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Liver Growth Associated with the Induction of Demethylase Activity after Injection of 3-Methylcholanthrene in Immature Rats[1]

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SUMMARY

One intraperitoneal injection of 1 mg of 3-methylcholanthrene into immature male and female rats results in an increase in azo dye demethylase activity. This is followed by an increase in wet and dry liver weight and in total protein, which reaches a peak of about 30–40% above control levels within 3 or 4 days. Cell proliferation contributes to the increase in liver weight and protein as indicated by an increase in the total DNA and nuclear count. Some of the increase in cell number is probably due to hepatocyte proliferation since there is an increase in heptaocyte mitotic activity.

Between 5 and 7 days after injection of 3-methylcholanthrene, the liver weight, protein, DNA, and nuclear count begin to decrease to control values, which they reach by 14 days. The decline in these parameters of liver growth is closely associated with a decrease in the azo dye demethylase activity.

INTRODUCTION

In rats, the administration of drugs like phenobarbital or 3-methylcholanthrene results in an increase in liver weight and total protein which is associated with an increase in drug-metabolizing enzyme activity of the liver (1, 2, 4–10). The mechanism by which this increase in liver weight and protein is brought about has been investigated after phenobarbital administration in both immature male and female rats (2, 4). However, no detailed studies of the daily changes in liver weight and protein after 3-methylcholanthrene treatment are available for rats, except for the investigation of Arcos *et al.* (1) on the daily changes in total protein, and this study is limited to the first four days after 3-methylcholanthrene injection. Nor are there any reports on the contribution of cell proliferation and cell enlargement to the increase in liver size after 3-methylcholanthrene treatment although it has been reported, in passing, that cell proliferation occurs (6).

This report presents the results of our investigation, in immature rats, on the changes in liver weight, protein, DNA, nuclear count, and hepatocyte mitotic activity following the injection of 1 mg of 3-methylcholanthrene, not only during the period of liver growth but also during the period in which the liver returns to its normal size. A preliminary report has appeared (13).

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MATERIALS AND METHODS

The immature rats used in this investigation came from the colony of Dr. Richard Levy, Department of Bacteriology and Botany, Syracuse University, or were purchased either from Holtzman Company, Madison, Wisconsin, or from Miller Farms, Cazenovia, New York. Rats were placed in pairs, in wire-bottom slung cages, in an air-conditioned animal room with a photo-period of 12 hours light and 12 hours dark. The rats were fed a 27% protein synthetic diet (Normal Protein Test Diet, sold by Nutritional Biochemicals, Cleveland, Ohio).

The rats were allowed 5–7 days for acclimatization, and during this period they were weighed daily. If they did not show expected weight gains they were discarded. Body weight and food and water intake were measured daily in one group of rats injected with 3-methylcholanthrene or corn oil throughout the acclimatization and experimental periods. There were no significant differences in body weight or food or water consumption during these periods.

Rats were injected intraperitoneally with 1 mg of 3-methylcholanthrene (Eastman Chemicals, Rochester, New York) or with 0.5 ml corn oil. At intervals the rats were killed by light anesthesia followed by decapitation. After thorough bleeding the livers were removed, blotted on paper towels, placed in aluminum dishes, and weighed with a Mettler balance to the nearest mg. In the experiments in which the livers were used for dry weight determinations, the livers were cut up and dried to constant weight in a drying oven.

All injecting and sacrificing were done between 9 and 10 A.M. to avoid differences due to diurnal rhythms.

The azo dye demethylase activity, protein, nuclear count, and DNA determinations were made using ten percent homogenates in 0.25 M sucrose as previously described (3–5). The substrate used for determining demethylase activity, 3-methyl-4-monomethylaminoazobenze, was a gift from Dr. J. A. Miller, University of Wisconsin, Madison, Wisconsin. For any one day the results represent the sum of three separate determinations in which 2 experimental and 2 control rats were used for each determination.

For the study of hepatocyte mitotic activity, rats were injected with 0.3 mg of colcemide (CIBA Pharmaceutical Co.) five hours prior to sacrifice. Upon sacrifice, a small piece of the left lateral lobe was removed and processed for histologic study (4). Hepatocyte mitotic activity was determined by counting the number of colcemide-arrested metaphases under oil immersion (X 970) in a standard area outlined by a square

reticule. The number of mitotic figures and the number of hepatocyte nuclei in 50 fields in at least three separate non-adjacent sections were counted.

