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Ornithine Decarboxylase Induction in Liver- and Hepatoma-Derived Cell Cultures

No Detectable Differences between Control and 3-Methylcholanthrene-Treated Cells

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SUMMARY

KANO, I., AND D. W. NEBERT. Ornithine decarboxylase induction in liver- and hepatoma-derived cell cultures: no detectable differences between control and 3-methylcholanthrene-treated cells. *Mol. Pharmacol.* **20**:172-178 (1981).

Ornithine decarboxylase (ODC) activity is markedly stimulated in fetal rat primary hepatocyte cultures by the addition of fresh growth medium and in hepatoma-derived cell cultures 2-6 hr following the addition of fresh serum-containing or serum-free medium. The three hepatoma-derived continuous cell lines studied were: H-4-II-E (rat), Hepa-1 (mouse), and HTC (rat "minimal deviation" hepatoma). When ice-cold fresh medium is added and the cultures are then incubated at 37°, ODC induction is greater than when fresh medium, prewarmed to 37°, is added. ODC induction is less in confluent cultures than in logarithmically growing cultures, and less in frozen postmitochondrial supernatant fractions than in freshly prepared fractions. No relationship is apparent between ODC induction and total cellular content of either cyclic AMP or cyclic GMP. Whereas 3-isobutyl-1-methylxanthine stimulates cyclic AMP levels, this inhibitor of phosphodiesterase does not enhance ODC activity. These results do not support the hypothesis that stimulation of monooxygenase and ODC activities by various inducers of P-450 is mediated by the increased amount of cyclic AMP. ODC induction is never found to be greater in 3-methylcholanthrene-treated cultures than in control cultures. "Basal" ODC activities in nonstimulated liver- or hepatoma-derived cells in culture are 2-25 times greater than those in liver of the nonstimulated adult intact animal. Therefore, a difference in ODC induction between 3-methylcholanthrene-treated and control cultures (a) may be obscured by the already elevated ODC activity, (b) may not be a necessary prerequisite for the induction of aryl hydrocarbon hydroxylase to occur, or (c) may be necessary if the starting ODC activity is very low but unnecessary if the starting ODC activity is already high. These data point out the difficulties in using cell culture to study the relationship between drug-metabolizing enzyme induction and ODC induction.

INTRODUCTION

ODC[1] is a rate-limiting enzyme in the biosynthesis of polyamines (putrescine, spermidine, and spermine). Numerous lines of evidence indicate that polyamines play an essential role in the regulation of various cell functions and metabolism including DNA synthesis, transcription, translation, and the modulation of membrane function and numerous enzyme activities (reviewed in ref. 1). ODC induction occurs during various conditions under which gene activation and tissue growth are enhanced (e.g., regenerating liver, developing embryos, tumorigenesis, lymphoblast formation in response to mitogens, differentiation of target organs in response to sex steroids or growth hormone, etc.).

Increases in ODC activity are not always correlated with a large degree of gene activation and rapid tissue growth. Phenobarbital treatment does not cause rapid tissue growth but is known to cause proliferation of the smooth endoplasmic reticulum (2) and also stimulates

[1] The abbreviations used are: ODC, ornithine decarboxylase (EC 4.1.1.1); MC, 3-methylcholanthrene; AHH, aryl hydrocarbon (benzo-[*a*]pyrene) hydroxylase (EC 1.14.14.1).

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polyamine biosynthesis (3–5). Treatment with polycyclic aromatic hydrocarbons causes little, if any, histological change (2) and is generally regarded as a process involving the activation of a very small number of genes. Polycyclic hydrocarbon pretreatment has also been shown, however, in rat (4, 5) and mouse (6, 7) liver to enhance polyamine biosynthesis. It therefore remains to be determined how many genes need to be activated before there is a detectable stimulation of ODC activity.

ODC induction appears to represent an early event during the sequence of subcellular changes between the time that the inducer first enters the cell and the time that induced drug-metabolizing enzyme activities become maximal (3–7). When rats and mice are given oral 1,3-diamino-2-propanol, an indirect inhibitor of ODC, the induction of various drug-metabolizing enzyme activities in the liver by several inducers is markedly inhibited (8), suggesting that ODC induction is an essential step during the process of drug-metabolizing enzyme induction.

