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DIRECT MEASUREMENT OF AMINOPYRINE *N*-DEMETHYLASE AND ANTIPYRINE HYDROXYLASE ACTIVITIES IN A MONOLAYER RAT PRIMARY ISOLATED HEPATOCYTE SYSTEM*

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Abstract—This paper describes a simple method for monitoring changes in aminopyrine *N*-demethylase and antipyrine hydroxylase activities in isolated primary hepatocyte monolayer culture. Aminopyrine *N*-demethylase activity was determined by monitoring the rate of formation of $^{14}CO_2$ derived from the *N*-demethylation of [dimethylamino-^{14}C]aminopyrine (AP). The rate of AP *N*-demethylation increased linearly with time for 60 min and proportionately with cell concentrations between 4.1×10^5 to 1.67×10^6 cells/incubation. As expected, non-linear AP *N*-demethylase kinetics were observed with hepatocytes as well as with microsomal preparations derived from control rats. Hepatocytes prepared from phenobarbital (PB)-pretreated animals exhibited increased AP *N*-demethylase activity and typical Michaelis–Menten kinetics. In contrast, microsomal preparations from PB-treated animals exhibited non-linear *N*-demethylase kinetics that differed from the kinetics of preparations derived from control animals. Antipyrine hydroxylase activity was determined by monitoring the rate of formation of non-extractable conjugated 4-hydroxyantipyrine from [*N*-^{14}C-methyl]antipyrine. Antipyrine hydroxylase activity was found to increase linearly for 120 min and proportionately with cell concentrations. Antipyrine hydroxylation by hepatocytes prepared from control and PB-pretreated animals followed typical Michaelis–Menten kinetics. AP *N*-demethylase activity immediately after plating was 10 per cent lower than at 4 hr, whereas antipyrine hydroxylase activities were similar. Culturing hepatocytes for 24 hr resulted in a decline to 40 and 60 per cent of control for AP *N*-demethylase activity and antipyrine hydroxylase activity respectively.

Isolated hepatocytes prepared from adult animals have been used extensively in the study of intermediary metabolism and endocrinology. Their use in drug biotransformation studies has been increasing rapidly. The expanding volume of literature in which isolated hepatocytes have been used in drug metabolism studies has been reviewed recently by Fry and Bridges [1]. Isolated hepatocyte preparations have been reported to be capable of exhibiting a number of Phase I reactions: aromatic hydroxylations [2, 3], alkyl hydroxylation [4], and numerous *N*- and *O*-demethylations [5–7]. Simultaneously, Phase II transformation processes, such as glucuronide and glutathione conjugations, take place [2].

The use of isolated hepatocytes in the study of drug metabolism has the advantage of eliminating the influences of many extrahepatic variables that may influence drug biotransformation kinetics, while retaining most of the physiological and structural characteristics that are destroyed by subcellular fractionation of liver. Previously reported studies carried out in suspension systems suffer from a number of disadvantages: (1) drug biotransformation studies are limited to short-term investigations due to a rapid decline in hepatocyte viability; and (2) hepatocytes have commonly been used immediately after isolation and, therefore, have had insufficient time to recuperate from preparative enzymatic and mechanical procedures. Hepatocyte suspensions also lack the cell–cell interactions that often play an important role in cellular function.

This paper describes a monolayer culture system of rat hepatocytes that maintain the capacity to *N*-demethylate aminopyrine and to hydroxylate antipyrine, thus circumventing the objections to the use of hepatocyte suspensions. Methods are described for the determination of aminopyrine *N*-demethylase and antipyrine hydroxylase activities by these primary cultured hepatocytes. Various aspects of the kinetics of aminopyrine *N*-demethylation and antipyrine hydroxylation are also discussed.

