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The Binding of 3-Methylcholanthrene to Macromolecular Components of Rat Liver Preparations

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SUMMARY

If [^{14}C] 3-methylcholanthrene (3-MC) is incubated *in vitro* with the 9000 × *g* supernatant fraction of a rat liver homogenate in the presence of a pyridine nucleotide, and with the 100,000 × *g* supernatant liquid from the incubation mixture fractionated on Sephadex G-100, two ultraviolet-absorbing, radioactively labeled peaks are observed. Peak A is eluted in the void volume and has been shown to be at least partially composed of RNA. Peak B is partially excluded and is proteinaceous. Its formation is prevented by omission from the reaction mixture of microsomes or the pyridine nucleotide; the formation of peak A is unaffected by these procedures. The formation of peak B is greatly enhanced by treatment of the animals with 3-MC 24 hr before death, whereas the formation of peak A is only slightly stimulated. Under the same conditions some formation of peak A is observed in heart and kidney, but not in spleen; peak B formation, however, is completely absent in heart and spleen and very low in kidney. The relevance of the formation of these complexes is discussed with reference to enzyme induction by 3-MC in liver.

INTRODUCTION

3-Methylcholanthrene is a potent carcinogen for most rodent tissues, although liver is relatively resistant to this action unless the drug is administered to fetal or newborn animals. In adult rat liver, however, the polycyclic hydrocarbon causes an increase in the activities of several drug-metabolizing enzymes located in the microsomal fraction (1–7). The latter effect is prevented by the simultaneous injection of puromycin (4) or actinomycin D (3, 4) or by certain amino acid analogues (6, 7), suggesting that increased enzyme synthesis is involved.

It has been established that the administration of 3-MC[1] to rats causes activation of the "aggregate" RNA polymerase system in liver (8, 9). Furthermore, the chromatin prepared from the livers of 3-MC-treated rats proved a more efficient template for *Micrococcus lysodeikticus* RNA polymerase than chromatin from untreated rats (10, 11). The nature of the RNA formed with the chromatin from 3-MC-treated rats as template was substantially different from that synthesized in the presence of control chromatin (12). These data suggested the activation of deoxyribonucleoprotein in liver as a result of 3-MC administration.

The manner in which 3-MC can produce these effects is not known. We had shown previously that within a short time after

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[1] The abbreviation used is: 3-MC, 3-methylcholanthrene.

the injection of either [^{14}C] or [^{3}H] 3-MC, binding to rat liver protein occurred (13). Since a relationship might exist between the formation of a complex between 3-MC (or metabolite) and this protein and the subsequent activation of template efficacy of liver chromatin, the binding phenomenon was studied in greater detail.

The present communication presents evidence for binding to cellular components when [^{14}C] 3-MC is incubated with the 9000 × *g* supernatant fraction of a rat liver homogenate in the presence of a reduced pyridine nucleotide. The conditions necessary for the formation of these complexes are discussed, and preliminary evidence is given as to the nature of these complexes. Furthermore, we show that binding to a protein fraction requires the presence of microsomes and is enhanced when the microsomes are isolated from the livers of rats previously treated with 3-MC. In this regard, we wish to point out the similarity of the findings presented in this report with those reported by Gelboin (14) and by Grover and Sims (15), who showed that various polycyclic hydrocarbons, including 3-MC, are bound to nucleic acids and/or protein in the presence of rat liver microsomes. Gelboin (14) also found that binding of ^{3}H-benzpyrene to DNA or RNA of rat liver was enhanced by using microsomes isolated from rats treated with 3-MC.

MATERIALS AND METHODS

Materials. Male albino rats, 80–100 g, were obtained from the Cheek-Jones Company, Houston, and were routinely deprived of food for 24 hr before death. 3-MC was obtained from Eastman Organic Chemicals, and [6-^{14}C] 3-MC, from the New England Nuclear Corporation. The 1-keto and 1-hydroxy derivatives of 3-MC were generously supplied by Dr. R. A. Seibert of this department. Pyridine nucleotides of highest purity, the disodium salt of ATP, and yeast alcohol dehydrogenase were obtained from Sigma Chemical Company. Pronase and bovine pancreatic ribonuclease were purchased from Calbiochem.