Induced ODC activity has an extremely short half-life, on the order of 11–17 min (9); hence, it is necessary to study animals every 2–3 hr, or less, in order to detect the rapid rise and fall of this very short-lived enzyme activity (7). It therefore would appear advantageous from several standpoints to study ODC induction in cell culture.

MATERIALS AND METHODS

Materials. Cyclic AMP and cyclic GMP determination kits were purchased from Amersham Company (Arlington Heights, Ill.). 3-Isobutyl-1-methylxanthine was bought from Aldrich Chemical Company (Milwaukee, Wisc.). All other chemicals were obtained from sources previously cited (7, 10, 11).

Cell culture methodology. Livers from fetal rats estimated to be 18–20 days of gestational age were used in the preparation of primary cultures of dispersed cells, as described in detail previously (11). Three continuous lines were also used in this study. H-4-II-E is a rat cell culture derived (12) from Reuber hepatoma H-35 and in 1973 was a generous gift of Dr. E. Brad Thompson, National Cancer Institute (Bethesda, Md.). Hepa-1 was derived from a transplanted hepatoma BW 7756 produced in the C57L/J mouse (13) and in 1971 was a generous gift from Dr. Gretchen Darlington, Department of Biology, Yale University (New Haven, Conn.). HTC is a hepatoma tissue culture line derived from an ascites tumor which in turn had been derived from the rat "minimal deviation" hepatoma 7288C (14); this line was kindly provided in 1973 by Dr. E. Brad Thompson. Fetal rat primary hepatocytes and H-4-II-E cells were grown in Eagle's minimal essential medium with 10% calf serum and 10% fetal calf serum. HTC cells were grown in Eagle's minimal essential medium containing 5% fetal calf serum. Hepa-1 was grown in Waymouth MAB medium with 10% fetal calf serum. The cells were plated at 3×10^6 per 3 ml in 60-mm tissue culture dishes for enzyme assays and at 18×10^6 per 20 ml in 150-mm dishes for cyclic nucleotide determinations. Unless otherwise indicated, the cultures were studied during logarithmic growth phase (generally 1–2 days after plating). Procedures involving the addition of MC or the dimethyl sulfoxide vehicle alone (control medium) have been previously described (11).

Enzyme assays. Both AHH activity and protein concentration were determined in triplicate for the cellular homogenate as previously described (11). One unit of AHH activity is defined as that amount of enzyme catalyzing in 1 min at 37° the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo[a]pyrene recrystallized standard. Specific activity is expressed in units per milligram of protein of total cellular homogenate.

ODC activity was determined by measuring $^{14}CO_2$ liberation from DL-[1-^{14}C]ornithine, as previously described (7, 15). The enzyme was assayed in triplicate at each time point. In 25-ml Erlenmeyer flasks, the incubation mixture in 2.0 ml at 4° included 50 mM Trizma/chloride (pH 7.5), 5 mM dithiothreitol, 4 mM ethylenediaminetetraacetic acid, 40 μM pyridoxal phosphate, 1 μM DL-[1-^{14}C] ornithine (59 mCi/mmole from Amersham Searle Corporation, Arlington Heights, Ill.), and 0.1 or 0.2 ml (approximately 2–4 mg of protein) of the postmitochondrial supernatant fraction (following centrifugation of the cellular homogenate at 12,000 × *g* for 20 min). First-order kinetics is observed at 1 μM ornithine concentrations. One unit is defined as that amount of enzyme catalyzing in 1 min at 37° the formation of 1.0 pmole of $^{14}CO_2$. Specific activity is expressed in units per milligram of postmitochondrial supernatant protein.

