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DRUG METABOLISM IN CELL CULTURE—I

IMPORTANCE OF STERIC FACTORS FOR ACTIVITY IN PORPHYRIN-INDUCING DRUGS

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Abstract—3,5-Diethoxycarbonyl-2,4,6-trimethylpyridine-^{14}C, a compound in which the ester groups are sterically hindered from hydrolysis, was synthesized and found to be slowly metabolized in chick embryo liver cell cultures. On the other hand, however, 3,5-diethoxycarbonyl-2,6-dimethylpyridine-^{14}C, a compound in which the ester groups are not sterically hindered from hydrolysis was rapidly metabolized in this system. Differences in δ-aminolevulinic acid synthetase induction and porphyrin accumulation can be explained on the basis of the rates of metabolism of these compounds.

A LARGE number of compounds have been found to produce elevated levels of hepatic δ-aminolevulinic acid (δ-ALA) synthetase, the rate-controlling enzyme in protoheme biosynthesis.[1] In addition, most of these compounds increase the activity of the liver microsomal enzymes and the amount of cytochrome P-450.[2] On the basis of these observations Granick[1] has suggested that the increased δ-ALA formed in response to these drugs, following increased synthesis of δ-ALA synthetase, is utilized for the formation of protoheme. This increased protoheme would then serve as the prosthetic group of cytochrome P-450, which functions in the oxidative metabolism of drugs. Utilizing the above ideas, Hirsch *et al.*[3] have suggested that the critical feature for activity in analogues of allylisopropylacetamide (AIA) and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) is an ester or amide sterically hindered from hydrolysis. These workers also suggested that drugs which could not be metabolized by a hydrolytic mechanism were oxidatively metabolized. Racz and Marks[4] examined the metabolism of the sterically hindered compound DDC, and its inactive, sterically unhindered analogue, 3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine (4-desmethyl-DDC), in 17-day-old chick embryos.[4] The results indicated but did not prove that DDC, whose ester groups are hindered from hydrolysis, was metabolized at a much slower rate than 4-desmethyl-DDC, whose ester groups are not sterically hindered from hydrolysis. However, the above studies were complicated by the instability of 4-desmethyl-DDC. In the present study, the ability of the sterically hindered 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (Ox-DDC; Fig. 1a) and the sterically unhindered 3,5-diethoxycarbonyl-2,6-dimethylpyridine (4-desmethyl-Ox-DDC; Fig. 1b) to induce δ-ALA synthetase has been examined and correlated to the metabolic rate of the two compounds.

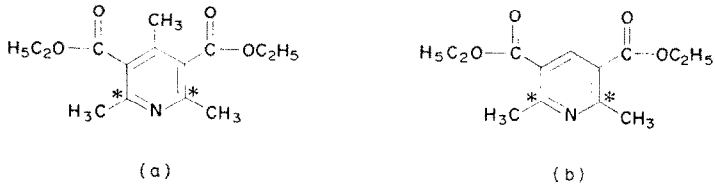

FIG. 1. Chemical structure of: (a) 3,5-diethoxycarbonyl-2,4,6-trimethylpyridine (Ox-DDC), and (b) 3,5-diethoxycarbonyl-2,6-dimethyl-pyridine (4-desmethyl-Ox-DDC). The asterisks indicate the positions of the labeled carbon atoms.

EXPERIMENTAL

The fertilized eggs used were of a White Leghorn strain obtained from a local hatchery, and stored at 10° for no longer than 7 days prior to incubation. The age of the embryo was taken as the number of days from onset of incubation. Ethyl acetoacetate-3-^{14}C (4·17 mCi/m-mole) was purchased from New England Nuclear Corp. Powdered basal medium containing Earle's salts and glutamine and 2·5% trypsin in saline were purchased from Grand Island Biological Co. Pooled bovine serum was purchased from Pentax, Inc., Winley–Morris Co., Ltd.

Instrumentation. All radioactive samples were counted in a Nuclear Chicago Mark II liquid scintillation system. Counts were corrected for quenching by the sample channels ratio method and the background count was subtracted. The thin-layer chromatograms containing radioactive samples were scanned using a Nuclear Chicago Actigraph III radiochromatography system. All radiochromatograms were scanned at a detector voltage of 960 V and gas pressure of 7 lb.

