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SHORT COMMUNICATIONS

Induction of Drug Metabolism

V. Independent Formation of Cytochromes P-450 and P_1-450 in Rats Treated with Phenobarbital and 3-Methylcholanthrene Simultaneously

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SUMMARY

The administration of 3-methylcholanthrene to rats causes the formation of cytochrome P_1-450, a variant of cytochrome P-450. One concept holds that cytochrome P_1-450 is formed when 3-methylcholanthrene or one of its metabolites combines irreversibly with the type I binding site of previously existing cytochrome P-450. Another concept submits that cytochrome P_1-450 is synthesized independently of cytochrome P-450 and very probably does not contain 3-methylcholanthrene or one of its metabolites. The current study supports the second concept. When phenobarbital, an agent known to induce the synthesis of cytochrome P-450, and 3-methylcholanthrene are administered simultaneously, microsomal levels of cytochrome P-450 hemoprotein, the binding of hexobarbital and aniline to this hemoprotein, and microsomal 3-methyl-4-methylaminoazobenzene *N*-demethylase activity are elevated nearly to the sums of each of these measurements obtained when the inducing agents are given singly. It was reasoned that if cytochrome P_1-450 results simply from the formation of a stable cytochrome P-450–polycyclic hydrocarbon complex, the P-450 hemoprotein obtained after simultaneous administration of phenobarbital and 3-methylcholanthrene should be entirely in the form of cytochrome P_1-450. Studies of the induction of cytochromes P-450 and P_1-450 led to the conclusion that the two hemoproteins are synthesized independently of each other. If 3-methylcholanthrene or one of its metabolites is incorporated into cytochrome P_1-450, the process must occur during the synthesis of new hemoprotein.

Previous publications from our laboratory showed that the administration of 3-methylcholanthrene and other polycyclic hydrocarbons to rats caused the induction of a microsomal P-450 hemoprotein which differed in certain of its physical and biochemical characteristics from that found in hepatic microsomes obtained from untreated animals or animals in which the microsomal drug hydroxylase system had been induced by phenobarbital administration (1–6). This hemoprotein was named cytochrome P_1-450 to designate a previously unrecognized species of cytochrome P-450. Because it is not found in meaningful amounts in the livers of untreated rats and because of its apparent lack of a type I binding site, cytochrome P_1-450 might be thought of as an aberrant cytochrome (4). Cytochrome P_1-450 is frequently referred to as cytochrome P-448 because it gives a maximum absorption peak at 448 mμ rather than at 450 mμ when it is reduced and combined with carbon monoxide

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(7). Schenkman and associates (8) proposed that the changes seen in microsomal hemoprotein after administration of polycyclic hydrocarbons result simply from the irreversible addition of the inducing agent, or one of its metabolites, to the type I binding site of native cytochrome P-450. Others have argued that cytochrome P-450 results from the biosynthesis of new hemoprotein without incorporation of the polycyclic hydrocarbon or one of its metabolites (4, 7, 9–12). The current studies support the latter view.

Simultaneous administration of maximal stimulatory doses of 3-methylcholanthrene and phenobarbital to rats causes additive or nearly additive stimulation of microsomal levels of P-450 hemoprotein and 3-methyl-4-methylaminoazobenzene *N*-demethylase activity (1–3). If cytochrome P_1-450 is simply a stable cytochrome P-450–polycyclic hydrocarbon complex, it should be expected that when 3-methylcholanthrene and phenobarbital are given simultaneously, the polycyclic hydrocarbon or its metabolite would combine with the cytochrome P-450 that formed as a result of phenobarbital administration as well as with that which was present initially or which resulted from 3-methylcholanthrene treatment. All of the newly formed P-450 hemoprotein should be in the form of cytochrome P_1-450. Instead, it appeared to be the mixture of cytochrome P-450 and cytochrome P_1-450 that one might expect to find if each of the hemoproteins were formed independently (3, 4). This in itself might dispose of the concept of a hemoprotein–polycyclic hydrocarbon complex as the explanation for the formation of cytochrome P_1-450, except that it can be argued that phenobarbital and 3-methylcholanthrene, both of which produce a type I binding spectrum with microsomes from the rat, may compete for the type I binding site *in vivo* and prevent all of the hemoprotein from combining with 3-methylcholanthrene or one of its metabolites. The unstable cytochrome P-450–phenobarbital complex thus formed would dissociate during preparation of the microsomes, with cytochrome P-450 being retained in the microsomes and phenobarbital being released to the supernatant fraction, whereas the stable hemoprotein complex formed with the polycyclic hydrocarbon (cytochrome P_1-450) would remain in the microsomes. The current study was designed in consideration of this possibility.