Separate groups of rats were used for the determination of the demethylase activity and mitotic counts and for the protein, DNA, and nuclear count determinations. In all cases the liver wet weights were determined, and although not identical, they were quite similar.

Statistical analyses were carried out using the small-sample *t* test, the paired comparison test, and the nonparametric Mann-Whitney *u* test (16). The *P* values reported were obtained using the small sample *t* test. *P* values of 0.05 or less were considered significant.

RESULTS

To be certain that the injection of 3-methylcholanthrene increases the drug-metabolizing enzyme activity of rat liver, we have injected a series of rats i.p. with 1 mg of 3-methylcholanthrene and have determined the azo dye-demethylase activity.

Chart 1 confirms previous observations of others (9) that the intraperitoneal injection of 1 mg of 3-methylcholanthrene into immature male rats increases the azo dye demethylase activity per gm of liver. Chart 1 also shows that the total azo dye demethylase activity, that is the enzyme activity/100 gm of body weight, increases, suggesting that an increase in liver mass has occurred.

Chart 2 indicates the effects of the intraperitoneal injection of 1 mg of 3-methylcholanthrene on liver weight. It can be seen that both wet and dry liver weight increase after treatment with 3-methylcholanthrene. The increase in liver wet and dry weight is highly significant by Day one ($P = 0.001$) and continues to be significant at this level through Day 4. At Days 7 and 10, the difference in wet and dry weight between the 3-methylcholanthrene-treated rats and the corn oil-treated rats is reduced, but it is still significant ($P = 0.01$). At Day 14, the difference between experimental and control liver wet and dry weight is no longer significant ($P = 0.56$).

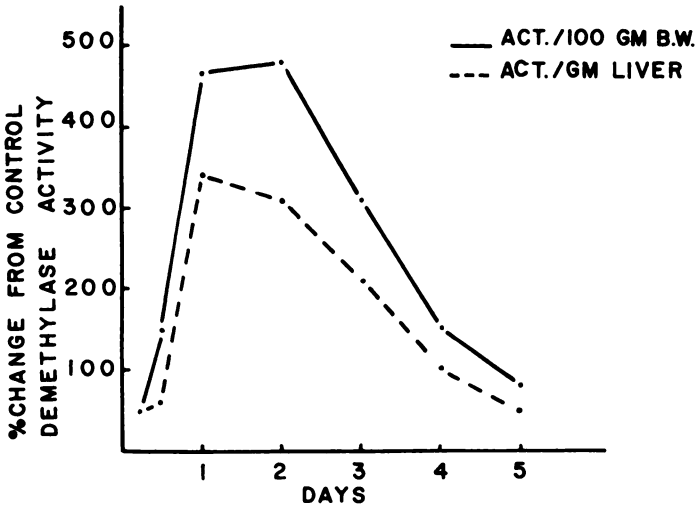

Chart 1. The azo dye-demethylase activity of livers from immature male rats injected intraperitoneally with 1 mg of 3-methylcholanthrene or corn oil. Each point is the ratio calculated from 6 experimental and 6 control rats. ACT., activity; B.W., body weight.