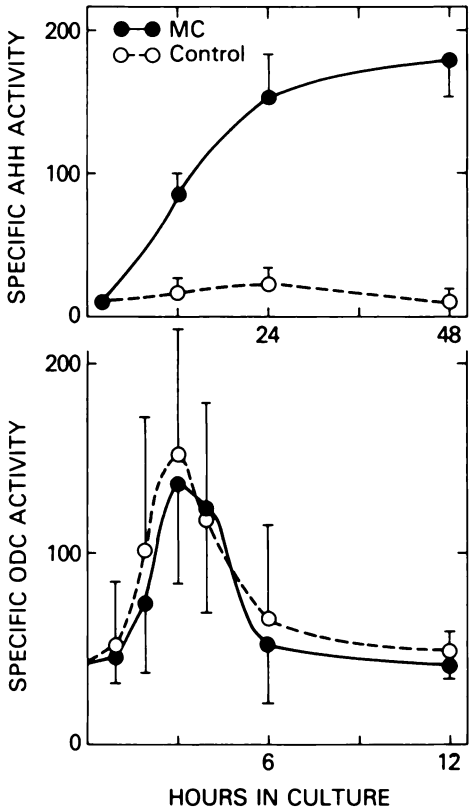

FIG. 1. *AHH (top) and ODC (bottom) activity in MC-treated and control primary hepatocyte cultures from fetal rats*

In this and all subsequent figures, the concentration of MC was 4.0 μM, and (unless otherwise specified) the growth medium had been warmed to 37° prior to adding it to the cultures. Note that the abscissa denotes 0–48 hr for AHH activity and only 0–12 hr for ODC activity. *Brackets* denote standard deviations from three experiments performed in different weeks.

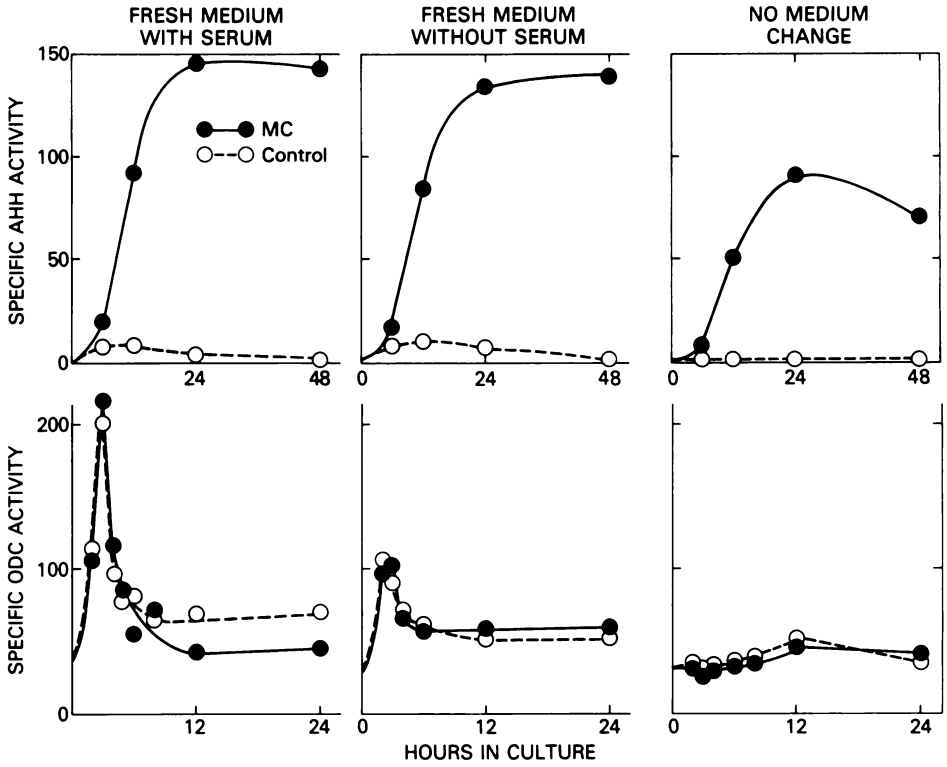

FIG. 2. *AHH (top) and ODC (bottom) activity in MC-treated and control H-4-II-E cultures in the presence of fresh serum-containing medium, fresh growth medium without serum, and without any medium change*

Note that the abscissa denotes 0–48 hr for AHH activity and only 0–24 hr for ODC activity. Cultures not having their medium changed at the time of MC addition had received fresh serum-containing medium 24 hr beforehand.

