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# CHLORCYCLIZINE *N*-DEMETHYLASE ACTIVITY IN CHICK EMBRYO HEPATIC CELL CULTURE*

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**Abstract**—Hepatic cells from chick embryo levers were grown in primary monolayer cell cultures. Homogenates of the cells were shown to contain cytochrome P-450 and chlorcyclizine *N*-demethylase *in vitro*. The addition of allylisopropylacetamide and phenobarbital increased the chlorcyclizine *N*-demethylase activity and cytochrome P-450 content, and this stimulation was prevented by the addition of inhibitors of protein and RNA synthesis.

HEPATIC microsomal mixed function oxygenase is a membrane-bound electron transport system requiring NADPH and molecular oxygen, which metabolizes a wide variety of drugs and endogenous compounds.[1,2] The overall enzymatic activity of this system is stimulated by the administration of a wide variety of xenobiotics.[1] This stimulation is blocked by a number of inhibitors of RNA and protein synthesis, suggesting that *de novo* protein synthesis is involved.

Induction of this system by phenobarbital (a prototype drug) consists of a proliferation of the smooth endoplasmic reticulum, including increased microsomal phospholipid and protein, and an increase in the activity of NADPH-cytochrome *c* reductase, cytochrome P-450 and in the overall microsomal oxygenase activity toward many, but not all, substrates.[1,3]

The turnover of cytochrome P-450 heme, most of which has a short half life,[4] accounts for a large fraction of the total heme synthesized in the liver of the untreated animal.[5,6] Induction of cytochrome P-450 to a higher level should create an increased demand for heme synthesis. The rate-controlling enzyme in heme synthesis is δ-aminolevulinic acid synthetase (ALA synthetase).[7] Induction of cytochrome P-450 is produced by insecticides, aromatic hydrocarbons, steroids and diverse therapeutic agents.[1] The list of agents which induce hepatic ALA synthetase is equally diverse and very similar.[8,9] Induction of cytochrome P-450 is accompanied by an increase in ALA synthetase in virtually all cases studied.[5,10–15] Suppression of ALA synthetase induction by heme or glucose administration[9,16–18] also prevents the induction of cytochrome P-450 and microsomal oxygenase activity.[19,20] These observations suggest that the ability to induce ALA synthetase is one of the factors determining the inducibility of cytochrome P-450.

In the present study, we examined microsomal oxygenase activity in chick hepatic cell culture. This system was selected because it has been shown to be very responsive to induction of ALA synthetase and porphyrin synthesis by a variety of drugs and

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steroids.[9,21] As a measure of microsomal oxygenase activity, we chose the *N*-demethylation of chlorcyclizine because of the sensitivity of the assay.[22]

## METHODS AND MATERIALS

*Materials.* Allylisopropylacetamide (AIA) was a gift from Hoffman-La Roche, Inc. Sodium phenobarbital was purchased from Mallinkrodt. Testosterone (17$\beta$-hydroxy-4-androsten-3-one) was purchased from CalBiochem and etiocholanolone (5$\beta$-androstan-3$\alpha$-ol, 3, 17-one) from Mann Research Laboratories. Estradiol (1,3,5-estratriene, 3, 17$\alpha$-diol) and 11-ketopregnanolone (5$\beta$-pregnane-3$\alpha$-ol, 11,20-dione) were specially synthesized.

Chlorcyclizine hydrochloride and norchlorcyclizine hydrochloride were gifts from the Burroughs Wellcome Co., Inc., U.S.A.

Glucose 6-phosphate dehydrogenase from Torula Yeast XI, glucose 6-phosphate, nicotinamide, adenosine triphosphate, NADH, NADP and NADPH were all purchased from Sigma Biochemicals. Viokase was purchased from Grand Island Biological Co.