MATERIALS AND METHODS

Chemicals. Leibovitz (L-15) tissue culture medium, Hi-WO5/BA2000 (serum-free medium), and fetal calf serum were obtained from International Scientific Industries, Inc., Cary, IL. Insulin was purchased from Eli Lilly & Co., Indianapolis, IN. HEPES‡ and bovine serum albumin (fraction V-fatty acid free) were purchased from the Sigma Chemical Co., St. Louis, MO. Collagenase type CLSII was obtained from Worthington Biochemical,

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‡ HEPES = 4-(2-hydroxyethyl)-1-piperagine-ethanesulphonic acid.

Freehold, NJ. Both [dimethylamino-^{14}C] aminopyrine and [*N*-^{14}C-methyl]antipyrine were purchased from the Amersham Corp., Arlington Heights, IL. Plastic culture plates were purchased from Falcon Plastics, Oxnard, CA. The Nalgene assay containers were obtained from the Nalge Co., Rochester, NY. Hyamine hydroxide was purchased from the Packard Instrument Co., Inc. Downers Grove, IL. Aquasol was obtained from the New England Nuclear Corp., Boston, MA. Angiocaths were purchased from the Desult Pharmaceutical Co., Sandy, UT.

Preparation of isolated rat hepatocytes. Male Sprague–Dawley rats (Laboratory Supply, Indianapolis, IN) weighing 170–250 g were maintained on a Tekland rat chow diet, were fed *ad lib.*, and were used to provide the cells for primary culture. The livers of control and phenobarbital (PB)-pretreated animals (40 mg/kg, i.p., daily for 4 days) were perfused, and the cells were isolated under sterile conditions, according to modifications of the method described by Zahlten and Stratman [8]. Each rat was anesthetized with ether, and 200 units of sodium heparin was injected into the inferior vena cava just above the renal veins. The portal vein was cannulated with a 166 Angiocath cannula, and the inferior vena cava was tied off just above the renal veins. The liver was cleared of blood with perfusion solution, and the inferior vena cava was immediately severed just below the heart. The perfusion solution contained 14 mM NaCl, 5 mM KCl, 0.4 mM Na_2HPO_4, 25 mM $NaHCO_3$, 20 mM HEPES, 11 mM glucose, and 10 mg/l phenol red. The solution was maintained at 37° at a pH of 7.37 by equilibration with 95% O_2/5% CO_2. The liver was then removed from the rat and placed in a bath with perfusion solution recirculated at 37° and equilibrated with 95% O_2/5% CO_2.

Collagenase (0.5 mg/ml) was then added to the recirculating solution and the perfusion was terminated after 8 to 10 min. The swollen liver was perfused and rinsed with an additional 30 ml of collagenase-free solution. The softened liver was placed in a petri dish with 10 ml of fresh perfusion solution and gently teased apart with two forceps. The dispersed hepatocytes were filtered through a double layer of sterile cotton gauze and then through a 60-μm nylon mesh. The hepatocytes were isolated by centrifugation (40 *g* for 2.5 min), and the pellet was resuspended in 30 ml of perfusion solution; the washing was repeated three times. The final pellet was resuspended in 30 ml of L-15 medium supplemented with 2% bovine serum albumin, glucose (1.5 mg/ml), insulin (13 milliunits/ml), gentamycin sulfate (50 μg/ml), HEPES (25 mM), and 5% fetal calf serum. Aliquots (10 ml) were placed in siliconized 250-ml Erlenmyer flasks that were gassed with 95% O_2/5% CO_2, capped, and shaken in a 37° water bath. The cells were counted and the membrane integrity was estimated by measuring trypan blue exclusion. Only hepatocyte preparations in which more than 90 per cent of the cells excluded trypan blue were utilized for our studies. Approximately 3.5 to 5.0 $\times$ 10^8 hepatocytes were obtained from each preparation. The hepatocytes were diluted with medium and plated on 35 mm collagen gel petri dishes prepared by the method of Michalopoulos and Pitot [9]. All studies were undertaken 4 hr after plating.