Preparation of fractions from rat organs. Homogenates of liver, spleen, kidney, and heart, obtained from three to five rats, were prepared in cold 0.25 M sucrose (1 g/4 ml) and were centrifuged for 15 min at 9000 × *g* at 4°. The supernatant fractions were stored frozen until used. In some experiments, livers were obtained from rats that had received 3-MC (20 mg/kg of body weight, intraperitoneally) 24 hr earlier. As a rule, unperfused liver was used in the preparation of homogenates. Occasionally livers were perfused with 0.9% NaCl via the portal vein and homogenates were prepared as described above. Aliquots of the 9000 × *g* supernatant fraction of liver homogenates were centrifuged at 100,000 × *g* for 1 hr at 4° to sediment the microsomes. The surface of the microsomal pellet was washed carefully with ice-cold 1.15% KCl. The pellet was stored frozen, covered with a layer of 0.1 M sodium phosphate buffer, pH 7.5. The 100,000 × *g* supernatant fraction was also stored frozen until used. Whole blood was obtained by heart puncture, the blood cells were removed by low-speed centrifugation in a table-top centrifuge, and the serum was stored frozen.

Assay for binding of 3-MC. Incubation mixtures contained the following components in a final volume of 1.5 ml: sodium phosphate buffer, pH 7.5, 100 μmoles; NAD^+, 0.13 μmole; $NADP^+$, 0.11 μmole; [^{14}C] 3-MC, 0.09 μmole (0.25 μCi) in 0.025 ml of dimethyl sulfoxide; and an appropriate amount of extract. After incubation at 37° for 10 min (unless otherwise stated), the reaction was stopped by plunging the tubes into an ice-water mixture. Particulate matter was removed by centrifugation at 100,000 × *g* for 1 hr. The supernatant liquid was applied to a column (2 × 24 cm) of Sephadex G-100 which had been equilibrated with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.05 M KCl; the column was eluted with the same buffer. The column was packed and eluted at room temperature. Approximately 1-ml fractions were collected and monitored for protein and nucleic acid by absorption at 280 nm. Fractions of 0.2 ml were counted in 10 ml of Bray's solution (16) in a Packard liquid scintillation spectometer. The counting efficiency was determined to be about 85% in

all cases. Protein was determined by the method of Lowry *et al.* (17).

Enzyme treatment. Ribonuclease, 2 mg/ml, was added to the reaction mixture immediately following incubation, and the tubes were kept at 37° for an additional 60 min. Binding was assayed as described above. Pronase, 2 mg/ml, was added to the reaction mixture, which was then incubated for 4 hr at 37°. The reaction mixtures were then centrifuged at 100,000 × *g* for 1 hr and the supernatant fluid was fractionated on Sephadex G-100 as previously described.

Molecular weight determination. Molecular weights were determined by elution from Sephadex G-100 columns, using proteins of known molecular weights as standards. Fractions containing the bound 3-MC (or metabolite) were applied to a column (3 × 40 cm) of Sephadex G-100 and eluted with 0.05 M Tris-HCl, pH 8.0, containing 0.05 M KCl. The void volume of the column was 65 ml, and 1-ml fractions were collected. The standards employed were cytochrome *c* (mol wt 12,000), hemoglobin (mol wt 68,000), purified regenerating rat liver deoxythymidine kinase (mol wt 81,000) (18), and yeast alcohol dehydrogenase (mol wt 126,000). It is known from the extensive studies of Whitaker (19), Andrews (20), and Ackers (21) that hemoglobin, under our conditions of gel filtration, dissociates into subunits and has an elution volume equivalent to its subunit molecular weight, i.e., 34,000. The elution volumes of cytochrome *c*, hemoglobin subunit, deoxythymidine kinase, and alcohol dehydrogenase in three to four runs were 154 ± 2 ml (mean ± standard error), 124 ± 2 ml, 95 ± 2 ml, and 81 ± 2 ml, respectively. Cytochrome *c* and hemoglobin were detected by their absorption at 412 nm; alcohol dehydrogenase, by the reduction of NAD^+ in the presence of ethyl alcohol; and deoxythymidine kinase, as described previously (18).