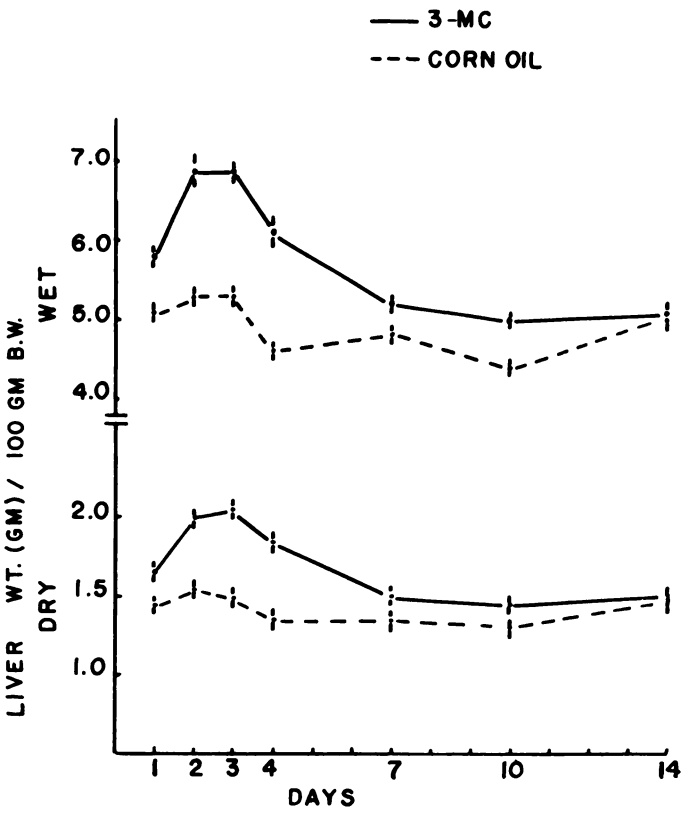

Chart 2. Liver wet and dry weight of immature male rats injected intraperitoneally with 1 mg of 3-methylcholanthrene (3-MC) or corn oil. Each point is the average of 6 rats. Vertical bars are the standard error of the mean. B.W., body weight.

If one calculates the increase in wet and dry liver weight as percent above control, the curves are very similar, indicating that the magnitude and pattern of the increases are similar for both wet and dry weights.

To determine if there is a comparable increase in total liver protein, another group of rats was injected with 1 mg of 3-methylcholanthrene. As in the previous group of male rats, there is an increase in wet weight (Chart 3). The increase in wet weight is similar but not identical to that seen in Chart 1. In Chart 3 we also see that the total homogenate protein increases after a single injection of 3-methylcholanthrene. The increase is significant by Day 2 ($P = 0.01$) and remains significantly increased through Day 4. The difference between the experimental and controls at Days 7, 10, and 14 is not significant ($P > 0.50$). Table 1 indicates that, although the total protein increases, the protein/gm liver does not significantly change throughout the experimental period ($P > 0.50$).

To determine if one injection of 3-methylcholanthrene will also result in a comparable increase in liver weight in immature female rats, a series of immature female rats have been injected intraperitoneally with 1 mg of 3-methylcholanthrene. Chart 4

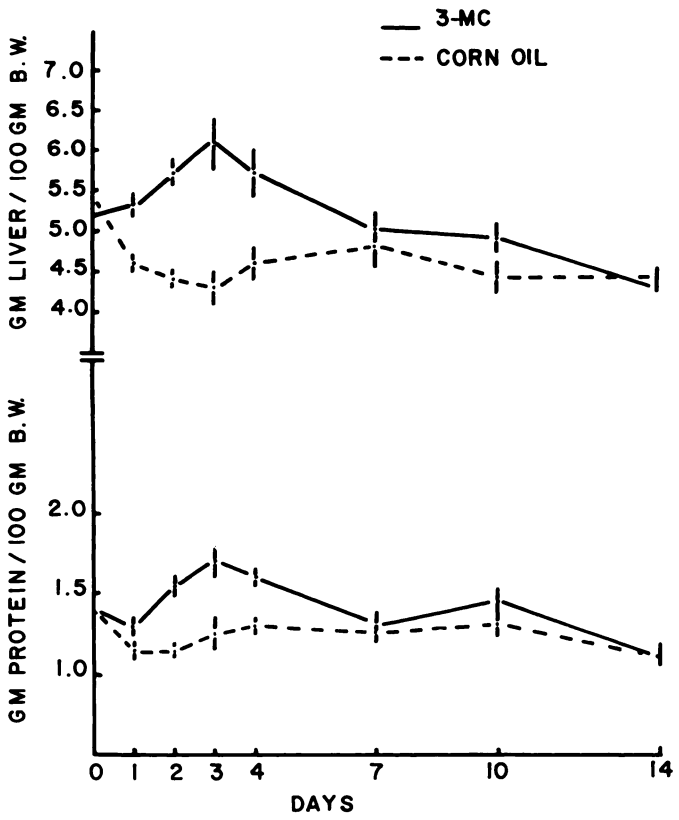

Chart 3. Wet weight and total homogenate protein of livers from immature male rats injected intraperitoneally with 1 mg of 3-methylcholanthrene (3-MC) or corn oil. Each point is the average of 6 rats. Vertical bars are the standard error of the mean. B.W., body weight.

indicates that 1 mg of 3-methylcholanthrene does increase liver weight in immature female rats. The increase in protein at 2 days is 23% (Table 2); this is significant ($P > 0.01$). At Days 3 and 4 the total protein is about 18% above control levels; this is also significant ($P < 0.01$). At Day 7 the total protein is not significantly different from control protein values ($0.05 > P < 0.10$). The protein/gm liver (Table 2) shows a slight decrease, which is significant only at Day 4 ($P = 0.02$).