Synthesis of labeled compounds. Ox-DDC-^{14}C was prepared by a method previously described.[5] 4-Desmethyl-Ox-DDC-^{14}C was prepared as previously described.[4]

Preparation of chick embryo liver cell cultures. Chick embryo liver cells were cultured in Petri dishes according to the procedure of Granick[1] as modified by Tyrrell and Marks.[6] Sixteen 17-day-old chick embryo livers were removed, and washed with calcium- and magnesium-free Earle's solution. A cell suspension was prepared by incubating the livers in a mixture of 10 ml trypsin (2·5%) in saline, and 10 ml calcium- and magnesium-free Earle's solution. The cell suspension was centrifuged at 250 *g* for 5 min; the supernatant was discarded, and the cells were resuspended in 30 ml of warm media. In experiments measuring δ-ALA synthetase, 1 ml of the cell suspension was added to 50 ml of growth medium in 150 mm Petri dishes. For the measurement of unchanged drug in the cell culture medium, 0·2 ml of cell suspension was added to 5 ml of growth media in 50 mm Petri dishes. The cultures were incubated in a Napco incubator at 37°, and constant gas flow (air 95%, CO_2 5%) of 8·4 l/min. Growth medium was prepared by dissolving powdered basal medium containing Earle's salts and glutamine in glass distilled water. To each liter of freshly prepared medium 100 ml of bovine serum was added. After 24 hr of incubation the medium was removed and replaced with fresh medium containing drugs.

Addition of drugs to cell culture medium. The drugs were dissolved in a quantity of ethanol calculated so that the amount of ethanol added to the medium did not exceed 2 μl/ml.

Determination of δ-ALA synthetase activity. The enzyme activity was determined by the procedure of Marver *et al.*[7] as modified by Tyrrell and Marks.[6] The protein content of the cell homogenates was determined by a colorimetric technique as described by Lowry *et al.*[8] The cells from a 150 mm dish yield approximately 4 mg of protein.

Determination of appropriate concentrations of Ox-DDC and 4-Desmethyl-Ox-DDC. Various concentrations of Ox-DDC or 4-desmethyl-Ox-DDC in ethanol were added to the cell culture medium. At 12 or 24 hr after addition of the drug, the medium was removed, the cells were harvested and δ-ALA synthetase activity was determined. The dose–response curves obtained at 12 hr after addition of the drugs are shown in Fig. 2.

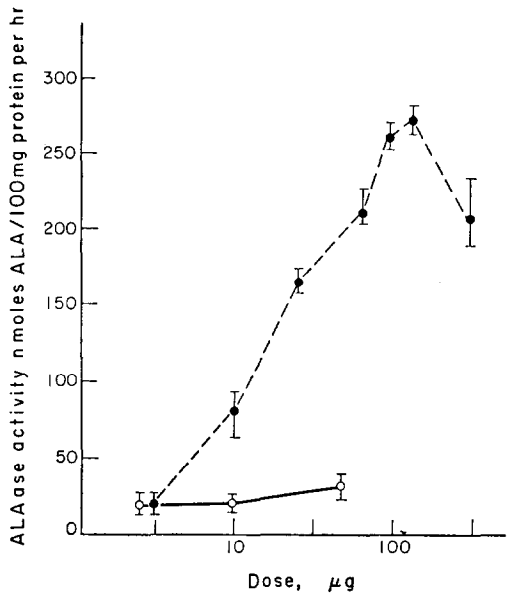

FIG. 2. Relationship between the dose of Ox-DDC (●– – –●) and 4-desmethyl-Ox-DDC (○——○) and δ-ALA synthetase activity. The enzyme activity was measured 12 hr after drug administration. Each point represents mean ± S.E. of mean.

Determination of δ-ALA synthetase activity at various periods of time after addition of drugs. Ox-DDC at 10 μg/ml (0·038 μmole/ml) and 100 μg/ml (0·38 μmole/ml) was added to cells in culture. After various periods of incubation, the medium was removed, and the cells were harvested for determination of δ-ALA synthetase activity.