RESULTS

Binding of 3-MC to macromolecular components of rat liver. Figure 1A shows a typical elution pattern obtained from chromatography on Sephadex G-100 of the 100,000 × *g* supernatant liquid from an incubation mixture containing the 9000 × *g* supernatant fraction of a rat liver homogenate, [^{14}C] 3-MC, and cofactors as described under MATERIALS AND METHODS. Three ultraviolet-absorbing, radioactively labeled peaks were observed. Peak A was totally

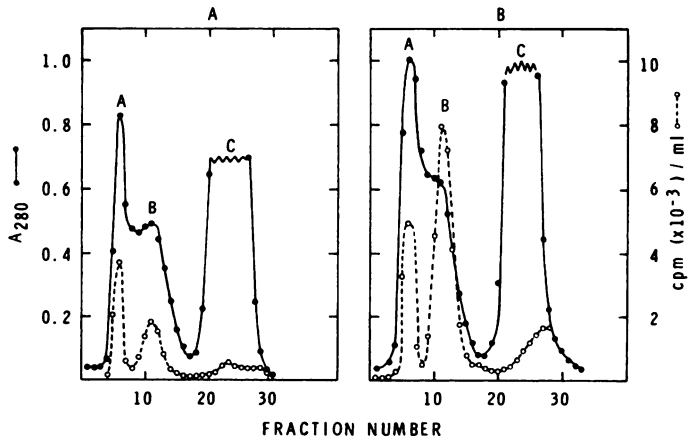

FIG. 1. *Binding of 3-MC to macromolecular components of rat liver*

Incubation mixtures contained the following components in a final volume of 1.5 ml: sodium phosphate buffer, pH 7.5, 100 μmoles; [^{14}C]3-MC, 0.25 μCi; NAD^+, 0.13 μmole; $NADP^+$, 0.11 μmole; and 0.4 ml of the 9000 × *g* supernatant fraction of a rat liver homogenate containing 21 mg of protein from control rats (A) or from rats treated with 3-MC as described under MATERIALS AND METHODS (B). The mixtures were incubated for 15 min, and the supernatant fraction was separated on Sephadex G-100 as described in the text.

excluded, and peak B was partially excluded from the Sephadex column. Peak C represents low molecular weight, ultraviolet-absorbing material present in the extract; the radioactivity eluted at this point is either unbound 3-MC or an unbound metabolite of this compound. These fractions were pooled and aliquots were taken for the determination of radioactivity as previously described.

Figure 1B shows the effect on the binding reaction of the intraperitoneal administration of unlabeled 3-MC, 20 mg/kg of body weight in corn oil, 24 hr prior to death. It can readily be seen that this treatment greatly enhanced peak B formation, whereas peak A formation was relatively unaffected. Since 3-MC treatment had such a pronounced effect on peak B formation, the animals were routinely treated with injections of 3-MC at 8:00 a.m., 24 hr prior to death.

Figure 2 shows that the rate of formation

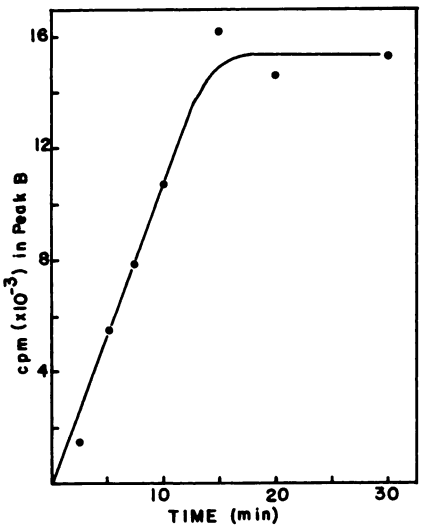

FIG. 2. *Kinetics of peak B formation*

Incubation mixtures contained the following components in a final volume of 1.5 ml: sodium phosphate buffer, pH 7.5, 100 μmoles; [^{14}C] 3-MC, 0.25 μCi; NADPH, 1.2 μmoles; and 0.2 ml of the 9000 × *g* supernatant fraction of a "stimulated" rat liver homogenate, containing 8.0 mg of protein. The mixtures were incubated for the times indicated and assayed for binding as described in the text.