Cyclic AMP and cyclic GMP determinations. Two 150-mm tissue culture dishes were used for each time point. After the cell surface had been washed several times with sodium phosphate-buffered saline (0.85% NaCl) at 37°, ice-cold 10% trichloroacetic acid (10 ml per dish) was added, and the cell precipitate was scraped with a rubber policeman and transferred to a volumetric tube; the final volume was adjusted to 11 ml. After the sediment was shaken vigorously for 2 min and suspended in an ice bath for 20 min, the insoluble protein was sedimented by brief centrifugation to form a pellet. The supernatant fraction (10.5 ml) was transferred to a 45-ml extraction tube and washed at least 10 times with 20 ml of diethyl ether to remove all acid. The supernatant fraction was then evaporated to dryness under a stream of nitrogen at 55°. The dried sample was then reconstituted by the addition of 0.05 M Trizma/chloride-1.0 mM ethylenediaminetetraacetic acid, pH 7.5 (cyclic nucleotide assay buffer), and aliquots were used for cyclic AMP and cyclic GMP determinations. From the original trichloroacetic acid-precipitated cellular homogenate protein, an aliquot was neutralized with 1 N NaOH and total cell protein determinations were made (11).

RESULTS

AHH and ODC induction in fetal rat primary hepatocyte cultures. MC induced AHH activity 15- to 20-fold during a 48-hr period (Fig. 1), whereas about a 2-fold rise in AHH activity was seen in control cultures. It is known (11) that freshly added control medium can induce detectable increases in AHH activity. Peak elevations in ODC activity occurred between 2 and 4 hr in both MC-

TABLE 1

Specific ODC activity in control and MC-treated H-4-II-E cultures following various changes in experimental protocol

Experimental changes in growth medium	Basal level (starting activity)	Maximally induced activity in control cultures[a]	Maximally induced activity in MC-treated cultures[a]
Usual protocol (Fig. 2)	30 ± 7	200 ± 50	210 ± 45
One-half the usual concentration of essential and nonessential amino acids	22 ± 6	160 ± 32	140 ± 19
Twice the usual concentration of essential and nonessential amino acids	41 ± 12	240 ± 58	180 ± 35
10% Calf serum omitted	26 ± 4	150 ± 17	160 ± 17
10% Fetal calf serum omitted	28 ± 7	160 ± 21	130 ± 28

[a] Values are expressed as the means ± standard deviation in experiments done during separate weeks ($N = 3$). Maximally stimulated ODC levels always occurred between 2 and 6 hr after the fresh medium (with or without MC) had been added.

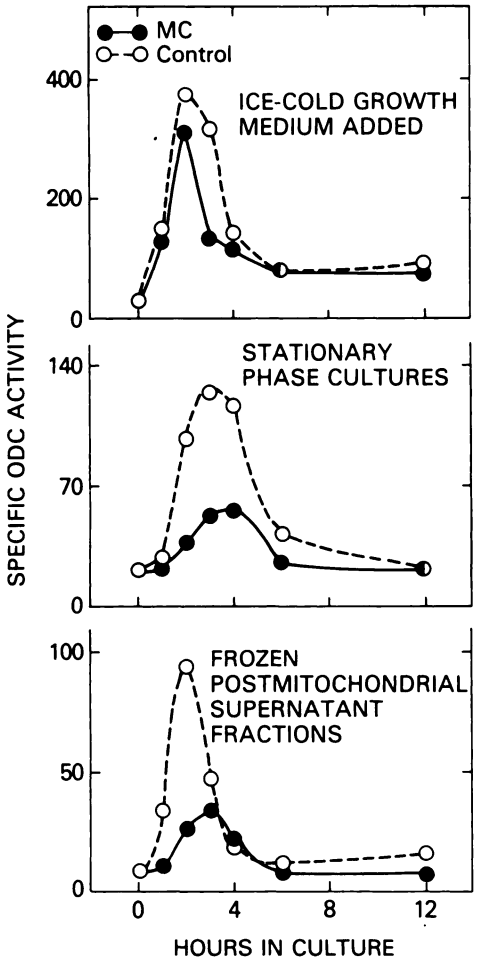

FIG. 3. *ODC activity in MC-treated and control H-4-II-E cultures under various experimental conditions*

