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N-DEMETHYLATION AS AN EXAMPLE OF DRUG METABOLISM IN ISOLATED RAT HEPATOCYTES

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Abstract—The capacity of isolated rat hepatocytes to carry out drug metabolism *in vitro* was studied. The aromatic hydroxylation of quinine sulfate was measured fluorometrically from two starting concentrations of the drug. The *N*-demethylation of dansylamide and antipyrine was measured by following the release of tritium into water after hydroxylation of ^{3}H-labeled methyl groups. The *N*-demethylation of antipyrine and dansylamide was inhibited by SKF-525A and ethylmorphine. The metabolism was rapidly increased (approximately 10 per cent) by the addition *in vitro* of 0.1 mM phenobarbital. Isolated rat hepatocytes are well suited for drug metabolism studies. They are readily prepared, retain their viability for hours, and allow many, well-controlled experiments from the same preparation of cells. The formation of ^3H_2O from suitably labeled precursors is a sensitive and promising technique to study certain aspects of drug metabolism.

A technique for the preparation of isolated rat hepatocytes involving the use of collagenase and hyaluronidase was first discussed by Howard *et al.* [1]. This method was then improved by Berry and Friend [2]. In recent years, many investigators have adopted and modified these procedures, and are currently isolating liver cells that are capable of many physiological functions. There have been recent reports of isolated hepatocytes synthesizing fatty acids and cholesterol [3], albumin [4] and glucose [5]. Fatty acid oxidation [6] and stimulation of gluconeogenesis by glucagon have been investigated in these cells [7]. Thus, the metabolic integrity of the isolated hepatocytes has been well established.

The liver is by far the most important organ for the metabolism of xenobiotics. Therefore, isolated mature hepatocytes would make an excellent model for the study of drug metabolism and drug–drug interactions. Despite the advantages of this system, there have been few attempts to use it to study drug metabolism. Berry [8] has successfully used isolated hepatocytes to study the action of pyruvate on ethanol metabolism. Holtzman *et al.* [9] have found that these cells will metabolize aniline, ethylmorphine and 3,4-benzo(a)-pyrene, while Corona *et al.* [10] and Zimmerman *et al.* [11] have studied hepatotoxicity by drugs in isolated liver cells. Recently, Moldéus *et al.* [12,13] have studied drug–cytochrome P-450 interactions and drug metabolism linked to cytochrome P-450 in isolated hepatocytes. Von Bahr *et al.* [14] have also studied the binding of drugs to cytochrome P-450 in this system. To our knowledge, this is a comprehensive list of drug metabolism experiments in isolated hepatocytes. We have worked with isolated rat hepatocytes in the past measuring gluconeogenesis and fatty acid oxidation and have found that the mechanics of preparation of these parenchymal cells greatly influence their metabolic activities.

In this paper, we will describe drug metabolism experiments in cells prepared by perfusion of isolated rat livers with collagenase. The advantage of such a drug-metabolizing system is obvious. While maintaining the integrity of the perfused liver system, one can now do many parallel experiments with aliquots of cells from one liver. These cells metabolize drugs for extended periods of time. If one wants to do experiments with relatively small aliquots of liver cells, one will have to use sensitive assay systems. We describe in this report the use of two assay systems which fulfill these requirements. One of these methods involves the use of ^{3}H-labeled drugs; the other, more classically, employs fluorescence measurements. One relatively simple approach for the measurement of hydroxylation reactions has not been used extensively in drug metabolism studies. This particular approach is based on the exchange of tritium with water from ^{3}H-labeled aliphatic substituents or side chains following the formal interjection of an oxygen atom into a C–H bond (Fig. 1). In the case of an aliphatic side chain, tritium is released on formation of a primary alcohol, and more tritium can be released on successive metabolism to an aldehyde or the acid. For a secondary alcohol, the only successive oxidation possible would lead to the formation of the ketone. In the case of alkyl substituents or heteroatoms, such as *S-O-* and *N-*, hydroxylation on the carbon next to the heteroatom leads to dealkylation and formation of aldehydes. Secondary tritium release occurs on further metabolism of these aldehydes to their corresponding acids.