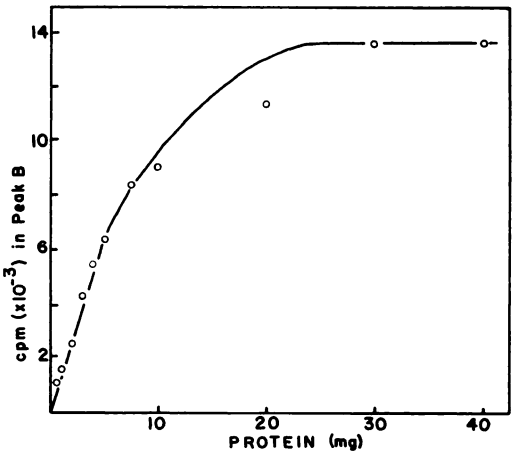

FIG. 3. *Effect of microsome concentration on peak B formation*

Incubation mixtures contained the following components in a final volume of 1.5 ml: sodium phosphate buffer, pH 7.5, 100 μmoles; NADPH, 1.2 μmoles; [^{14}C] 3-MC, 0.25 μCi; 0.2 ml of the 100,000 × *g* supernatant fraction of a rat liver homogenate, containing 6.5 mg of protein; and increasing amounts of a microsomal suspension containing the amounts of protein indicated. Both fractions were obtained from rats which had been treated with 3-MC 24 hr previously. The mixtures were incubated at 37° for 10 min and assayed for binding as described in the text.

of peak B was linear under the assay conditions for 10–15 min, after which it formed a plateau. Further incubation did not result in the breakdown of peak B.

Substitution of the 100,000 × *g* for the 9000 × *g* supernatant fraction of a rat liver homogenate prevented the formation of peak B, while peak A formation remained normal. Figure 3 shows the effect on peak B formation of adding increasing amounts of a microsomal fraction to incubation mixtures containing the 100,000 × *g* supernatant fraction and the appropriate cofactors. It can be seen that increasing amounts of microsomal protein caused an increase in peak B formation. Under the conditions of the assay, maximum binding was achieved with approximately 25 mg of microsomal protein.

Table 1 shows that the increase in peak B formation caused by prior treatment with 3-MC is a function of the microsomal fraction. When the 100,000 × *g* supernatant

TABLE 1

Effect on peak B formation of prior treatment of animals with 3-methylcholanthrene

Incubation mixtures contained the following components in a final volume of 1.5 ml: sodium phosphate buffer, pH 7.5, 100 μmoles; [^{14}C] 3-MC, 0.25 μCi; NADPH, 1.2 μmoles; and 0.2 ml of the 100,000 × *g* supernatant fraction of a rat liver homogenate and 0.2 ml of a suspension of microsomes isolated from rat liver. The animals were treated as indicated. The amount of protein in the 100,000 × *g* supernatant fractions (6 mg/assay) was approximately the same in each case, as was the protein concentration of the microsomal suspensions (2 mg/assay). The mixtures were incubated at 37° for 10 min and assayed for binding as described in the text.

Microsomes	100,000 × *g* supernatant fraction	Radioactivity in peak B
		cpm
Control	Control	2810
Control	3-MC	2080
3-MC	Control	6010
3-MC	3-MC	6420

fraction from 3-MC-treated animals and microsomes from control animals were employed, peak B formation was no greater than when both fractions were obtained from control rats. On the other hand, the amount of peak B formed when both the 100,000 × *g* supernatant fraction and the microsomal fraction were obtained from 3-MC-treated rats was only slightly greater than when only the microsomal fraction was obtained from 3-MC-treated rats.

We also found that incubation of [^{14}C] 3-MC with liver microsomes isolated from treated rats, rat serum, and NADPH resulted in the formation of ultraviolet-absorbing, radioactively labeled peaks in the same regions as peaks A and B. However, the labeling in peak B was not due to blood protein, since binding to liver preparations was quantitatively similar in perfused and unperfused livers. These results suggest either that the liver microsomes per se are the source of the macromolecules which bind the polycyclic hydrocarbon or that serum as well as liver contains possible binding proteins. Although we have not been able to rule out the former possibility definitively, certain evidence does not favor its acceptance. For example, no labeled peak A or B was observed if a washed microsomal preparation previously incubated with cofactors and labeled 3-MC was transferred to a 100,000 × *g* liver supernatant fraction.

TABLE 2

Effect of pyridine nucleotides on peak B formation

Incubation mixtures contained the following components in a final volume of 1.5 ml: sodium phosphate buffer, pH 7.5, 100 μmoles; [^{14}C] 3-MC, 0.25 μCi; and an appropriate amount of the 9000 × *g* supernatant fraction of a homogenate prepared from unperfused rat liver. NAD^+ or NADH, 2.7 μmoles; $NADP^+$ or NADPH, 2.4 μmoles; ATP, 0.83 μmole; and $K_3Fe(CN)_6$ or $K_4Fe(CN)_6$, 4.8 μmoles, were added as shown. Incubations were carried out at 37° for 5 min, and the supernatant fractions were eluted from Sephadex G-100 as described in the text.