*Culture of chick embryo liver cells.* The livers of 14- to 16-day-old chick embryos were removed under sterile conditions, and cultured according to the method of Granick[9] with a few modifications. The livers were pooled, washed three times in Earle's solution, without calcium or magnesium (modified Earle's solution), blotted dry and weighed. Then the livers were minced to very fine pieces with a sterile razor blade and digested in 12 ml of modified Earle's solution containing 100 mg of trypsin (Worthington) and 30 mg of pangestin (Difco) for 20 min at 37°. During this period the cells were gently aspirated into and out of a large bore pipette to aid in cell separation. The cell suspension was then diluted with modified Earle's solution to 150–200 mg/ml. One ml of cell suspension and 20 ml of medium were added to 10 cm plastic petri dishes; 2 ml of cell suspension and 40 ml of medium to 15 cm dishes (Falcon Plastics on Nunc). The medium consisted of 1 liter of Eagle's basal medium without phenol red, 100 ml of fetal bovine serum, 10 ml of 100 × concentrate of amino acid mixture; 10 ml of 0·2 M glutamine (all purchased from Microbiological Associates), 0·5 ml of mycostatin (2000 units), 6 mg of penicillin G, 10 mg of streptomycin and 3·0 g of $NaHCO_3$. The pH of the medium was adjusted to 7·4 with HCl. The cells were placed in an air–5% $CO_2$ atmosphere at 37°, and after 5–8 hr the medium was removed by aspiration and replaced with fresh medium.

To the treated plates were added the drugs under study; allylisopropylacetamide and phenobarbital were dissolved directly in the medium, and estradiol, 11-ketopregnanolone, testosterone and etiocholanolone were first dissolved in ethanol. The final ethanol concentration in the medium was 0·5 per cent.

After an additional 12 hr of culture, the medium was again removed, the cells were washed twice with 0·1 M $KH_2PO_4$–$K_2HPO_4$, pH 7·4 buffer or phosphate buffered-KCl (pH 7·4) and the cells scraped from the petri dish with a Teflon policeman. The cells were centrifuged at 1000 *g* for 10 min, weighed by difference in tared centrifuge tubes and homogenized in phosphate buffer. When cytochrome P-450 was measured, the homogenates were centrifuged at 2500 *g* and the cytochrome P-450 was measured in the supernatant fraction. Cytochrome P-450 content was quantified by the CO-induced difference spectrum according to the method of Omura and Sato[23] on a split beam Cary 15 spectrophotometer. The extinction coefficient of the Soret maximum of

the hemoprotein–CO complex was assumed to be 91 $mM^{-1}$ $cm^{-1}$ in all cases.[23]

*Enzyme assay.* Chlorcyclizine *N*-demethylase activity was assayed in a final volume of 2·5 ml containing $K_2HPO_4$–$KH_2PO_4$ buffer, pH 7·4, 100 μmole; NADP, 0·52 μmole; NADH, 0·28 μmole; glucose 6-phosphate, 3·3 μmole; $-MgCl_2 \cdot 6H_2O$, 5·0 μmole, glucose 6-phosphate dehydrogenase, 1 Kornberg unit; and chlorcyclizine hydrochloride, 126 μg ($1·5 \times 10^{-4}$ M). Usually homogenates containing 5–10 mg wet wt of cells (400–1000 μg of protein) were assayed. The reaction was incubated in a shaker bath for 30 min at 37° and terminated by the addition of 2·5 ml of 10% trichloroacetic acid. The deproteinized mixture was cooled in ice, centrifuged at 10,000 *g* for 20 min and the supernatant decanted and stored frozen until analysis.

In most cases, each homogenate was assayed in duplicate along with a blank (complete reaction mixture deproteinized prior to addition of chlorcyclizine).

In addition, duplicate flasks containing the complete reaction mixture (minus chlorcyclizine) and a known amount of product, norchlorcyclizine, were assayed to determine the counts/min/microgram of product formed.

In some experiments, alterations in the incubation mixture were used, and are so noted under the appropriate figure or table. Equivalent results were obtained when the enzyme was determined in a 1·25, 2·5 or 5·0 ml final volume (maintaining the same constituent concentrations) and terminated with an equal volume of 10% trichloracetic or metaphosphoric acid.