Determination of N-*demethylation of aminopyrine.* Culture plates containing freshly cultured hepatocytes were placed in 125-ml Nalgene straight side jars with gas tight seals and two resealable openings. A 3-cc glass vial was also placed in the container which was then flushed with 95% O_2/5% CO_2, sealed, and incubated at 37° with shaking at 37 oscillations/min. Following a 10-min equilibration period, aminopyrine (0.25 μCi/plate of [^{14}C-dimethyl] aminopyrine) was added in a volume of 100 μl to initiate the reaction. Incubation was continued at 37° for 30, 60, 90 or 120 min. The reaction was terminated by the addition of 1 ml of 20% trichloroacetic acid. One milliliter of 0.5 N hyamine hydroxide was placed in the glass vial through the resealable opening. The container was resealed and allowed to stand overnight for collection of $^{14}CO_2$. The hyamine hydroxide was removed and prepared for counting in Aquasol. The amount of *N*-demethylation occurring was quantified by determining the amount of $^{14}CO_2$ trapped by the hyamine hydroxide, using a Beckman LS 8100 scintillation spectrophotometer.

Determination of the rate and efficiency of $^{14}CO_2$ *trapping by hyamine hydroxide.* A standard solution of $NaH^{14}CO_3$ (1×10^6 dpm) was placed in the incubation system described above for the metabolism studies. $^{14}CO_2$ was liberated by acidifying the solution with 1 ml of 20% trichloroacetic acid. Hyamine hydroxide was exposed to the $^{14}CO_2$ under standard incubation conditions for various lengths of time and prepared for counting in a Beckman LS 8000 scintillation counter.

The efficiency of $^{14}CO_2$ trapping by hyamine hydroxide was studied at various times after generation of CO_2. More than 98 per cent of the $^{14}CO_2$ generated could be trapped by hyamine hydroxide, but long exposure was required to achieve this degree of trapping. At 16 hr of exposure to $^{14}CO_2$, trapping was nearly complete. Based on the results of this time course, samples were exposed to hyamine overnight to ensure complete trapping.

Microsomal aminopyrine N-*demethylation.* Hepatic microsomes were prepared as described by Sladek and Mannering [10]. Aminopyrine *N*-demethylase activity was determined using the method described by el Defrawy el Masry *et al.* [11], modified by the exclusion of nicotinamide. Microsomes were incubated in the presence of 0.2 to 10 mM aminopyrine, and formaldehyde was determined by the method of Nash [12]. Microsomal protein content was determined by the method of Lowry *et al.* [13] using purified bovine serum albumin as a standard.

Determination of antipyrine hydroxylation. Antipyrine hydroxylase activity was measured as described above for aminopyrine *N*-demethylase activity, with the following modifications. Antipyrine (0.5 μCi/plate) was used as the substrate and the addition of hyamine hydroxide was omitted from the procedure. The reaction was terminated by the addition of 0.5 ml of 1 N sodium hydroxide. Two successive extractions of the medium were then carried out to separate antipyrine from its hydroxylated

water-soluble conjugated metabolites. First, the aqueous medium was extracted with 5 ml of ether–dichloromethane–isopropyl alcohol (60:40:1) for 30 min. The tubes were centrifuged at 1500 *g* for 10 min, and the organic phase was discarded. The remaining aqueous medium was then extracted with 5 ml of chloroform for 10 min and centrifuged to separate the phases. The aqueous phase was removed and 200 μl was placed in a counting vial and acidified with 50 μl glacial acetic acid. Aquasol (10 ml) was added to each vial and the samples were counted by scintillation spectrometry.

The possible formation of non-conjugated 4-hydroxyantipyrine in our system was checked by analyzing the organic extracts obtained from a 60-min incubation of 1.25×10^6 cells/plate in the presence of 2 mM [^{14}C]antipyrine. To the combined organic extract, 250 μg of unlabeled antipyrine and 4-hydroxyantipyrine was added. The organic extract was evaporated under a stream of nitrogen and the residue was dissolved in 50 μl of methanol. The methanol extract was spotted on a silica TLC plate and chromatographed using a 5:1 mixture of chloroform–ether. The area of the chromatogram corresponding to 4-hydroxyantipyrine was removed, extracted with methanol, and analyzed by liquid scintillation spectrophotometry. When compared to time non-incubated controls, no significant level of 4-hydroxyantipyrine metabolite was detected.