The optical density measured for the basal enzyme activity varied from 0·005 to 0·02, while the optical density measured for the activity of the enzyme from cells incubated in the presence of 10 μg/ml of Ox-DDC varied from 0·12 to 0·15 and at 100 μg/ml of Ox-DDC from 0·16 to 0·48. Since the optical density measured for the basal enzyme activity is low, the confidence that can be placed in the enzyme activity at basal levels is much less than that placed in the values for the activity of the enzyme

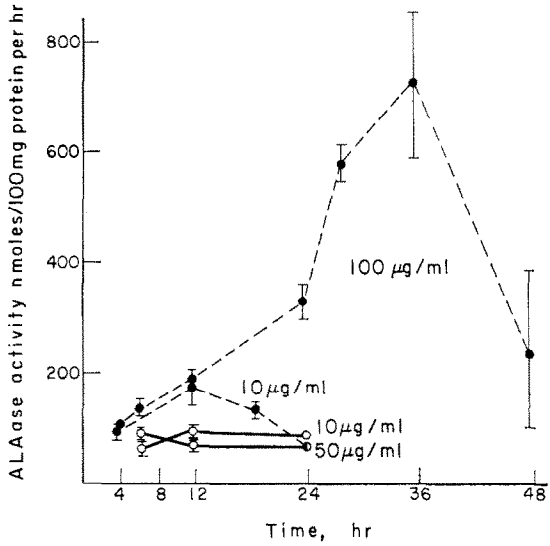

FIG. 3. δ-ALA synthetase activity in chick embryo liver cells in culture at different time intervals after addition of Ox-DDC (●- - -●) and 4-desmethyl-Ox-DDC (○——○) to the medium. Each point represents mean ± S.E. of mean.

in the induced state. The experiment was repeated with 10 μg/ml (0·039 μmole/ml) and 50 μg/ml (0·20 μmole/ml) of 4-desmethyl-Ox-DDC. The results are shown in Fig. 3.

Amount of unchanged drug in cell culture media after various periods of incubation. An accurately weighed amount of the labeled drug was dissolved in a quantity of redistilled ethanol calculated to give a dilution of drug so that the amount of ethanol added did not exceed 2 μl/ml of media. The labeled drugs were added to the medium of cells in culture and the cultures reincubated. After various periods of incubation, the media were collected, and the cells washed with calcium- and magnesium-free Earle's solution. The Earle's solution was allowed to remain in contact with the cells for 5 min at 37°, collected and added to the medium. The Petri dishes containing the cells were stored in a refrigerator for future extraction of the radioactive drug and determination of protein content.

The combined medium and calcium- and magnesium-free Earle's solution was transferred to a 60-ml separatory funnel and 0·1 ml of 1 N NaOH added. The unchanged drug was extracted from the aqueous medium with three 10-ml portions of ether. One ml of the combined ether extract, containing the unchanged drug, was added to 14 ml of toluene scintillation solution [consisting of 6 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP) in 1 l. of toluene] and counted. One ml of the aqueous fraction containing the metabolite(s) was added to 14 ml of Aquasol (New England Nuclear Corp.) and counted. From the counts in the two fractions, the amount of unchanged drug and metabolite(s) was estimated.

It was considered desirable to check the recovery of unchanged drug from the medium and to exclude the possibility of metabolic transformation of the drugs in

the medium. For this purpose, known amounts of Ox-DDC-^{14}C and 4-desmethyl-Ox-DDC-^{14}C were added to medium alone, and incubated for 2 hr. The ether extraction procedure described above recovered 98·5 $\pm$ 2·5 per cent (mean $\pm$ S.E. of mean, n = 10) of unchanged drug.

To ensure that the extraction procedure of the cell culture medium quantitatively separated the unchanged drugs from their metabolite(s), both the aqueous and ether layers were reduced to a volume of 1 ml, and 0·3 ml of the residue was spotted as a band on a 5 $\times$ 20 cm thin-layer plate coated with a 250 micron layer of Silica gel G. The plates were developed in benzene–methanol (14:1).[5] The *Rf* values for Ox-DDC and 4-desmethyl-Ox-DDC were found to be 0·72 and 0·71, respectively,[4,5] and the metabolite(s) remained at the origin. The corresponding radioactive areas of Silica gel were removed from the plate and counted in a liquid scintillation counter.[4,5] The ether phase contained only unchanged drug and the aqueous phase contained only metabolite(s).