*Determination of norchlorcyclizine by acetylation with $^3$H-acetic anhydride.*[24] One ml of supernatant, 2 ml of water and 1 ml of 2 N NaOH were shaken with 10 ml of hexane for 1 hr. After centrifugation, a 9·0-ml aliquot of the hexane phase was transferred into a 15-ml shaking tube. The hexane was evaporated to dryness at 45° under a stream of nitrogen. Two ml of hexane, 0·05 ml of 15% pyridine solution in hexane and 0·05 ml of acetic $^3$H-anhydride in benzene (2·5 mCi/ml; specific activity 50 mCi/m-mole) were added and the mixture was then heated with the tube closed for 1 hr at 60°. The tube was then opened and heated at 90° for 15 min to evaporate the contents to dryness. After the addition of 2 ml of 0·1 N NaOH, the tube was heated at 95° for 15 min. The tube was cooled to room temperature and 2·0 ml of heptane was added. After shaking for 15 min, the aqueous phase was removed and discarded. Two ml of 0·1 N HCl was then added and, after extraction for 10 min, the heptane phase was discarded. Two ml of fresh heptane were added and again, after a 5-min extraction, the heptane phase was discarded. The aqueous phase was then made basic by the addition of 0·1 ml of 5 N NaOH. Two ml of heptane was added and the mixture was extracted for 15 min. Ten ml of scintillation solution containing 0·5% 2,5-diphenyloxazole (PPO) and 0·01% 1,4-*bis*[2-(5-phenyl oxazolyl)]benzene (POPOP) in toluene was added to 1·5 ml of the heptane phase and the radioactivity in the samples was quantified.

*Protein determination.* The protein content was determined by the alkaline–copper method of Sutherland *et al.*[25] using bovine serum albumin (Armour) as a standard. Enzyme activity is expressed as micrograms of norchlorcyclizine formed per 30 min per milligram of whole cell protein or per milligram wet weight of tissue.

## RESULTS

The microsomal oxygenase system converts the tertiary amine substrate, chlorcyclizine, to the secondary amine, norchlorcyclizine, as seen in Fig. 1. The norchlor-

cyclizine in an aliquot of the deproteinized incubation mixture is acetylated with $^3$H-acetic anhydride and the radioactive product isolated, and quantified by liquid scintillation spectrometry.[20]

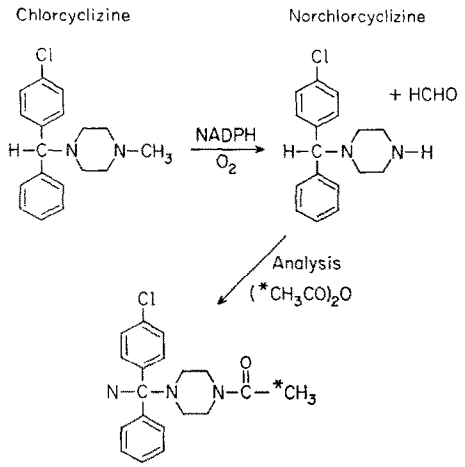


FIG. 1. Metabolism of chlorcyclizine to norchlorcyclizine and assay of the product. Chlorcyclizine is metabolized by the microsomal mixed function oxygenase system to norchlorcyclizine. Norchlorcyclizine is then acetylated with tritiated acetic anhydride, and quantified by liquid scintillation spectrometry.

As seen in Fig. 2, the addition of various amounts of the metabolite, norchlorcyclizine, to the complete reaction mixture (minus chlorcyclizine) before and after incubation resulted in similar recovery. This demonstrates: (1) the linearity of norchlorcyclizine added vs the acetylated radioactive product formed and (2) no loss of norchlorcyclizine during incubation. One $\mu$g of norchlorcyclizine added to the incubation yields approximately 40,000 net counts/min (above the blank) when acetylated with tritiated acetic anhydride. The radioactivity in the blank (blank = complete reaction mixture deproteinized at time zero) is primarily due to the amount of substrate added, and is independent of the other components. The blanks containing $1{\cdot}5 \times 10^{-4}$ M chlorcyclizine had about 7000 counts/min.