Top, at time zero, ice-cold growth medium (rather than 37° growth medium, as in Figs. 1 and 2) was added. After addition of ice-cold medium, the cells were incubated for the next 12 hr as usual at 37°. *Middle*, cultures allowed to grow to confluency were used (rather than cultures during logarithmic growth phase, as in Figs. 1 and 2). *Bottom*, postmitochondrial supernatant fractions were stored overnight at −80° before the ODC assay (rather than fresh samples being assayed within 1-2 hr of the cultures being harvested, as in Figs. 1 and 2). Note the different relative activities plotted on the ordinates.

treated and control cultures. Between 12 and 48 hr, ODC activity remained at basal levels between 10 and 60 units per milligram of protein (data not illustrated). The rise in ODC activity was never statistically greater in MC-treated than in control cultures. Considerably greater variability in ODC activity from week to week was observed with primary hepatocyte cultures than with the continuous hepatoma-derived cell lines.

AHH and ODC induction in H-4-II-E cultures as a function of the growth medium. MC induced AHH activity to similarly high levels, whether or not the freshly added medium contained fetal calf serum (Fig. 2). AHH induction by polycyclic hydrocarbons independent of the addition of serum to the growth medium has been noted previously (16). On the other hand, MC-induced AHH activity was very much lowered when the inducer was added to cells without a medium change. Frequent changes in the medium are known (17) to maximize AHH

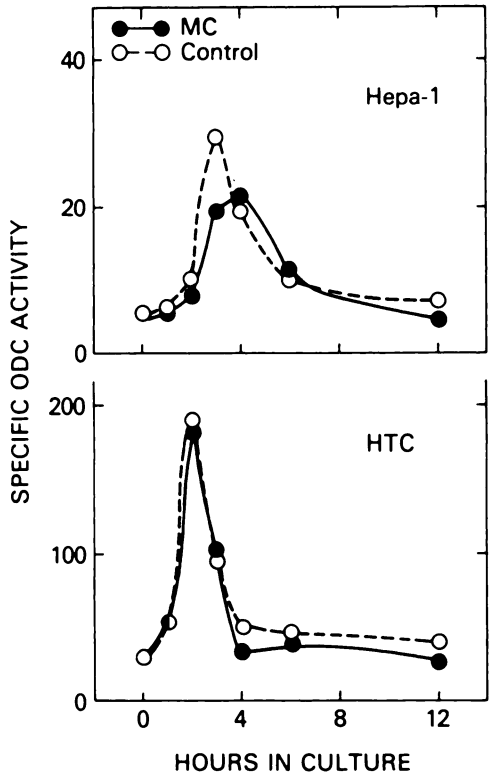

FIG. 4. *ODC activity in MC-treated and control Hepa-1 (top) and HTC (bottom) cultures*

Note the 5-fold difference in activities on the ordinates.

induction and the metabolism of polycyclic hydrocarbons to excreted products.

Specific ODC activity was markedly stimulated (maximum ≅ 3 hr) in cultures receiving fresh serum-contain-

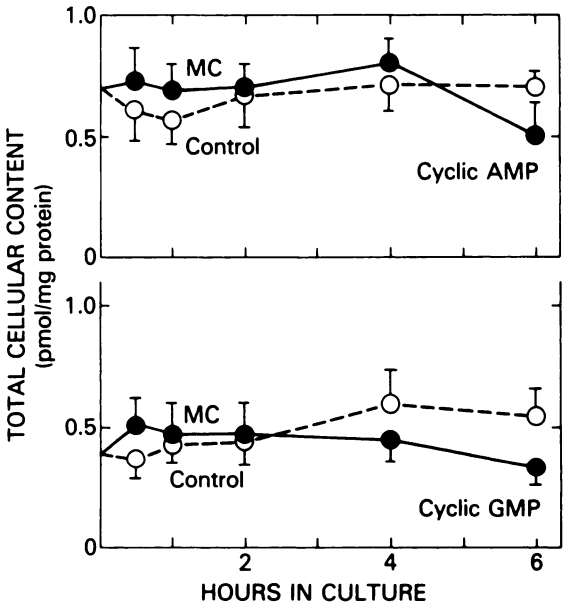

FIG. 5. *Lack of any change in total cellular content of cyclic AMP (top) or cyclic GMP (bottom) in MC-treated and control H-4-II-E cultures in the presence of fresh serum-containing medium*

No changes, or small decreases, were detected in cultures having fresh growth medium without serum and in cultures having no medium change.