To begin to understand something about the mechanism by which the increase in liver weight and protein is brought about, we have determined the changes in the liver DNA and nuclear count following a single intraperitoneal injection of 3-methylcholanthrene in immature male rats. Total DNA (Table 3) significantly increases by Day 3 ($P = 0.03$). By Day 7, the total DNA of the 3-methylcholanthrene-treated rats begins to decrease, and it is no longer significantly different from the controls ($P > 0.50$). Concomitant with the increase in DNA, there is an increase in the homogenate nuclear count (Table 3) which is significant at Day 2 ($P = 0.02$). From Day 7 onward, the differences between the experimental and control nuclear counts are not significant ($P > 0.05$). Table 1 presents the DNA and nuclear count/gm liver. Although they fluctuate throughout the experimental period, there is no significant difference between the phenobarbital- and the saline-treated rats.

Finally, Chart 5 demonstrates that, 2 days after 3-methylcholanthrene treatment, the hepatocyte mitotic activity is significantly increased and then returns to control levels.

DISCUSSION

A single intraperitoneal injection of 1 mg of 3-methylcholanthrene into immature male rats results in a significant increase in the azo dye demethylase activity of the liver. This confirms the results of many other investigations (for reviews see 5–7, 9).

Associated with the increase in azo dye demethylase activity in immature male rats is an increase in liver wet and dry weight and total protein. Immature female rats also show an increase in liver wet and dry weight and total protein. The increase in liver weight after treatment of immature male rats with 1 mg 3-methylcholanthrene has been previously noted (5, 6, 9, 12). However, we believe this is the first report of the daily changes in liver wet and dry weight in male and female immature rats. The daily changes in the total liver protein, for the first 4 days after 3-methylcholanthrene injection, have been previously reported by Arcos *et al.* (1).

The increase in liver wet and dry weight in the immature male rats is significant at Day 1, but the increase in total

Table 1

Days	No. of rats C/E[a]	mg protein/gm liver		mg DNA/gm liver		Nuclei ($\times 10^6$)/gm liver	
		Corn oil	3-MC	Corn oil	3-MC	Corn oil	3-MC
0	6/6	258 ± 8[b]	267 ± 6	2.4 ± 0.1	2.3 ± 0.2	160 ± 5	162 ± 9
1	7/7	258 ± 11	246 ± 10	2.4 ± 0.2	2.4 ± 0.2	178 ± 13	154 ± 11
2	6/6	259 ± 8	273 ± 7	2.5 ± 0.2	2.2 ± 0.2	183 ± 7	166 ± 5
3	6/6	293 ± 9	286 ± 10	2.5 ± 0.2	2.3 ± 0.2	176 ± 6	154 ± 11
4	7/6	287 ± 11	281 ± 11	2.4 ± 0.2	2.5 ± 0.2	171 ± 5	159 ± 6
7	6/5	258 ± 11	258 ± 11	2.1 ± 0.1	2.3 ± 0.1	141 ± 15	133 ± 7
10	5/5	290 ± 16	295 ± 13	1.9 ± 0.1	2.1 ± 0.1	164 ± 13	175 ± 11
14	6/5	255 ± 13	262 ± 18	2.4 ± 0.1	2.8 ± 0.2	165 ± 17	179 ± 11

The effect of an intraperitoneal injection of 3-methylcholanthrene (3-MC) on liver protein, DNA, and nuclear count in immature male rats.

[a] Control/experimental.

[b] Average ± standard error.