MATERIALS AND METHODS

Materials

Male Sprague–Dawley rats, 200–250 g, were maintained on a standard Purina rat chow diet at 22° in temperature-controlled rooms on a cycle of 12 hr of light and 12 hr of darkness. Animals used in gluconeogenesis experiments and citrate metabolism experiments were deprived of food for 48 hr. Collagenase (CLS-111, 132 U/mg) was obtained from

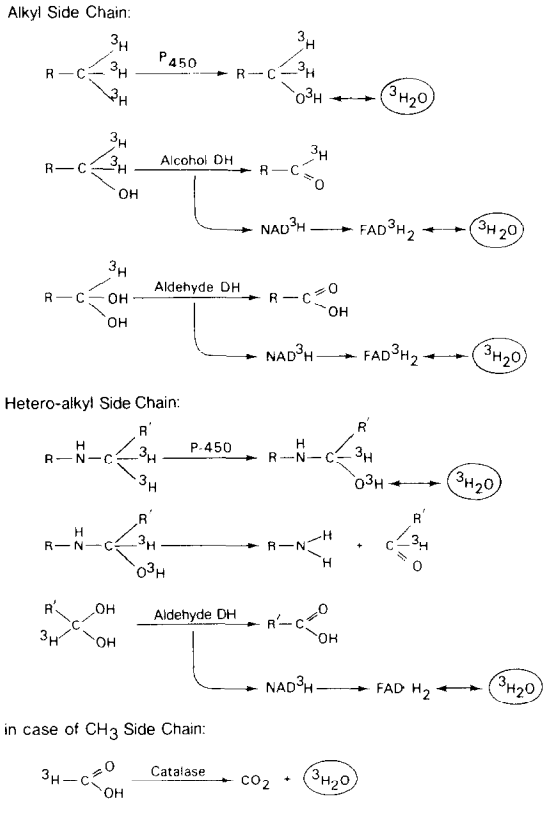

Fig. 1. Mechanism of tritium exchange into water from ^{3}H-labeled aliphatic substituents or side chains following α-hydroxylation and successive reactions.

Worthington Biochemical Corp. Antipyrine, EDTA,* HEPES, and bovine albumin, fraction V, were obtained from Sigma Chemical Co. Amino acids (100 X), L-glutamine (200 mM) and "antibiotic-antimycotic" were purchased from Grand Island Biological Co. Dextran T-70 was from Pharmacia and dextrose from Mallinckrodt. Lithium lactate was obtained from Nutritional Biochemicals Corp. and dansylchloride from Pierce Chemical Co. Dansylchloride[*N*-methyl-^{3}H], 1–5 Ci/m-mole, antipyrine-[*N*-methyl-^{3}H], 100–200 mCi/m-mole, and citric acid[1,5-^{14}C], 2–10 mCi/m-mole, were purchased from New England Nuclear. Ethylmorphine-HCl and quinine sulfate were purchased from Merck & Co. and sodium phenobarbital from Eli Lilly & Co. SKF-525A was obtained from Smith Kline & French Labs. The mitochondrial uncoupler FCCP was a generous gift of the E. I. Dupont De Nemours Co. Fresh cow blood was obtained from a local slaughterhouse, allowed to clot, and centrifuged. The serum was then poured off and frozen in 100-ml containers at −30°. Dansylamide was synthesized by reacting dansylchloride with excess ammonia in tetrahydrofurane water. After standing overnight, the reaction mixture was taken to dryness on a rotary evaporator and the residue recrystallized from alcohol water.