TABLE 2

Specific ODC activity and cyclic AMP content in H-4-II-E cultures treated with 3-isobutyl-1-methylxanthine (IMX)

	IMX added	Basal level (starting amount)	Maximally induced amount in control cultures[a]	Maximally induced amount in MC-treated cultures[a]
	mM			
Freshly added medium				
Specific ODC activity	0	27 ± 5	190 ± 44	150 ± 32
	0.5	34 ± 7	180 ± 28	170 ± 31
Cyclic AMP concentration	0	0.66 ± 0.11	0.68 ± 0.07	0.62 ± 0.09
(pmoles/mg protein)	0.5	1.6 ± 0.36	1.6 ± 0.36	2.1 ± 0.34
No medium change				
Specific ODC activity	0	23 ± 2	48 ± 8	50 ± 5
	0.5	22 ± 4	53 ± 12	49 ± 9
Cyclic AMP concentration	0	0.56 ± 0.07	0.59 ± 0.06	0.62 ± 0.08
(pmoles/mg protein)	0.5	1.5 ± 0.21	1.7 ± 0.26	1.8 ± 0.31

[a] Values are expressed as the means ± standard deviation in experiments done during separate weeks ($N = 3$). Maximally stimulated ODC and cyclic AMP levels always occurred between 2 and 12 hr after the addition of fresh medium (with or without MC) or no medium change.

ing medium (Fig. 2). Maximal ODC specific activities ranged between 140 and 260 among approximately 10 time-course experiments using H-4-II-E cells. ODC induction was much less in cells receiving fresh medium without serum, and in cells receiving no medium change there was no detectable increase during the first few hours after MC or dimethyl sulfoxide (control) had been added to the cultures. The reason for a small yet significant and reproducible rise in ODC activity in these latter cultures at 12 hr is not understood.

Unexpectedly, ODC induction in MC-treated cells was never found to be greater than that in control cultures. ODC is known (18–21) to be enhanced by the addition of serum and amino acid concentrates to cell cultures, possibly due to the proliferating state of cultured cells when DNA and protein synthesis are stimulated. Several concentrations of essential and nonessential amino acids were tested (Table 1), and growth medium with 10% calf serum or 10% fetal calf serum omitted was examined. ODC was stimulated to higher levels (Fig. 3) when ice-cold rather than 37° growth medium was added to the cultures. ODC activities were also higher (Fig. 3) during logarithmic growth phase than during the stationary phase (confluency), and frozen postmitochondrial supernatant fractions exhibited about one-half or one-tenth as much ODC activities as freshly prepared fractions. This latter finding illustrates the high degree of lability of ODC activity to freezing and thawing. With the addition of other compounds, we probably could have found factors successful in preventing this lability of the frozen enzyme, but we chose instead to study only freshly prepared samples because of better reproducibility and consistently higher activities. However, in all instances in which ODC was stimulated by these changes in growth conditions or in which ODC activity remained relatively stable, we found no experimental conditions under which MC-induced ODC activity was significantly greater than control ODC activity.

AHH and ODC activities in Hepa-1 and HTC cultures. AHH activity is greatly induced by MC in Hepa-1 cultures and is stimulated only very slightly by MC in HTC cultures (22). We found that the basal and inducible levels of ODC in HTC cells (Fig. 4) were similar to those in H-4-II-E cells; the basal and inducible ODC levels in Hepa-1 cells were about 6 times less than those in H-4-II-E cultures. Again, maximal ODC stimulation occurred 2–4 hr after the addition of serum-containing medium, and no differences in ODC activities were found between MC-treated and control Hepa-1 or HTC cultures.