In order to determine the amount of unchanged drug and metabolite(s) remaining in the cells after removal of the medium, 3 ml of methanol was added to the Petri dishes, and the cells were dislodged with the aid of a Teflon policeman. The cells in methanol were pipetted into a Potter–Elvehjem apparatus, homogenized, and transferred to a centrifuge tube. After centrifugation, the supernatant was decanted into a small flask, and the residue was extracted once more in an additional 3 ml of methanol followed by homogenization and centrifugation. The extraction procedure was repeated, and the three supernatants were combined. The tissue residue was solubilized in 1 ml of NCS (Nuclear Chicago Corp.); 14 ml of toluene scintillation solution was added, and the sample was counted. No significant amount of radioactivity was found in this residue. The methanol extract was reduced to a volume of 0·5 ml, and the radioactive materials were separated by thin-layer chromatography and estimated as described above. The amount of unchanged drug recovered from the methanolic extract was added to the unchanged drug recovered from the medium by the extraction procedure described above. The protein content of the cells in culture was determined for five identical Petri dishes. The cells from a 50 mm dish yield approximately 1·5 mg of protein. The results are shown in Fig. 4 and are plotted as nmoles of unchanged drug/mg of protein.

RESULTS AND DISCUSSION

The first objective of this study was to examine the ability of various doses of Ox-DDC and 4-desmethyl-Ox-DDC to induce δ-ALA synthetase. This would provide a rational basis for selecting doses to compare the two compounds. Figure 2 shows the dose–response curves for the two compounds. Ox-DDC at 10 μg/ml (0·038 μmole/ml) significantly elevated δ-ALA synthetase activity above control values, and a maximum effect was observed at 100 μg/ml (0·38 μmole/ml). With 4-desmethyl-Ox-DDC, on the other hand, no significant increase in δ-ALA synthetase activity was observed over control values. Concentrations of 4-desmethyl-Ox-DDC greater than 50 μg/ml (0·20 μmole/ml) inhibited cell growth and, therefore, could not be employed. The finding that 4-desmethyl-Ox-DDC is completely inactive is in accordance with previous findings.[9] From these results, 10 μg/ml (0·038 μmole/ml) and 100 μg/ml (0·38 μmole/ml) of Ox-DDC, and 10 μg/ml (0·039 μmole/ml) and 50 μg/ml (0·20 μmole/

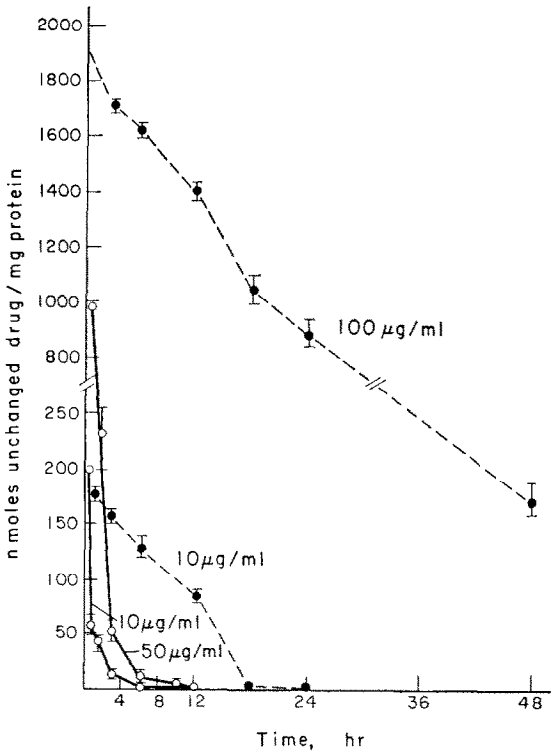

FIG. 4. Amount of unchanged drug in chick embryo liver cells and cell culture medium at different time intervals after addition of Ox-DDC (●- - -●) and 4-desmethyl-Ox-DDC (○——○) to the medium. Each point represents mean ± S.E. of mean.

ml) of 4-desmethyl-Ox-DDC were selected as the concentrations at which to study the induction of δ-ALA synthetase and the metabolic rate of the drugs.