The assay was linear for at least 45 min. A 30-min incubation time was routinely employed. The apparent Michaelis constant with respect to chlorcyclizine was $7{\cdot}5 \times 10^{-5}$ M as seen in the double reciprocal plot in Fig. 3. In most assays the chlorcyclizine concentration was $1{\cdot}5 \times 10^{-4}$ M.

*Induction experiments.* The following studies were designed to investigate the induction of *N*-demethylase activity in hepatic cell culture by a variety of compounds.

Chick embryo hepatic cells were cultured for 8 hr, the medium was changed, and the treated cultures were exposed to allylisopropylacetamide (AIA, 50 $\mu$g/ml) or phenobarbital (100 $\mu$g/ml) for 12 hr. As shown in Fig. 4, AIA-treated cultures had a 2·5-fold increase and phenobarbital-treated cells, a 1·5-fold increase in *N*-demethylase activity compared to control cultures. This figure also demonstrates the linearity of product formation with enzyme concentration.

A rise in cytochrome P-450 accompanied the stimulation of microsomal oxygenase

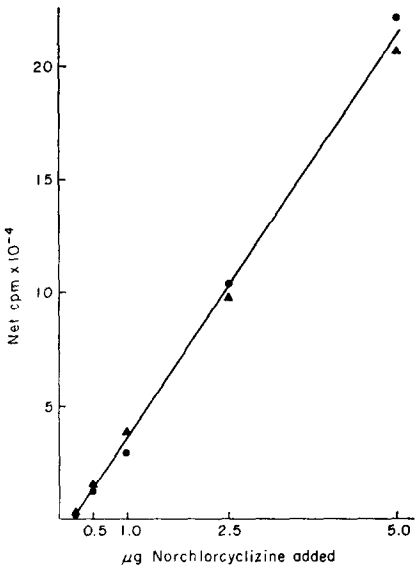


FIG. 2. Linearity of added norchlorcyclizine and formation of radioactive acetylated product. Norchlorcyclizine in varying amounts was added to flasks containing the complete reaction mixture minus chlorcyclizine. The incubation mixture contained 6·5 μmoles of glucose 6-phosphate; 2 units of glucose 6-phosphate dehydrogenase; 0·52 μmole of NADP; 0·28 μmole of NADH; 120 μmole of nicotinamide; 2·27 μmole of ATP; 196 μmole of KCl; 10 μmole of $MgCl_2.6H_2O$; 200 μmole of $K_2HPO_4$–$KH_2PO_4$, pH 7·4 buffer, and homogenate containing 25 mg wet wt of whole cells in a 5-ml volume. The flasks were deproteinized at time zero (●) or incubated for 30 min before deproteinization (▲). An aliquot of the mixture was then extracted, acetylated, and the radioactive product quantified in the usual way.

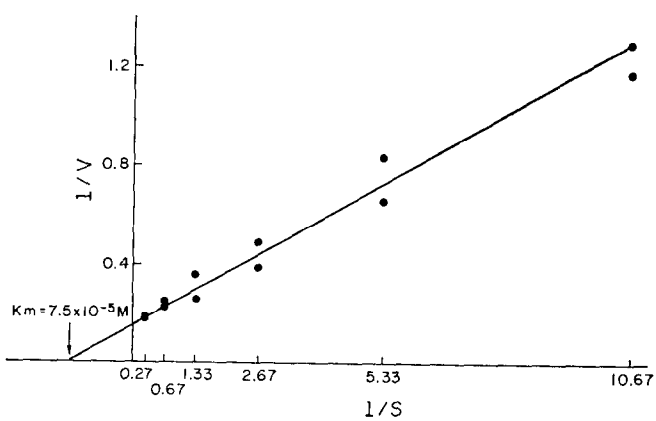


FIG. 3. Double reciprocal plot of Michaelis kinetics. 1/S is the reciprocal of the chlorcyclizine concentration ($1/10^{-4}$ M chlorcyclizine) and 1/V is the reciprocal of the rate of norchlorcyclizine formation (1/μg norchlorcyclizine formed per 30 min). The apparent Michaelis constant $K_m$ is 7·5 × $10^{-5}$ M for chlorcyclizine. (The assay conditions are the same as those listed in Fig. 2.)