Phenobarbital sodium and 3-methylcholanthrene were given to male Simonsen rats (100–160 g) intraperitoneally in amounts known to produce maximal or nearly maximal inductive effects within 4 days. Groups of rats received either (a) phenobarbital sodium (40 mg/kg/day in 0.9% NaCl solution), (b) 3-methylcholanthrene (20 mg/kg/day in corn oil), (c) both of these inducing agents, or (d) 0.9% NaCl or corn oil (control group) for 4 days. Phenobarbital administration was then stopped and daily 3-methylcholanthrene administration was continued in all animals that had been receiving 3-methylcholanthrene, whether with or without phenobarbital, until the rats were killed on successive days of the remainder of the 9-day experiment. Livers were removed 20 hr after the last injection, and microsomes (105,000 × *g* pellet) were prepared as described previously (2) and used on the same day. The microsomal preparations were assayed for total protein and total P-450 hemoprotein (P-450 + P_1-450) contents, the ratio of the ethyl isocyanide binding peaks at pH 7.4 (A_{455}:A_{430}), hexobarbital (type I) and aniline (type II) binding, and 3-methyl-4-methylaminoazobenzene and ethylmorphine *N*-demethylase activities by methods described previously (2–4). These measurements were selected because they reflect the relative amounts of cytochromes P-450 and P_1-450 in the microsomes. Thus, aniline binding, the A_{455}:A_{430} ratio, and 3-methyl-4-methylaminoazobenzene *N*-demethylase activity increase with the formation of cytochrome P_1-450 that follows 3-methylcholanthrene administration, while hexobarbital binding is reduced and the rate of ethylmorphine *N*-demethylation is unaffected or reduced slightly (1–6). Phenobarbital administration increases all of these measurements with the exception of the A_{455}:A_{430} ratio, which remains unchanged. The effects of both inducing agents are essentially complete after 4 days of injection. From previous experience it was expected that changes in the micro-

somes that had occurred as a result of 4 days of phenobarbital administration would gradually disappear after its discontinuation and would no longer be evident by the 9th day of the experiment. The properties of microsomes from rats that had been treated with 3-methylcholanthrene for 9 days were expected to remain essentially unchanged from the 4th through the 9th days of the experiment. At the end of the experiment the properties of the microsomes from animals which had received both inducing agents were expected to depend upon whether or not cytochrome P_1-450 results from the direct combination of hemoprotein and a polycyclic hydrocarbon to form a stable complex. Shortly after the discontinuation of phenobarbital administration, residual phenobarbital should be removed from the animal by biotransformation, thereby eliminating any competition it might have exerted previously in preventing the polycyclic hydrocarbon from combining with the type I binding site of cytochrome P-450. The reaction between cytochrome P-450 and 3-methylcholanthrene would then be unimpaired by phenobarbital, and the newly formed cytochrome P-450 which resulted from phenobarbital administration would be converted to cytochrome P_1-450. The level of cytochrome P_1-450 in the microsomes at the end of the experiment would be higher than that seen in animals which had received 3-methylcholanthrene, but no phenobarbital, by an amount corresponding approximately to the cytochrome P-450 formed as a result of phenobarbital administration minus the amount of cytochrome P-450 lost during the biotransformation occurring while phenobarbital was depleted. On the other hand, if 3-methylcholanthrene or one of its metabolites does not combine directly with cytochrome P-450 to form cytochrome P_1-450, and the two cytochromes are formed by independent processes, at the end of the experiment the cytochrome P-450 that had been caused to appear as a result of phenobarbital administration would have disappeared, leaving only the cytochrome P_1-450 that had been caused to appear as a result of 3-methylcholanthrene administration. The latter should correspond approximately to the amount of cytochrome P_1-450 observed in microsomes from rats which had received 3-methylcholanthrene only throughout the experiment. The data presented in Fig. 1 show the latter alternative to be the case.