The second objective of the study was to examine the time course of induction of δ-ALA synthetase activity after addition of the drugs to the medium. From Fig. 3 it can be seen that 4-desmethyl-Ox-DDC does not elevate δ-ALA synthetase activity. This is in accordance with previous findings that this compound does not cause accumulation of porphyrins in cell culture.[9] On the other hand, Ox-DDC at 10 μg/ml (0·038 μmole/ml) causes a significant elevation of δ-ALA synthetase, which reached a maximum at 12 hr and at 24 hr had returned to control values. Ox-DDC at 100 μg/ml (0·38 μmole/ml) caused a marked increase in δ-ALA synthetase activity, which reached a maximum at 36 hr, and was still elevated at 48 hr after addition of the drug. These observations are in accordance with the expectation that 4-desmethyl-Ox-DDC would be readily metabolized by a hydrolytic process and inactivated. However, the sterically protected esters in Ox-DDC would not be as readily hydrolyzed, and the drug would not be as rapidly inactivated as the sterically unhindered 4-desmethyl-Ox-DDC.

The third objective of the study was to examine the rate of the metabolism of the two compounds in the cell culture system. After various periods of incubation, the unchanged drug was extracted from the medium with ether. This method afforded quantitative recovery of known amounts of radioactive drugs added to medium.

Furthermore, thin-layer chromatography of both the ether and aqueous fractions demonstrated that the ether removed all the intact drug, leaving the water-soluble metabolite(s) in the aqueous phase. If the ether extract contained a metabolite, it would have to migrate with the parent compound in the chromatographic system to escape detection. This possibility has not been eliminated, but would appear rather unlikely. The cells were extracted and found to contain, after washing, approximately 1 per cent of the total radioactivity added to the system. From Fig. 4 it can be seen that 4-desmethyl-Ox-DDC is very rapidly metabolized. Seventy-five per cent of the 10 μg/ml (0·039 μmole/ml) concentration was removed in 30 min, and by 6 hr no intact drug remained. Ox-DDC (10 μg/ml, 0·038 μmole/ml), on the other hand, is metabolized at a much slower rate, only 15 per cent being metabolized at 1·5 hr and 30 per cent at 6 hr. 4-Desmethyl-Ox-DDC at 50 μg/ml (0·20 μmole/ml) is again very rapidly metabolized, and 6 hr after addition of drug to the medium virtually no intact drug remains. After the addition of 100 μg/ml (0·38 μmole/ml) of Ox-DDC, intact drug levels remain elevated at high levels up to and beyond 48 hr (Fig. 4). These results clearly demonstrate that the inactive, sterically unhindered 4-desmethyl-Ox-DDC undergoes a much more rapid metabolic transformation than does the active, sterically hindered Ox-DDC. These results support the hypothesis that one of the critical features for activity in this series of compounds is an ester sterically hindered from hydrolysis. They suggest that the sterically unhindered 4-desmethyl-Ox-DDC is very rapidly hydrolyzed, whereas the sterically hindered Ox-DDC cannot be hydrolyzed and is metabolized by an oxidative process or, due to steric factors, is hydrolyzed at a much slower rate. These results also lend support to the suggestion that for a compound to cause porphyrin accumulation it must be present in sufficient quantity and for an adequate period of time, in order to elevate δ-ALA synthetase levels high enough and long enough so that the δ-ALA produced cannot all be converted to protoheme.[5] It has recently been suggested that DDC and allylisopropylacetamide must undergo a metabolic transformation to an active metabolite in order to induce porphyrin biosynthesis.[10] The results presented above indicate that Ox-DDC and 4-desmethyl-Ox-DDC rather than their metabolites must be present for induction of δ-ALA synthetase activity.

To further clarify the difference in activity of Ox-DDC and 4-desmethyl-Ox-DDC it will be of interest to elucidate the nature of the metabolites formed. These studies are in progress.

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