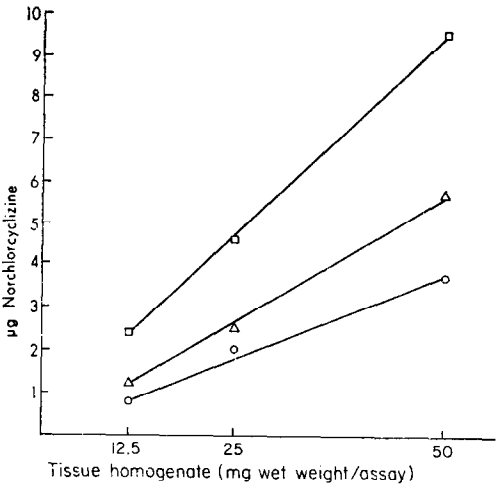


FIG. 4. Induction of chlorcyclizine *N*-demethylase activity in cell culture. Cell cultures were grown for 8 hr, and the medium was changed. To the treated cultures was added either phenobarbital (100 μg/ml) or AIA (50 μg/ml). After 12 hr of further incubation the cells from several identically treated petri dishes were pooled and homogenized. *N*-demethylase activity was assayed on the 2500 *g* supernatant equivalent to 12·5, 25 and 50 mg wet wt of cells. The assay conditions were the same as in Fig. 2 with a chlorcyclizine concentration of $7{\cdot}5 \times 10^{-5}$ M. Control (○); phenobarbital-treated (△); allyisopropylacetamide-treated (□).

activity. As seen in Table 1, the induction of cytochrome P-450 was inhibited by actinomycin D. Similarly, the stimulation of chlorcyclizine *N*-demethylase activity by AIA was prevented by the addition of actinomycin D, 0·1 μg/ml; puromycin, 1·5 μg/ml and cyclohexamide, 0·2 μg/ml to the culture medium. This evidence supports the concept that the stimulation of enzyme activity involves *de novo* ribonucleic acid and protein synthesis. It has been shown that both AIA and phenobarbital induce ALA synthetase in the chick embryo hepatic cell culture.[9] AIA was found to be superior

TABLE 1. INDUCTION OF CYTOCHROME P-450 IN CHICK EMBRYO HEPATIC CELL CULTURE*

| Treatment | Δ O.D. (450–490 nm)† | Cytochrome P-450 (nmoles/g liver)‡ |
|---|---|---|
| Control | 0·014 | 3·9 |
| AIA | 0·027 | 7·4 |
| Phenobarbital | 0·022 | 5·9 |
| AIA + actinomycin D | 0·013 | 3·7 |

* Cell cultures were grown for 8 hr, the media was changed and the cultures were exposed to AIA (50 μg/ml), phenobarbital (150 μg/ml) or AIA (50 μg/ml) and actinomycin D (0·1 μg/ml). After 11 hr, the cells were harvested and the cytochrome P-450 content was measured as in Methods and Materials.

† Observed CO-difference spectrum.

‡ Computed cytochrome P-450 content per gram of liver.

to phenobarbital as an inducer of both microsomal oxygenase (Fig. 4) and ALA synthetase[9] and was used as a standard inducer (a positive control) in all subsequent experiments.

There are numerous reports in the literature on the morphologic and biochemical development of the endoplasmic reticulum during embryonic development which in general suggests that microsomal oxygenase activity increases with gestation.[26,27]

We examined the basal level and inducibility of chlorcyclizine *N*-demethylase activity with respect to gestational age of the embryo at the time of culture. Liver cell cultures were prepared from livers of 12-, 14-, 16-, 18- and 20-day embryos. Cells from 18-day embryos adhered to the petri dish poorly and cells from the 20-day embryos did not adhere at all. As seen in Table 2, the ability to induce enzyme activity (ratio of treated to control cultures) was almost the same at each age. However, the basal activity fell off sharply at 18 days, coincident with poor cell growth. *In vivo*, the microsomal oxygenase activity in the fetus appears to rise with gestational age.[26,27] In contrast, in cell culture, the basal activity appears to be more dependent on good cell attachment and growth.