Addition	Radioactivity in peak B
	cpm/mg protein
None	39
NAD	403
NADP	386
NADH	533
NADPH	527
ATP	53
NADPH + $K_3Fe(CN)_6$	40
NADPH + $K_4Fe(CN)_6$	412

The nucleotide requirement for the formation of peaks A and B was determined, and these results are presented in Table 2. Omission of pyridine nucleotide resulted in a decrease of approximately 90% in the amount of peak B formed. Peak A, however, was not affected by the omission of pyridine nucleotide. All the pyridine nucleotides were active, the reduced forms possibly being more so. This observation was confirmed by keeping the system in the oxidized state by the addition of $K_3Fe(CN)_6$; no radioactivity could be detected in the region corresponding to peak B. Addition of $K_4Fe(CN)_6$ with NADPH did not affect activity appreciably. Addition of ATP resulted in the formation of only a slight amount of peak B. The results obtained with preparations from perfused

liver were comparable with those shown in Table 2 for unperfused liver.

The requirement of the microsomal fraction and a pyridine nucleotide for peak B formation suggested that metabolism of 3-MC occurs before binding takes place. However, two oxidized derivatives of 3-MC, the 1-keto and 1-hydroxy compounds, were found to be no more competitive than unlabeled 3-MC in the binding reaction in which peak B is formed.

Tissue specificity of the binding reaction. No significant formation of peak B was observed when [^{14}C] 3-MC was incubated with the 9000 × *g* supernatant fractions of rat heart or spleen in the presence of the cofactors required for its formation in liver. Some bound polycyclic hydrocarbon was noted in the peak A region. However, when 9000 × *g* supernatant fractions of rat kidney were employed, radioactivity could be detected in the areas corresponding to both peaks A and B, although the amount in the latter was less than 5% of that found in the liver. Furthermore, only in liver was increased peak B formation observed following 3-MC treatment.

Characterization of peaks A and B. Table 3 shows the effect of incubating the reaction mixtures with RNase and Pronase as described under MATERIALS AND METHODS. It can be concluded that peak B is substantially protein in nature, whereas peak A appears to be composed, at least in part, of RNA. The molecular weight of the protein moiety of peak B was determined by gel filtration as described under MATERIALS AND METHODS. The elution volume (Fig. 4) of peak B was found in four separate runs to be 107 ± 1 ml (mean ± standard error), corresponding to a molecular weight of 59,000 ± 1,000.

TABLE 3

Effect of ribonuclease and Pronase treatment on binding of 3-methylcholanthrene

Incubation mixtures contained the following components in a final volume of 1.5 ml: sodium phosphate buffer, pH 7.5, 100 μmoles; [^{14}C] 3-MC, 0.25 μCi; NAD^+, 0.14 μmole; $NADP^+$, 0.11 μmole; and 0.4 ml of the 9000 × *g* supernatant fraction of a rat liver homogenate containing 21 mg of protein. Ribonuclease A (five times recrystallized; 50 Kunitz-McDonald units/mg), 2 mg/ml, and Pronase (3 units/mg), 2 mg/ml, were added as indicated. The control tubes and the tubes to which the enzymes had been added were incubated for an additional 60 min at 37°. The amount of binding was assessed as described under MATERIALS AND METHODS.

Treatment	Radioactivity in	
	Peak A	Peak B
	cpm	*cpm*
None	7,860	15,000
Ribonuclease	3,380	14,000
Pronase	5,870	2,000

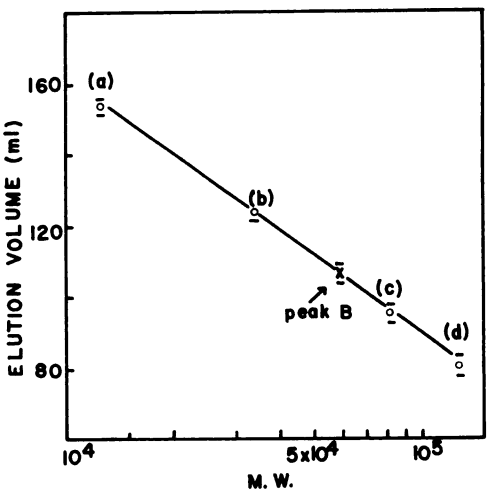

FIG. 4. *Determination of molecular weight of peak B*

Peak B was subjected to gel filtration on a column (3 × 40 cm) of Sephadex G-100, and the elution volume was determined. Four proteins of known molecular weights—cytochrome *c* (mol wt 12,000) (*a*), hemoglobin subunits (mol wt 34,000) (*b*), deoxythymidine kinase (regenerating rat liver) (mol wt 81,000) (*c*), and alcohol dehydrogenase (mol wt 126,000) (*d*)—were used as standards. Under the conditions of the separation, hemoglobin dissociates into its subunits. The figure shows a plot of elution volume with respect to the logarithm of molecular weight (M.W.). The molecular weight of peak B was determined to be 59,000 ± 1,000. Three to four determinations of the elution volume of each of the standards, and four of peak B, were made. The bars at each point indicate the standard error.