Cyclic AMP and cyclic GMP levels in cell cultures. It has been reported (5, 23–25) that, during ODC induction by either phenobarbital or benzo[a]pyrene, cyclic AMP and cyclic AMP-dependent protein kinase activity are significantly increased in rat liver; these increases range between 6% and 50%, however. AHH induction by dibutyryl cyclic AMP in mouse 3T3 cultured fibroblasts (26) and by dibutyryl cyclic AMP, aminophylline, theophylline, and papaverine in hamster BHK cultures (27) has been reported. We therefore searched for changes in cyclic AMP and cyclic GMP content in H-4-II-E cells (Fig. 5). Neither freshly added serum-containing medium nor MC caused any significant changes in cyclic AMP or cyclic GMP total cellular content during 6-hr experiments, such as the one illustrated. Similar results were found with Hepa-1 and HTC cultures (data not shown).

Lack of effect by 3-isobutyl-1-methylxanthine. This chemical is a specific inhibitor of phosphodiesterase, thereby causing increases in cyclic AMP (28). We added 3-isobutyl-1-methylxanthine to MC-treated and control H-4-II-E cultures that had received fresh serum-containing medium and to MC-treated and control H-4-II-E cultures that had received no medium change (Table 2). Although cyclic AMP stimulation was observed, we found no significant differences in ODC levels. We conclude that cyclic AMP increases are not intimately associated with detectable differences in ODC induction.

DISCUSSION

Because of the apparent inhibition of rat and mouse liver drug-metabolizing enzyme induction by 1,3-diamino-2-propanol [an inhibitor of ODC activity (8)] and the inconvenience of killing animals every 2 to 3 hr in order to detect rapid rises and falls in ODC activity *in vivo* (7), it seemed appropriate to study ODC induction in culture. However, by using fetal rat primary hepato-

cyte cultures and H-4-II-E, Hepa-1, and HTC cell cultures, we found no strict relationship between AHH induction and ODC induction by MC. These data are discouraging with regard to the promise of using tissue culture to study ODC activity.

In mouse liver and kidney of the intact adult animal, specific ODC activities are generally two or less under nonstimulated conditions; in the fetus or newborn, however, these activities range between 5 and 500, presumably due to hormonal changes, cell proliferation, and such events during gestation and the neonatal period (7). The fetal rat primary hepatocyte cultures and the three continuous cell lines used in this study had basal ODC specific activties ranging between about 4 and 50, apparently considerably increased from basal activities in the nonstimulated intact animal. The fact that ODC activity is increased at all times in these cultures may simply reflect the active division of the cells and therefore an increased requirement for polyamines critical for cell division functions. Hence, unless a cell culture possesses very low (e.g., 2.0 or 0.2) specific ODC activity under resting conditions, differences between MC-treated and control cultures, as in this study, can probably be buried beneath the abnormally high basal ODC activity. It will be of interest to see if one can find any actively dividing cell culture line with basal ODC levels similar to those in the intact nonstimulated adult animal.

It has been proposed, based exclusively on an apparent parallelism between ODC stimulation and the enhancement of cyclic AMP-dependent protein kinase activity, that the stimulation of monooxygenase and ODC activties by various inducers of cytochrome P-450 is mediated by the increased activity of the kinase (5, 23–25). The present data (Fig. 5) and data gathered by others (7, 29) do not support this hypothesis.

A strict correlation has been reported (7) between the Ah^b allele and ODC induction in the liver and kidney of genetically *Ah*-responsive mice treated with polycyclic aromatic inducers. It was therefore proposed (7) that ODC induction is an essential early event during the sequence of subcellular changes between the time that the inducer first enters the cell and the time that induced drug-metabolizing enzyme activities become maximal. However, in the present report, we cannot detect the strict relationship between AHH induction and ODC induction. Three possible interpretations of the data are: (a) differences between the MC-treated and control cultures are obscured by the abnormally elevated "basal" ODC activity; (b) ODC induction (although it may occur) is not an essential prerequisite during the enhancement of drug-metabolizing enzyme activities by various inducers; (c) whereas a very low ODC basal level will give rise to an obligatory striking increase in ODC activity, high basal ODC levels are sufficient to "cover" such stimuli as AHH induction and therefore no further enhancement of ODC activity is required. Further studies are indicated to prove which of these possibilities is correct. In any event, our study emphasizes the difficulties in attempting to study the relationship between ODC induction and the *Ah* locus in liver- and hepatoma-derived cell cultures.

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