TABLE 2. BASAL ACTIVITY AND INDUCIBILITY OF CHLORCYCLIZINE *N*-DEMETHYLASE ACTIVITY AS A FUNCTION OF EMBRYO AGE AT THE TIME OF CELL CULTURE*

| Treatment | Age of embryo (days) | *N*-demethylase activity (μg norchlorcyclizine formed/mg protein/30 min) | Ratio of activity (treated/control) |
|---|---|---|---|
| Control | 12 | 2·20† | 2·16 |
| AIA | | 4·76 | |
| Control | 14 | 2·01 | 2·24 |
| AIA | | 4·48 | |
| Control | 16 | 1·50 | 2·39 |
| AIA | | 3·61 | |
| Control | 18 | 0·51 | 2·27 |
| AIA | | 1·16 | |

* Livers from chick embryos of the indicated gestational ages were pooled and prepared for cell culture. After 8 hr of culture the medium was changed and AIA (50 μg/ml) was added to the treated cultures. The cells were harvested 12 hr later and homogenates prepared from two pooled, identically treated petri dishes. The enzyme assay was performed in a 2·5-ml vol. as described in Methods and Materials.

† Mean of duplicate determinations.

The induction of ALA synthetase has been suggested as a necessary part of the induction of microsomal oxygenase activity to provide the heme needed for the synthesis of cytochrome P-450.[14] Various steroids, especially those with a 5 β-hydrogen configuration, have been demonstrated to induce ALA synthetase in hepatic cell culture.[21] Representative steroids were chosen: etiocholanolone and 17-ketopregnanolone, both 5 β-H steroids and good inducers of ALA synthetase; and testosterone and estradiol, both $\Delta^4$ steroids. As seen in Fig. 5, testosterone produced a dose-related increase in *N*-demethylase activity, roughly a 2-fold increase at a concentration of 20 μg/ml. Etiocholanolone and 11-ketopregnanolone also produced significant, but

smaller stimulation of microsomal oxygenase activity. Estradiol produced a small, dose-related decrease in enzyme activity.

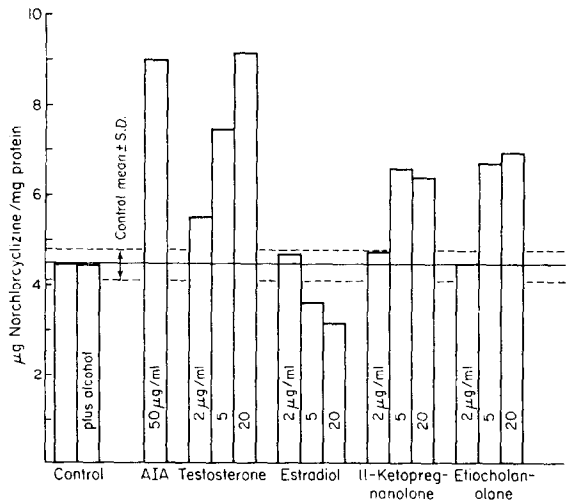


FIG. 5. Stimulation of chlorcyclizine *N*-demethylase activity by various steroids. Cell cultures were grown for 8 hr and then the medium was changed and the cultures were exposed to the drugs indicated for the next 12 hr. The enzyme assay was performed in a final volume as described in Materials and Methods. The reaction was started with the addition of homogenate equivalent to 10 mg of wet wt of cells. The results, presented as bar graphs, are the mean of duplicate determinations.

Cultures prepared on different weeks varied in their response to AIA and other drugs. In some cultures chlorcyclizine *N*-demethylase was not inducible at all. In contrast, ALA synthetase as measured by porphyrin accumulation was always inducible, thus dissociating the response of the two enzymes. Induction of microsomal oxygenase appears to be more demanding metabolically, requiring synthesis of substantial amounts of protein and phospholipids for new membrane formation, and requiring prosthetic groups for the flavoprotein and hemoprotein formed. In an attempt to find optimal culture conditions which would permit consistent induction, the medium was systematically enriched. The addition of vitamins, glutamine, amino acids, ferrous citrate, testosterone and various sera did not improve the variability of induction. However, recent experiments sugget that the problem lies in the method of enzymatic cell separation and density of plating the cells. The use of 0·1% Viokase[28] and plating at less than 100 mg of liver/dish has yielded consistently inducible cultures.