Buffers. The composition of the main buffers were as follows: (a) perfusion buffer: 30 g dextran, 4.05 g NaCl, 465 ml of 0.154 M HEPES (pH 7.45), 30 ml of 0.154 M KCl, 15 ml of 0.154 M $MgSO_4 \cdot 7\,H_2O$, 4.6 ml of 0.825 mM EDTA, 15 ml of 0.154 M K_2HPO_4, 60 mg penicillin, and 375 ml H_2O; (b) wash buffer: 30 g dextran, 7.65 g NaCl, 65 ml of 0.154 M HEPES (pH 7.45), 30 ml of 0.154 M KCl, 15 ml of 0.154 M $MgSO_4 \cdot 7\,H_2O$, 4.6 ml of 0.825 mM EDTA, 15 ml of 0.154 M K_2HPO_4, 60 mg penicillin, and 775 ml H_2O; and (c) incubation buffer: 150 mg dextrose, 100 mg lithium lactate, 0.5 ml of 0.154 M $CaCl_2$, 1 ml amino acid mixture (100 ×), 1 ml L-glutamine (200 mM), 2 g bovine albumin, 1 ml antibiotic-antimycotic (100 ×), add perfusion buffer to 100 ml. All buffers had a final pH of 7.4. Before the above three buffers were used, they were filtered through a Millipore filter of 0.45 μm pore size. The dextrose, lithium lactate and glutamine were excluded from the gluconeogenesis and citric acid metabolism experiments.

Perfusion system. The perfusion system incorporated the following features: isolated organ *in vitro*; a circulating perfusate volume of 100 ml; enclosed silastic membrane oxygenating system; constant volume nonpulsatile pump; perfusion pressure monitoring; and the canulated organ submerged in buffer inside the thermostated perfusion vessel [15]. The ischemic period was no longer than 15 sec.

Preparation of hepatocytes

The rats were anesthesized with ether throughout the surgical procedure. The abdominal cavity was opened, the bile duct isolated, and a polyethylene catheter (PE 10, Intramedic) inserted. The portal vein was then isolated and the canula of the perfusion apparatus (grooved 15 gauge SS needle) inserted and tied into the portal vein at the general locus of the lienal branch. The perfusion was started (6 ml/min) and the liver transferred from the abdominal cavity to the perfusion apparatus. After the transfer, the perfusion rate was adjusted to a flow rate of 10 ml/g min. The entire procedure takes less than 5 min, and the ischemic period was usually less than 15 sec.

The perfusion apparatus was filled prior to the liver isolation with 100 ml of the perfusion buffer, and equilibrated with oxygen at 32°. About 25 ml of the perfusate was used to clear the liver of red cells at a flow rate of 10 ml/min. The liver was then placed in the apparatus, the perfusate recirculated, and 50 mg collagenase in 25 ml perfusion buffer added to bring the total perfusing volume back to 100 ml. The perfusion time in the presence of collagenase was 15–20 min at a flow rate of 10 ml/g/min. When the liver had significantly increased in size and started to leak perfusate out of the lobes, the system was changed to noncirculating and the collagenase-containing buffer was washed out of the liver. Three 25-ml washes of clean buffer were perfused through the liver to wash out remaining collagenase. The liver was disconnected from the canula, the tissue gently separated by opening scissors, and poured onto a sieve of 1-mm mesh size. The liver was washed into a second plastic beaker with bovine serum and the

*Abbreviations used: EDTA, ethylenediamine tetraacetic acid; HEPES, *N*-2-hydroethyl piperazine-*N*-2-ethane sulfonic acid; dansylchloride, B-dimethylamine-1-napthalene sulfonyl chloride; SKF-525A, 2-diethylaminoethyl-2, 2-diphenylvalerate HCl; FCCP, *P*-trifluoromethoxyphenylhydrazone of carbonyl cyanide.