At the end of 4 days of combined administration of phenobarbital and 3-methylcholanthrene, the increase in microsomal content of CO-binding pigment (P-450 + P_1-450) was about 80% of the sum of that seen in microsomes from rats which received the two inducing agents singly (Fig. 1A). By the end of 9 days, levels declined to normal in microsomes from animals which had received phenobarbital alone for 4 days. The content of CO-binding pigment of microsomes from animals which had received both inducing agents declined only to the level observed in microsomes from rats which had received 3-methylcholanthrene alone. Significantly, parallel rates of decline in CO-binding pigment were seen in the microsomes from these two groups of rats. Concomitantly, the ratio of the 455 mμ and 430 mμ peaks rose in microsomes from the group receiving both inducing agents, to the level seen in microsomes from animals which had received 3-methylcholanthrene only (Fig. 1B). Since the elevation of this ratio is indicative of the formation of cytochrome P_1-450, it is apparent from the figure that at 4 days the microsomes from the rats receiving both inducing agents contained a mixture of cytochromes P-450 and P_1-450 and that during the period from 4 to 9 days the cytochrome P-450 that had resulted from phenobarbital induction disappeared, leaving only the cytochrome P_1-450 that had resulted from 3-methylcholanthrene induction. Thus, when parts A and B of Fig. 1 are compared, it is seen that both quantitative and qualitative changes occur in the hemoprotein when the two inducing agents are used in combination, and that these changes favor the view that phenobarbital and 3-methylcholanthrene inductions are separate phenomena.

Both phenobarbital and 3-methylcholanthrene increase aniline binding to hemoprotein (4). In Fig. 1C it may be seen that, when used in combination, the two inducing agents caused an increase in aniline binding which was about 75% of the sum of the

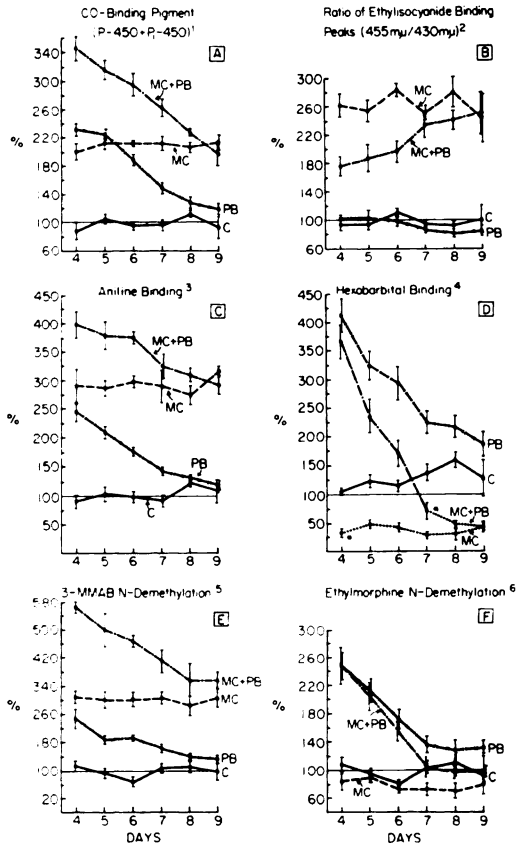

FIG. 1. *Induction of cytochrome P-450 and P_1-450 by phenobarbital and 3-methylcholanthrene*

Rats were given phenobarbital sodium (PB), 40 mg/kg daily, intraperitoneally, for 4 days and/or 3-methylcholanthrene (MC), 20 mg/kg daily, intraperitoneally, for 4–9 days. Livers were removed 20 hr after the last injection, and microsomes (105,000 × *g* pellet) were prepared and used in the six determinations on the same day. Preparations for spectral studies contained 1.0 mg of microsomal protein per milliliter. Incubation mixtures contained 0.4–0.7 mg of microsomal protein per milliliter. The concentrations of aniline and hexobarbital used in the binding studies were 6.67 and 2.5 mM, respectively. The concentrations of ethylmorphine and 3-methyl-4-methylaminoazobenzene (3-MMAB) used in the *N*-demethylation studies were 2.0 and 0.2 mM, respectively. Results are given as percentages of control values, which were obtained using livers from 12 untreated rats at the beginning of the experiment. Group C consisted of rats which received only 0.9% NaCl or corn oil injections during the first 4 days of the experiment. Each point represents the mean value obtained from five rats, and the vertical bars indicate the standard errors. The number of days refers to the days on which injections were given, not the day the animal was killed which was 20 hr after the last injection.