## DISCUSSION

In this report studies are presented on microsomal oxygenase activity in chick embryo hepatic cell culture. The assay of chlorcyclizine *N*-demethylase is linear with time and enzyme concentration, and the enzyme has a relatively high apparent affinity for the substrate ($K_m = 7{\cdot}5 \times 10^{-5}$ M). The assay for norchlorcyclizine is quite sensitive and *N*-demethylase activity can be determined easily in 5–10 mg wet wt of chick embryo liver cells.

The microsomal oxygenase activity is doubled by AIA, as is the cytochrome P-450 content. This stimulation appears to involve *de novo* protein and RNA synthesis, because it is blocked by actinomycin D, puromycin and cycloheximide. The *N*-demethylase activity is also induced by phenobarbital, testosterone, etiocholanolone and 11-ketopregnanolone. The variability of induction noted between different cultures appears to be related to the conditions of cell separation and cell density. The use of Viokase[28] and lower density plating has resolved this problem.

There are several reports of studies of microsomal oxygenase activity in cell culture or isolated cell systems,[28–37] but only a few of these investigations have concentrated on hepatic cell cultures. Mohri *et al.*[29] reported phenobarbital induction of aminopyrine *N*-demethylase activity in a suspension culture of adult rat liver cells. This system appeared unstable and activity in control cultures fell with time. Henderson and Dewaide[30] reported on *N*-demethylation of aminopyrine, *p*-hydroxylation of aniline and glucuronidation of *p*-nitrophenol in isolated whole rat cells in suspension. For brief periods of time, these cells maintained a linear metabolism of the above mentioned drugs, and for the oxygenation reactions the intact cells metabolized the drugs as well as or better than sonicated homogenates. We have previously reported that cultures of chick embryo liver cells *N*-demethylate aminopyrine added to the culture.[31] The formation of 4-aminoantipyrine is linear with time over a 16-hr period. This metabolism of aminopyrine is reversibly inhibited by a number of type I substrates and a carbon monoxide atmosphere.

The most thorough investigation of microsomal oxygenase activity in cell culture has been reported in a series of papers by Gielen, Nebert *et al.*[28,33–35] Initially Nebert and Gelboin[33] reported the induction of aryl hydrocarbon hydroxylase (benzo[a]-pyrene hydroxylase) in whole hamster cell culture by a variety of aromatic compounds. More recently Gielen and Nebert[28,35] found that microsomal oxygenase activity was inducible by phenobarbital and pp'DDT in fetal rat hepatic cell culture.

In this report, we present some preliminary observations on the inducibility of microsomal oxygenase activity in chick embryo hepatic cell culture. This appears to be the first report of induction of microsomal oxygenase activity other than aryl hydrocarbon hydroxylase in a stable culture of liver cells. Such a culture system offers potential usefulness in studying drug metabolism in a number of areas:

(1) the culture promises to provide a simple screening system for testing the inducibility of microsomal oxygenase by xenobiotics;

(2) unlike the whole animal, the hepatic cell culture is an isolated system, ideal for studying hormonal and nutritional influences; and

(3) easy access to the media allows isolation of metabolites and removal of inducing agent or antimetabolite after a fixed period of time.

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## REFERENCES

1. A. Conney, *Pharmac. Rev.* **19**, 317 (1967).
2. G. Mannering, in *Selected Pharmacological Testing Methods* (Ed. A. Burger), Vol. 3, p. 51. Marcel Dekker, New York (1968).
3. S. Orrenius, M. Das and Y. Gnosspelius, in *Microsomes and Drug Oxidations* (Eds. J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts and G. J. Mannering), p. 251. Academic Press, New York (1969).