gentle stirring motion of a soft rubber bulb. The bovine serum contains an α_2-macroglobulin, which irreversibly binds to and inhibits collagenase [16], and thereby helps in the removal of remaining enzyme, which can be deleterious in long time incubations of isolated liver cells. The crude liver cell suspension was gently drawn into a 100-ml pipet through a 3-mm fire-polished opening. This was repeated two times to dispense the cells. The suspension was then placed in the top conical tube of two stacked 50-ml plastic centrifuge tubes. The bottom tube was prefilled with 45 ml of clean oxygenated wash buffer and the upper tube was a conical centrifuge tube with a 6-mm opening in the bottom. The tubes were centrifuged for 2 minutes at 40 *g*, forming a loosely packed pellet. The supernatant was discarded and the pellet was resuspended in wash buffer, and the washing procedure repeated two more times. The final pellet was resuspended in 100 ml of the incubation buffer. The cell yield was 90–95 per cent.

Each incubation reaction contained 5-ml cells which were pipetted into 25-ml Erlenmeyer flasks, the bottoms of which had been heated and pushed inward to form a conically raised center which kept the cells from settling in the center of the flask. The opening of the pipet tip used for the dispension of the cells was enlarged to 3 mm and fire polished. The 100-ml cell suspension was swirled continuously while pipetting to insure equal aliquots of cells in each reaction. The concentration was 45–60 mg wet weight cells/ml or about 4.4 to 5.9 $\times$ 10^6 cells/ml. All incubations were carried out at room temperature. The reaction flasks were swirled in a gyrating incubator at 120 rev/min.

Assays

The fluorescence assay of quinine and its metabolites has been previously described [17]. In our experimental procedure, it was somewhat modified. Two ml of a 10-ml reaction containing liver cells was added to 10 ml benzene + 0.2 ml NaOH. The mixture was sonicated and centrifuged. Five ml of the benzene layer was then sonicated with 4 ml of 7% $HClO_4$. The fluorescence of the resulting aqueous layer containing quinine was measured. One ml of the original aqueous phase containing guinine metabolites was adjusted to 7% $HClO_4$ and its fluorescence determined. This separation of parent compound and metabolite is based on the differences in solubility after glucuronide conjugation of hydroxylated quinines.

The radioactive assay involved the formation of tritiated water from molecules which were tritiated in a position such that the tritium was labilized upon hydroxylation by the P-450 enzyme system. Tritium (CH_3–N)-labeled antipyrine and dansylamide were used in these experiments. The appropriate concentrations of drug plus 0.5 μCi of very high specific radioactivity drug were added to each reaction. One-half ml of the incubation medium containing cells was taken at various time intervals and centrifuged, and 200 μl of the supernatant was placed on a short Dowex-50 cation exchange column in the H^+ form. The column was eluted with 2 ml water, and the effluent counted in 14 ml of a solution containing 333 ml Triton X-100 (Rohm & Haas), 667 ml toluene and 3.8 g Omnifluor. The vials were then counted in a Beckman LS 250 liquid scintillation counter.

RESULTS AND DISCUSSION

Cell viability

The trypan-blue exclusion test (0.25% trypan blue in perfusion buffer) indicated that about 95 per cent of the cells were intact. The isolated liver cells utilized oxygen at a linear rate (Fig. 2) and synthesized glucose from various precursors at rates comparable to the isolated perfused liver (Fig. 3). They also metabolized [1,5-^{14}C]citrate in a linear fashion for 8 hr (Fig. 4).

Metabolism of quinine sulfate

Quinine is metabolized primarily in the liver. Many of the metabolic degradation products have been identified as hydroxy derivatives [18], with the main metabolite arising from hydroxylation in the 2-position of the quinoline ring. The metabolites are soluble in water at high pH, whereas the parent compound is soluble in benzene under these conditions. Metabolism of quinine and the formation of its fluorescing metabolites are shown in Fig. 5.