RESULTS

Hepatocyte aminopyrine N-*demethylation.* The effect of mechanical agitation on the capacity of hepatocytes to metabolize drugs was examined. A range of 15–56 oscillations was found to be without effect on the rate of aminopyrine *N*-demethylation, which appeared to be linear, with cell concentrations of 0.41, 0.83, 1.25, and 1.67×10^6 cells/plate, 30 and 60 min after addition of 2 mM substrate, as shown in Fig. 1. Incubation times of 10 and 20 min were monitored at 1.25×10^6 cell/plate and the rates were found to increase linearly with time and are in agreement with rates determined at 30 and 60 min. At 120 min after substrate addition, a slight deviation from linearity was observed at all cell concentrations studied. Figure 2 shows a proportional increase in the rate of $^{14}CO_2$ production with increasing cell concentrations between 0.41 and 1.25×10^6 cells/plate; a small but significant increase in the rate of metabolism was observed with 1.67×10^6 cells/plate.

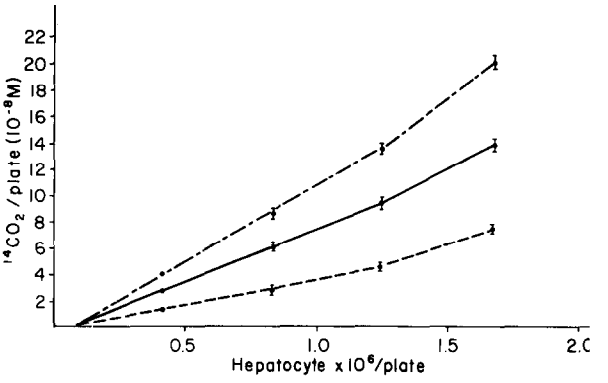

Fig. 2. Effect of hepatocyte concentration on aminopyrine *N*-demethylation. Hepatocytes plated at a concentration of 0.41 to 1.67×10^6 cells/plate were incubated in the presence of 2 mM [4-*N*,*N*-^{14}C-dimethylamino]aminopyrine for 30 (---), 60 (——), and 120 (————) min. The amount of aminopyrine *N*-demethylated per plate was determined by monitoring the amount of $^{14}CO_2$ produced. The data are expressed as the mean of three experiments ± S.E.

Hepatocytes prepared from control and PB-pretreated rats were plated at a concentration of 8.3×10^5 cells/plate and incubated for 30 min in the presence of 0.083 to 8.0 mM aminopyrine. The Lineweaver–Burk plot of the rate of aminopyrine *N*-demethylation was non-linear for hepatocytes derived from control rats (Fig. 3). A low K_m of

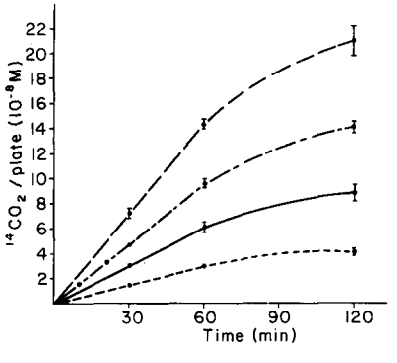

Fig. 1. Effect of incubation time on aminopyrine *N*-demethylation. Hepatocytes, 0.41×10^6 (---), 0.83×10^6 (——), 1.25×10^6 (————), and 1.67×10^6 (——) cells/plate, were incubated in the presence of 2 mM [^{14}C]aminopyrine. The amount of aminopyrine *N*-demethylated per plate was determined by monitoring the amount of $^{14}CO_2$ produced. The data are expressed as the mean of three experiments ± S.E.