4. W. LEVIN and R. KUNTZMAN, *J. biol. Chem.* **244,** 3671 (1969).
5. O. WADA, Y. YANO, G. URATA and K. NAKAO, *Biochem. Pharmac.* **17,** 595 (1968).
6. R. SCHMID and H. MARVER, *Biochim. biophys. Acta* **24,** 319 (1966).
7. S. GRANICK and G. URATA, *J. biol. Chem.* **238,** 821 (1963).
8. F. DEMATTEIS, *Pharmac. Rev.* **19,** 523 (1967).
9. S. GRANICK, *J. biol. Chem.* **241,** 1359 (1966).
10. T. TEPHLY, E. HASEGAWA and J. BARON, *Metabolism* **20,** 200 (1971).
11. J. CREIGHTON and G. MARKS, *Biochem. Pharmac.* **18,** 2040 (1969).
12. H. MARVER, R. SCHMID and H. SCHÜTZEL, *Biochem. biophys. Res. Commun.* **33,** 969 (1968).
13. L. KAUFMAN, A. SWANSON and H. MARVER, *Science, N.Y.* **170,** 320 (1970).
14. H. MARVER and R. SCHMID, *Gastroenterology* **55,** 282 (1968).
15. A. POLAND, J. GOLDSTEIN, P. HICKMAN and V. BURSE, *Biochem. Pharmac.* **20,** 1281 (1971).
16. S. SASSA and S. GRANICK, *Proc. natn. Acad. Sci. U.S.A.* **67,** 517 (1970).
17. N. HAYASHI, Y. KURASHUMA and G. KIKUCHI, *Archs Biochem. Biophys.* **148,** 10 (1972).
18. D. P. TSCHUDY, F. H. WELLAND, A. COLLINS and G. HUNTER, *Metabolism* **13,** 396 (1964).
19. K. J. JERVELL, T. CHRISTOFFERSEN and J. MORLAND, *Archs Biochem. Biophys.* **11,** 15 (1965).
20. H. MARVER, in *Microsomes and Drug Oxidations* (Eds. J. R. GILLETTE, A. H. CONNEY, G. J. COSMIDES, R. W. ESTABROOK, J. R. FOUTO and G. J. MANNERING, p. 495. Academic Press, New York (1969).
21. S. GRANICK and A. KAPPAS, *J. biol. Chem.* **242,** 4587 (1967).
22. M. JACOBSON, I. TSAI, A. POLAND and R. KUNTZMAN, *J. Pharmac. exp. Ther.*, **82,** 312 (1972).
23. T. OMURA and R. SATO, *J. biol. Chem.* **239,** 2370 (1964).
24. R. KUNTZMAN, I. TSAI and J. BURNS, *J. Pharmac. exp. Ther.* **158,** 332 (1967).
25. E. SUTHERLAND, C. CORI, R. HAYNES and N. OLSEN, *J. biol. Chem.* **180,** 825 (1949).
26. G. DALLNER, P. SIEKEVITZ and G. PALADE, *J. Cell Biol.* **30,** 97 (1966).
27. C. STRITTMATTER and F. UMBERGER, *Biochim. biophys. Acta* **180,** 18 (1969).
28. J. E. GIELEN and D. W. NEBERT, *J. biol. Chem.* **246,** 5189 (1971).
29. T. MOHRI, T. NARUGE and H. KITAGAWA, *J. pharm. Soc. Japan* **90,** 528 (1970).
30. P. HENDERSON and J. DEWAIDE, *Biochem. Pharmac.* **18,** 2087 (1969).
31. A. POLAND and A. KAPPAS, *Molec. Pharmac.* **7,** 697 (1971).
32. J. KOWAL, E. SIMPSON and R. ESTABROOK, *J. biol. Chem.* **245,** 2438 (1970).
33. D. NEBERT and H. GELBOIN, *J. biol. Chem.* **243,** 6242 (1968).
34. D. NEBERT, *Biochem. biophys. Res. Commun.* **36,** 885 (1969).
35. J. GIELEN and D. NEBERT, *Science, N.Y.* **172,** 167 (1971).
36. E. CANTRELL and B. BRESNICK, *J. Cell Biol.* **52,** 316 (1972).
37. J. HOLTZMAN, V. ROTHMAN and S. MARGOLIS, *Biochem. Pharmac.* **21,** 581 (1972).