There is not an equal amount of fluorescence appearing to account for the disappearance of quinine fluorescence. This may indicate that some of the metabolites are not fluorescent or that the molar fluorescence for the metabolites is lower than that of the parent compound. Quinine disappears from the preparation in a logarithmic fashion with a half-life of 66 min (Fig. 6). The half-life was the same when starting from either 3.7 or 1.4 μg/ml of quinine sulfate. This experiment shows that aromatic hydroxylation

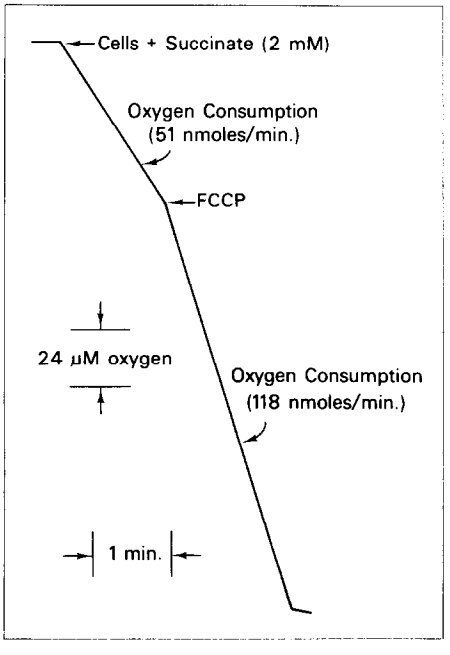

Fig. 2. Oxygen consumption of isolated hepatocytes as measured with a Clark-type oxygen electrode. Liver cell concentration was 50 mg/ml wet weight. Measurements were made at 37° in a volume of 2 ml of buffer. The cells were "uncoupled" with 0.25 μM FCCP.

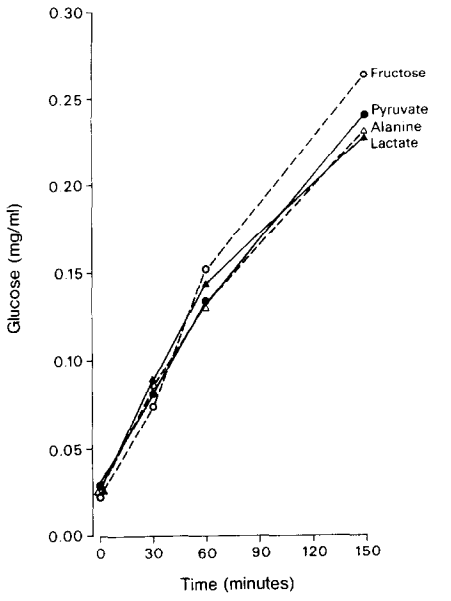

Fig. 3. Cumulative glucose production by isolated hepatocytes from 10 mM of gluconeogenic substrates. Incubations were carried out at 23° with liver cell concentrations of 50 mg/ml wet weight.

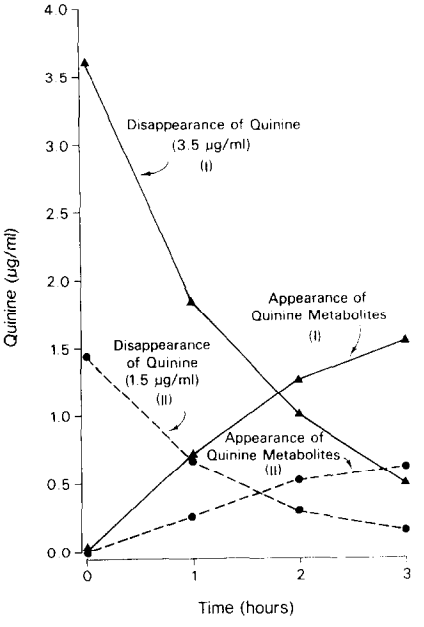

Fig. 5. Disappearance of quinine sulfate and the appearance of fluorescing metabolites in preparations of isolated hepatocytes. Reaction flasks contained 50 mg/ml of liver cells and were incubated at 23°. Samples were taken at 0, 1, 2 and 3 hr and assayed as described in the text.

followed by glucuronidation takes place in our preparation of isolated liver cells, which is in agreement with findings in other laboratories.