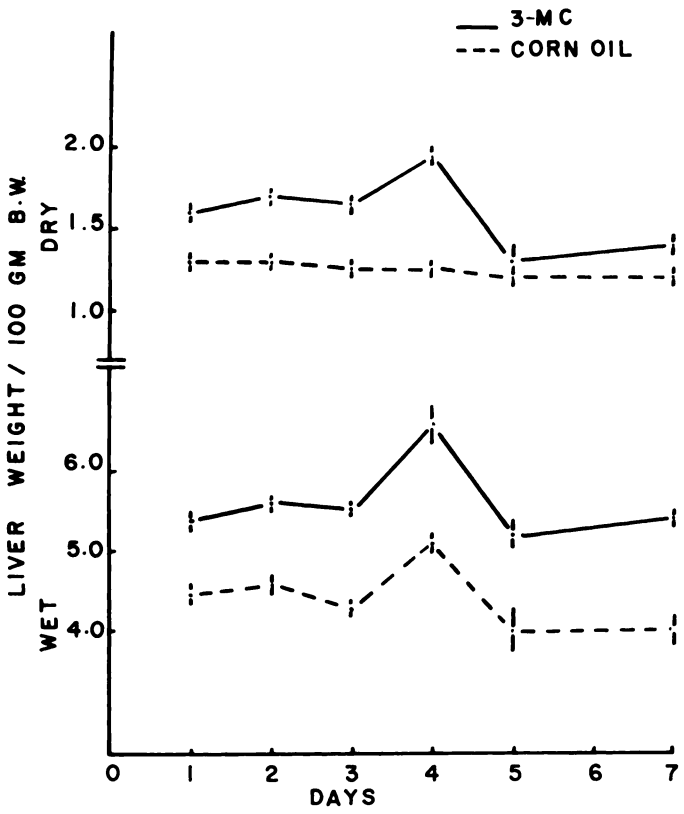

Chart 4. Liver wet and dry weight of immature female rats injected intraperitoneally with 1 mg of 3-methylcholanthrene (3-MC) or corn oil. Each point is the average of 6 rats. Vertical bars are the standard error of the mean. B.W., body weight.

Table 2

Day	No. of rats C/E[a]	mg protein/gm liver Corn oil	mg protein/gm liver 3-MC	gm protein/100 gm body weight Corn oil	gm protein/100 gm body weight 3-MC
0	4/4	259 ± 6[b]	265 ± 2	1.12 ± 0.03	1.25 ± 0.08
1	6/6	256 ± 6	255 ± 4	1.49 ± 0.10	1.57 ± 0.17
2	6/6	259 ± 3	254 ± 1	1.18 ± 0.06	1.45 ± 0.04
3	6/6	267 ± 6	254 ± 3	1.13 ± 0.09	1.33 ± 0.04
4	6/6	266 ± 3	250 ± 5	1.16 ± 0.002	1.37 ± 0.03
7	6/6	263 ± 4	252 ± 4	1.17 ± 0.03	1.25 ± 0.05

Liver protein of immature female rats injected intraperitoneally with 1 mg of 3-methylcholanthrene (3-MC).
[a]Control/experimental.
[b]Average ± standard error.

protein is not significant until Day 2. This suggests that at Day 1 there is an increase in nonvolatile substances other than proteins. The nature of these substances is unknown.

Within 4 to 7 days after the administration of 3-methylcholanthrene, liver wet and dry weight and total liver protein begin to return to control levels, reaching them by Day 14. The decreases in liver weight and protein follow the decrease in the azo dye demethylase activity closely.

Table 3

Days	No. of rats C/E[a]	Nuclei × 10^6/100 gm body weight Corn oil	Nuclei × 10^6/100 gm body weight 3-MC	mg DNA/100 gm body weight Corn oil	mg DNA/100 gm body weight 3-MC
0	6/6	857 ± 35[b]	851 ± 22	12.7 ± 0.6[c]	11.8 ± 1.0
1	7/7	803 ± 62	815 ± 56	11.0 ± 0.9	12.7 ± 1.1
2	6/6	803 ± 40	945 ± 32	11.0 ± 0.9	12.4 ± 1.2
3	6/6	751 ± 52	915 ± 33	10.4 ± 0.8	13.6 ± 1.0
4	7/6	781 ± 30	909 ± 52	10.6 ± 1.0	14.4 ± 1.2
7	6/5	629 ± 41	668 ± 39	9.7 ± 1.0	11.6 ± 0.6
10	5/5	723 ± 55	858 ± 40	8.2 ± 0.5	10.6 ± 1.2
14	6/5	719 ± 78	767 ± 44	10.4 ± 0.7	11.9 ± 0.9

Total nuclear count and DNA of livers from immature male rats intraperitoneally injected with 1 mg of 3-methylcholanthrene (3-MC).
[a]Control/experimental.
[b]Average ± standard error.