[1] A. $\Delta(A_{448\text{-}450} - A_{490})$; 100% = 0.070 absorbance unit/mg of protein. [2] B. Ratio of $\Delta(A_{455} - A_{490})$ to $\Delta(A_{430} - A_{490})$; 100% = 0.70. [3] C. $\Delta(A_{435} - A_{400})$; 100% = 0.023 absorbance unit/mg of protein. [4] D. $\Delta(A_{390} - A_{420})$; 100% = 0.016 absorbance unit/mg of protein. [5] E. 100% = 62.0 mμmoles of formaldehyde formed per milligram of protein per hour. [6] F. 100% = 487 mμmoles of formaldehyde formed per milligram of protein per hour.

* Dotted portion of curve indicates that a modified spectrum was observed; values were based on $\Delta(A_{415} - A_{430\text{-}435})$.

increases observed when each agent was administered separately. Aniline binding decreased during the 4–9-day period about as rapidly in microsomes from rats which had received both inducing agents as in microsomes from animals that had received phenobarbital alone. Moreover, the level at the end of the experiment was the same as that found in microsomes from animals which had received 3-methylcholanthrene only, which again supports the view that cytochrome P-450 had disappeared during the 4–9-day period, leaving only the cytochrome P_1-450 that resulted from 3-methylcholanthrene administration. The data present in Fig. 1D confirm the observation that phenobarbital increases and 3-methylcholanthrene decreases hexobarbital binding to hemoprotein. The elevated hexobarbital binding value seen in microsomes from animals which had received only phenobarbital declined rapidly after withdrawal of the drug and almost reached the control level by the end of the experiment. Hexobarbital binding by microsomes from animals which had received both inducing agents declined to the subnormal level seen in microsomes from animals which had received 3-methylcholanthrene only. Not only was the magnitude of binding altered, but a qualitative change in binding characteristics also occurred. Hexobarbital gave a typical type I binding spectrum with microsomes from untreated and phenobarbital-treated animals, with a

peak at about 390 mμ and a trough at about 420 mμ. The hexobarbital binding spectrum seen with microsomes from animals treated with 3-methylcholanthrene gave a spectrum with a peak at about 415 mμ and a trough at 430–435 mμ. When both inducing agents were administered, microsomes showed a typical type I binding spectrum with hexobarbital at the end of the 4-day period, but 3 days after withdrawal of the phenobarbital the spectrum changed to the modified type seen in microsomes from animals which had received 3-methylcholanthrene but no phenobarbital. This is interpreted to mean that a mixture of cytochromes P-450 and P_1-450 existed in the microsomes of the animals given both inducing agents for 4 days, and that after withdrawal of the phenobarbital, cytochrome P-450 gradually disappeared, until by the 7th day its concentration in the microsomes was reduced to a degree such that the presence of cytochrome P_1-450 could be revealed by the modified spectrum given with hexobarbital.

Phenobarbital induces increased microsomal ethylmorphine and 3-methyl-4-methylaminoazobenzene *N*-demethylation, but 3-methylcholanthrene induces the *N*-demethylation of the latter compound only (1–3). In Fig. 1E it can be seen that the increase in 3-methyl-4-methylaminoazobenzene *N*-demethylation obtained after 4 days of administration of both inducing agents was about equal to the sum of the increases obtained when each of the agents was given separately. After discontinuation of phenobarbital, the level of 3-methyl-4-methylaminoazobenzene *N*-demethylase declined to that observed in microsomes from animals which had received 3-methylcholanthrene only. This decline was essentially parallel to that seen in microsomes from animals which had received phenobarbital only. Ethylmorphine *N*-demethylase activity was somewhat less than normal in microsomes from 3-methylcholanthrene-treated rats during the 4–9-day period (Fig. 1F). This was reflected in the decline of ethylmorphine *N*-demethylase seen after phenobarbital withdrawal in microsomes from rats which received both inducing agents, the decline being somewhat greater than that observed in microsomes from rats which had received phenobarbital only.

These studies support the view that cytochromes P-450 and P_1-450 are synthesized independently of each other and that cytochrome P_1-450 does not result from a direct combination of cytochrome P-450 with 3-methylcholanthrene or one of its metabolites. If polycyclic hydrocarbons or their metabolites are incorporated into cytochrome P_1-450, the process must accompany the synthesis of new hemoprotein.

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