Metabolism of antipyrine and dansylamide

Antipyrine, a drug that is distributed in total body water and does not bind appreciably to plasma proteins, has been used to measure drug metabolic activity. Conney *et al.* [19] have shown that dogs pretreated with chlordane had increased metabolism of antipyrine. In contrast, the rate of metabolism of antipyrine is slowed when dogs are treated chronically with bishydroxycoumarin, a competitive inhibitor of drug metabolism. Although the main metabolite of antipyrine is 4-hydroxy-antipyrine and hydroxylation of the C-methyl group has been reported [20], *N*-demethylated antipyrine has been shown to contribute 10–20 per cent of the total metabolites [21]. We have found in our experiments that *N*-demethylation of antipyrine definitely contributes to the overall metabolism, up to 20 per cent of the total. The demethylated metabolite has been identified and the

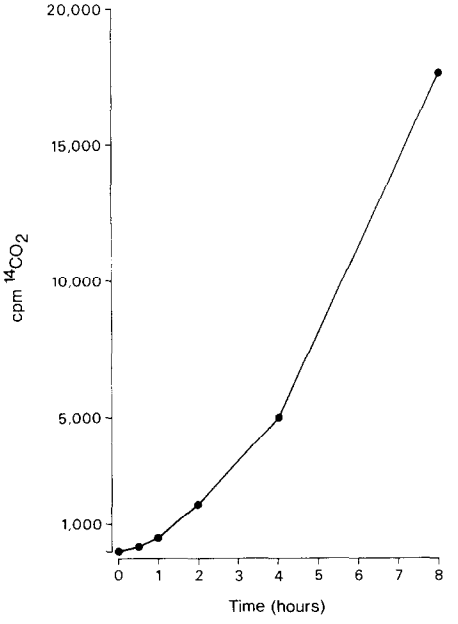

Fig. 4. Citrate metabolism by isolated hepatocytes. Tracer amounts of [1,5-^{14}C]-citric acid were used as the substrate. The $^{14}CO_2$ production was measured by continuous trapping of $^{14}CO_2$ evolved from reaction mixtures in an oxygen atmosphere at 23°. Incubation flasks contained 50 mg/ml of liver cells and 0.05 µCi of substrate.

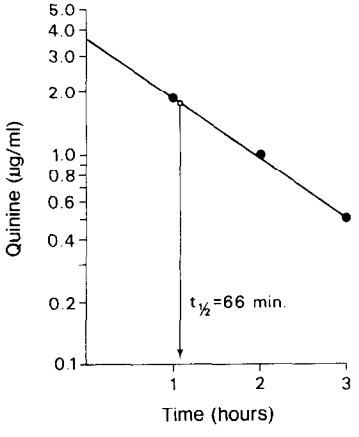

Fig. 6. Semilog plot of quinine sulfate disappearance from isolated hepatocyte reaction media. A half-life time of 66 min was obtained from a starting concentration of 3.5 µg/ml of quinine. Similar results are obtained by plotting the disappearance of drug from a starting concentration of 1.5 µg/ml.

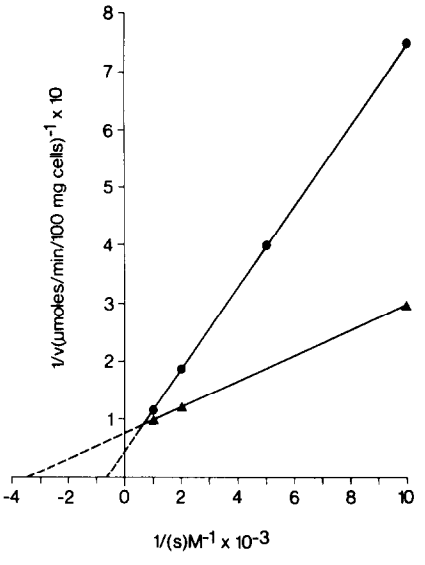

Fig. 7. Lineweaver–Burk plot of the *N*-demethylation of antipyrine (●) and dansylamide (▲).

