vivo*, it is not known whether the parent compound, 3-MC, or one or more of its metabolites is the active "inducer" of the microsomal hydrocarbon hydroxylase system. In this regard, Bresnick *et al.*[4] reported the 1-keto derivative to be less effective than 3-MC after administration *in vivo* as an "inducer" of zoxazolamine and BP-hydroxylases; the 1-hydroxy derivative was inactive.

All four derivatives of 3-MC (1-keto, 1-hydroxy, trans-1,2-dihydroxy, and cis-11,12-dihydroxy-11,12-dihydro) induced the hydroxylase enzyme in fetal liver explants, although to varying degrees. It is of interest that the trans-1,2-dihydroxy-3-MC, which is a normal metabolite of 3-MC in liver homogenates,[8] was a potent stimulator of the hydroxylase system and, under the employed conditions, was more active than the parent compound. However, at the present time, the possibility cannot be ruled out that the differential effect of derivatives may be due partially to a difference in solubility of the drug or the intracellular saturation level, or both.

The differential effects of the derivatives of 3-MC upon the drug-metabolizing system in fetal liver explants are also interesting in the light of the studies of Sims[12] upon the carcinogenic activities of some metabolites of 3-MC. In his studies, the 1-hydroxy-3-MC and the 1-one-3-MC were active carcinogens, producing the same number of tumors as did the parent compound after subcutaneous injection into mice,

while the trans-1,2-dihydroxy-3-MC showed only weak activity. In this regard, no positive relationship was noted between the carcinogenic action of the metabolite of 3-MC in the intact animal and the "inducing" effect upon the drug-metabolizing system in fetal liver grown in culture.

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