DISCUSSION

The results described above indicate that 3-MC (or a derivative of it) can bind to

macromolecular components of the 9000 × *g* supernatant fraction of a rat liver homogenate. Binding of this compound to an RNA-containing fraction has been observed in rat spleen, heart, liver, and kidney; binding to a protein fraction was observed in kidney and liver. However, the extent of binding in kidney was very much less than that in liver. In addition, binding in kidney was not simulated by prior treatment of the animals with 3-MC, as was the case in liver. The significance of this finding is not known at present.

At this time, it is not clear whether or not metabolism of 3-MC occurs prior to binding. Since the formation of peak A appears to be independent of added cofactors, it seems probable that in this case 3-MC is not metabolized prior to binding. However, the possibility that the cofactors required for metabolism are present in the 100,000 × *g* supernatant fraction cannot be ruled out. The formation of peak B may involve the prior metabolism of 3-MC, since both a microsomal fraction and a pyridine nucleotide (probably NADPH) are required. The metabolism of many agents takes place in the microsomal fraction of liver; it involves the mixed-function oxidase system and requires both NADPH and oxygen as cofactors.

It is of interest that microsomes isolated from animals treated with 3-MC increased the quantitative aspects of peak B formation. This stimulation could be predicted if 3-MC is metabolized prior to binding, since 3-MC is a potent stimulator of its own metabolism (reviewed in ref. 22).

Several groups of workers have demonstrated the binding of polycyclic hydrocarbons to DNA (14, 15, 23–29), RNA (28, 29), and protein (29–35). Furthermore, Gelboin (14) and Grover and Sims (15) have shown that the binding of various polycyclic hydrocarbons, including 3-MC, to DNA requires the presence of a microsomal fraction and NADPH. In addition, Gelboin (14) found that the binding of ^{3}H-benzpyrene to DNA *in vitro* was increased when microsomes from 3-MC-treated animals were used.

If 3-MC is metabolized prior to binding, metabolites of the polycyclic hydrocarbon should compete with 3-MC for binding sites on the protein in peak B. Preliminary experiments were conducted along these lines with the 1-keto and 1-hydroxy derivatives of 3-methylcholanthrene. If either of these two derivatives were on the pathway leading to bound polycyclic hydrocarbon, preferential dilution of the labeled 3-MC would have been anticipated, which would result in a reduction in the amount of the latter fixed in peak B. Unfortunately, neither derivative was any more effective in this respect than unlabeled 3-MC itself. Two possible explanations are that (a) 3-MC is not metabolized prior to binding or (b) these metabolites are not directly on the pathway to bound hydrocarbon. It is not possible at present to determine which of these possibilities is correct.

It is not known at this time whether the receptor for 3-MC or derivative is initially present in the microsomes and is then transferred to the soluble compartment as a result of binding, or whether the protein is in the soluble state. Several attempts involving incubation of the microsomal fraction with [^{14}C] 3-MC, followed by incubation of both the particulate and soluble fractions of these preliminary incubation mixtures with the 100,000 × *g* supernatant fraction, in the hope of demonstrating metabolism followed by binding, have met with no success. This might indicate that the whole reaction takes place bound to the protein and that free metabolites cannot participate in the binding reaction. Furthermore, the lack of specificity of the pyridine nucleotide requirement might indicate that a pyridine nucleotide is required for binding as well as for metabolism.

One can only speculate at this time about the function of the bound 3-MC (or derivative). It is interesting that the reaction is fast and appears to occur *in vivo* (13). It is conceivable that the binding serves as a means by which a lipophilic substance may be selectively steered into a particular hepatic organelle, i.e., nucleus, ultimately to activate or amplify a specific genomic region.

Experiments are now in progress to determine the nature of the bound polycyclic hydrocarbon and to ascertain whether this complex can increase the efficacy of normal

liver chromatin as a template in the synthesis of RNA.

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