formation of ^3H_2O from [*N*-^{3}H-methyl]antipyrine is only possible after hydroxylation of the *N*-methyl group. The question arises whether the appearance of tritiated water reflects the rate of drug hydroxylation, since further ^3H_2O is released on secondary metabolism of formaldehyde. The rate of formaldehyde metabolism by liver cells, i.e. soluble and mitochondrial aldehyde dehydrogenase, is faster than drug metabolism as concluded from the following experiments. The rate of ^3H_2O formation from 0.5 mM antipyrine was not retarded by the addition of 65 mM methanol, which produces high levels of intracellular formaldehyde. Also, when formaldehyde was added to the reaction medium after centrifugation, and precipitated with dimedone and the precipitate counted in a liquid scintillation counter, no radioactivity was found to be incorporated, which attests to the very low formaldehyde pool during *N*-demethylation. This indicates that the P-450-catalyzed hydroxylation reaction is the rate-limiting step in the overall sequence of events leading to the formation of ^3H_2O. The rate of ^3H_2O release from the two radioactive drugs is concentration dependent and a Lineweaver–Burk plot shows the Michaelis constants to be 1.4 mM for antipyrine and 0.3 mM for dansylamide respectively (Fig. 7).

Isolated liver cells rapidly metabolized antipyrine and dansylamide. The metabolism of 0.5 mM dansylamide and 0.5 mM antipyrine was inhibited by 19 and 17 per cent, respectively, with 1.5 mM ethylmorphine, a drug which is also *N*-dealkylated. Antipyrine and ethylamorphine are type I binding drugs, while dansylamide has not been categorized. SKF-525A is a compound that inhibits the metabolism of many type I drugs [22]. At a concentration of 50 μg/ml, SKF-525A inhibited the *N*-demethylation of 0.5 mM antipyrine by 50 per cent, and inhibited the *N*-demethylation of 0.5 mM dansylamide by 62 per cent (Figs. 8 and 9). Heat inactivation of the cells for 1 min at 100° prior to incubation completely stopped the release of ^3H_2O from both drugs (shown for antipyrine only, Fig. 8). SKF-525A is a type I binding drug [23,24] and may inhibit drug metabolism by irreversibly binding to the type I binding site of the cytochrome moiety (Bidleman and Mannering, unpublished results). This might indicate that dansylamide *N*-demethylation is preceded by type I binding.

The *N*-demethylase activity of the liver cells was slightly but reproducibly increased when the cells were incubated simultaneously with 0.5 mM antipyrine and 0.05 or 0.1 mM phenobarbital. Higher concentrations of phenobarbital inhibited antipyrine *N*-demethylation. We have no explanation for the stimulation phenomenon at this time, although this might be due to enzyme induction *in vitro*. On the other hand, this effect is evident within the first 30 min indicating the likelihood of a stabilization of existing enzyme activity [25]. Phenobarbital is a good inducer of drug metabolism *in vivo* [26,27] but has not been used to induce drug metabolism in isolated liver cells. However, Nebert and Gielen [25] have successfully induced aryl hydrocarbon hydroxylase with phenobarbital in cultured mammalian liver cells.

There are several advantages for the use of isolated liver cells in the study of drug metabolism. The endoplasmic reticulum of adult rat hepatocytes is fully developed, while this is not true of embryonic liver cells which would be used in hepatocyte cell culture. In addition, isolated hepatocytes can be obtained in large quantities. It is also possible to pretreat rats prior to cell isolation in order to manipulate conditions affecting drug metabolism. Thus, it becomes possible to do parallel experiments with cells from one animal, thereby eliminating biological variation. Because of the greater stability of isolated suspended cells as compared to microsomes, drug metabolic