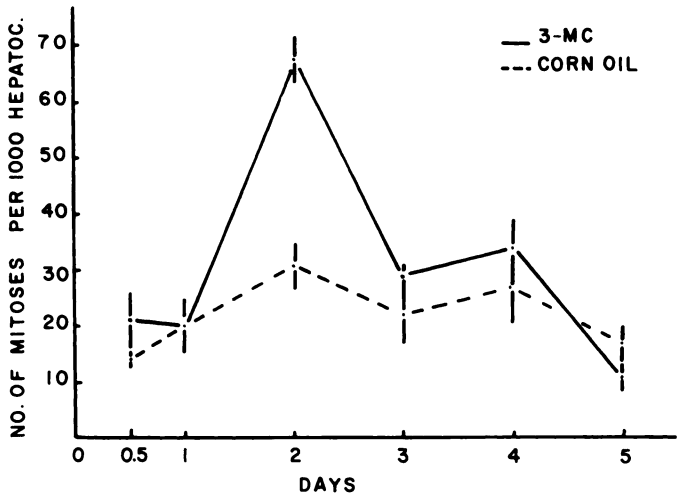

Chart 5. Hepatocyte (HEPATOC.) mitotic activity of livers from immature male rats injected intraperitoneally with 1 mg of 3-methylcholanthrene (3-MC) or corn oil. Each point is the average of 6 rats. Vertical bars are the standard error of the mean.

Our investigation also demonstrates that, in immature male rats, cell proliferation plays a significant role in bringing about the increase in liver weight and protein produced by the injection of 1 mg of 3-methylcholanthrene since there is an increase in both the total DNA and nuclear count. Our data do not permit us to say how much each cell population of the liver proliferates. We assume that some of the increase in the nuclear count is due to an increase in the number of hepatocytes. This possibility is supported by the fact that we find an increase in hepatocyte mitotic activity. Again, as in the case of liver weight and protein, the nuclear count and DNA return to control levels by Day 14. We do not know which cell populations have decreased nor if the cells which initially proliferated are the ones which decrease in number. We also do not know how the cell numbers are decreased. We have examined many sections and have seen no obvious pathology. We are currently investigating this question.

It is of interest to compare the effects of 3-methylcholanthrene on liver growth in immature male rats with those produced by phenobarbital, especially since 3-methylcholanthrene is reported to induce a much smaller number of enzymes than phenobarbital (for reviews see 5–7).

A single injection of 1 mg of 3-methylcholanthrene or 4 injections of 50 mg/kg. body weight of phenobarbital both result in an increase in liver wet and dry weight, total protein, DNA, nuclear count, and heptaocyte mitotic activity, although the increase in liver weight is greater after 3-methylcholanthrene treatment (4). In both cases, the increase in demethylase activity precedes the increase in the various parameters of liver growth. Similarly, in both cases, after cessation of treatment, the demethylase activity, liver weight, protein, DNA, and nuclear count, return to control levels (4).

It should be clear that the increase in liver size after 3-methylcholanthrene injection is not simply due to the increase in the amount of the microsomal drug-metabolizing enzymes, since the microsomal protein/gm liver does not increase (5, 6). Even after phenobarbital treatment when the microsomal protein/gm liver does increase (5, 6, 15), the homogenate protein/gm liver does not increase (4). The increase in liver size is due to an increase in all the relevant cellular constituents, as in any growth situation.

The mechanism by which either 3-methylcholanthrene or phenobarbital stimulate liver growth is unknown. It may be that the drugs have separate effects on the machinery for the induction of the drug-metabolizing enzymes and on the machinery concerned with liver growth. It is also possible that the induction of the drug-metabolizing enzymes is causally related to the triggering of liver growth. Perhaps turning on enzyme synthesis somehow triggers liver growth (2, 4, 11). Whatever the mechanism is by which these drugs stimulate liver growth, it is probably not related to the increase in the endoplasmic reticulum (6, 10, 14, 15) since, in rats, 3-methylcholanthrene causes as much or more increase in liver size as does phenobarbital, but it reportedly does not significantly increase the amount of smooth endoplasmic reticulum (10). It is possible, however, that the mechanism for liver growth after phenobarbital treatment is different from that produced by 3-methylcholanthrene and that the increase in the smooth endoplasmic reticulum is a prerequisite for liver growth after phenobarbital treatment.

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