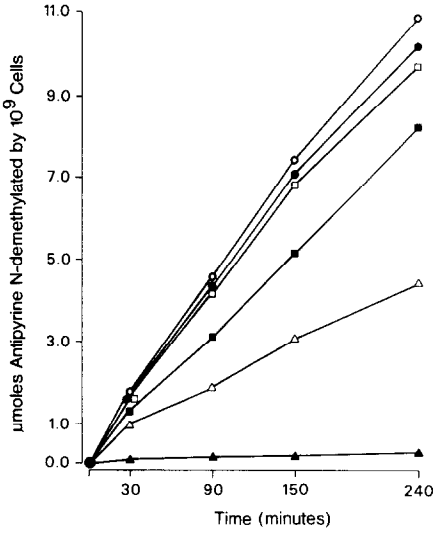

Fig. 8. Effect of inhibitors and stimulators on antipyrine *N*-demethylation by isolated hepatocytes. Key: *N*-demethylation of 0.5 mM antipyrine or 0.5 mM antipyrine + 65 mM methanol (□); 0.5 mM antipyrine + boiled cells (▲); 0.5 mM antipyrine + 1.5 mM ethylmorphine (■); 0.5 mM antipyrine + 50 μg/ml of SKF-525 A (△); 0.5 mM antipyrine + 0.05 mM phenobarbital (●); and 0.5 mM antipyrine + 0.1 mM phenobarbital (○). Incubations were carried out at 23° with 50 mg/ml wet weight liver cells.

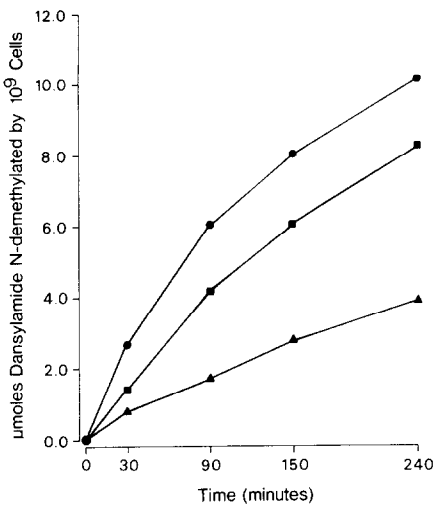

Fig. 9. Effect of inhibitors on *N*-demethylation of dansylamide by isolated hepatocytes. Key: *N*-demethylation of 0.5 mM dansylamide (●); 0.5 mM dansylamide + 1.5 mM ethylmorphine (■); and 0.5 mM dansylamide + 50 μg/ml of SKF-525 A (▲). Incubations were carried out at 23° with liver cell concentrations of 50 mg/ml wet weight.

reactions may be carried out over relatively long periods of time and yield linear kinetics.

In conclusion, isolated rat hepatocytes prepared by a modification of the Berry and Friend technique are highly suitable for drug metabolism studies *in vitro*. They retain their viability for long periods of time and allow many, well-controlled experiments from the same batch of cells.

It is possible to demonstrate the inhibition and stimulation of drug metabolism in isolated liver cells. The *N*-demethylation of both antipyrine and dansylamide is inhibited to about the same degree with SKF-525A. Ethylmorphine also inhibited the *N*-demethylation of both compounds, probably by competing with the two labeled drugs for the type I binding site. SKF-525A may have a similar mechanism of action. The metabolism of antipyrine was stimulated by adding small concentrations of phenobarbital. Dansylamide metabolism demonstrated a similar, but less consistent stimulation by small additions of phenobarbital. Since, in both cases, the effects are seen at the 30-min time points of incubation, it is likely that these increased metabolic rates are not due to induction of new enzyme synthesis but are due to stimulation or stabilization of existing enzymes. The release of ^3H_2O from suitably labeled precurors appears to be a promsing technique to study certain aspects of drug metabolism.

The fluorescent properties of quinine make it a convenient alternate tool to study effects on drug metabolism. In isolated liver cells, one can follow the disappearance of the fluorescent parent compound and simultaneously the appearance of the fluorescing metabolites. The sensitivity is comparable to a radioactive assay.

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