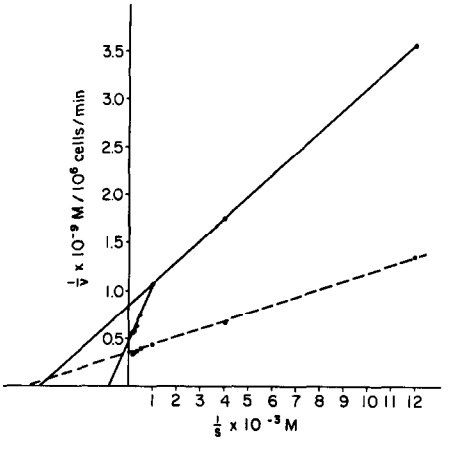

Fig. 3. Lineweaver–Burk plot of isolated hepatocyte *N*-demethylation of aminopyrine by isolated hepatocytes. Cultured hepatocytes (0.83×10^6 cells/plate) derived from control (——) and phenobarbital-pretreated (---) rats were incubated in the presence of 0.083 to 8.0 mM (0.25 μCi/plate) aminopyrine for 30 min. The rate of aminopyrine *N*-demethylation/10^6 cells per min was determined by monitoring the rate of $^{14}CO_2$ production.

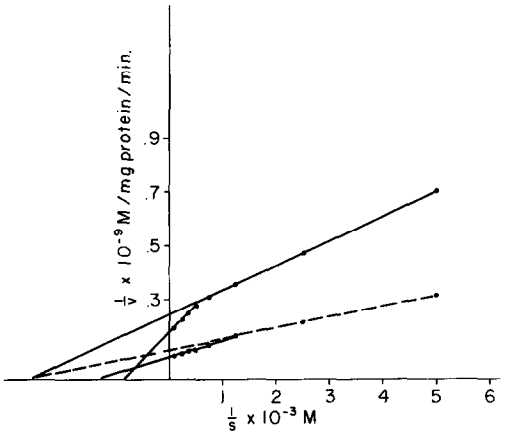

Fig. 4. Lineweaver–Burk plot of hepatic microsomal *N*-demethylation of aminopyrine. Hepatic microsomes from control (——) and phenobarbital-pretreated (---) rats were incubated in the presence of 0.2 to 10 mM aminopyrine. The rate of aminopyrine *N*-demethylation was determined by monitoring the rate of formaldehyde production (see Materials and Methods).

0.27 ± 0.02 mM and a high K_m of 1.39 ± 0.01 mM with corresponding V_{max} values of $1.18 \times 10^{-9} \pm 0.06$ and $2.22 \times 10^{-9} \pm 0.10$ M/10^6 cells per min, respectively, were derived from the kinetic plot. Such non-linear kinetics suggest the presence of two enzymes reponsible for *N*-demethylation of aminopyrine, with different substrate affinities. Hepatocytes prepared from phenobarbital-pretreated animals displayed linear aminopyrine *N*-demethylation kinetics (Fig. 3) with a K_m of 0.23 ± 0.01 mM and V_{max} of $2.88 \times 10^{-9} \pm 0.03$ M/10^6 cells per min.

Microsomal aminopyrine N-*demethylation.* The kinetics of aminopyrine *N*-demethylation by hepatic microsomes from control and phenobarbital-pretreated rats are shown in Fig. 4. Microsomes were incubated in the presence of 0.2 to 10 mM aminopyrine, and the rate of aminopyrine *N*-demethylation was determined by monitoring the rate of formaldehyde production. Microsomal preparations derived from control animals gave apparent K_m values of 1.25 ± 0.7 mM and 0.48 ± 0.03 mM and apparent V_{max} values of 4.39 ± 0.32 and 5.91 ± 0.37 nmoles formaldehyde · (mg protein)$^{-1}$ · min^{-1} respectively. Microsomal preparations derived from PB-pretreated animals gave apparent K_m values of 0.823 ± 0.09 and 0.410 ± 0.013 mM and apparent V_{max} values of 9.71 ± 0.50 and 12.50 ± 0.73 mM formaldehyde · (mg protein)$^{-1}$ · min^{-1} respectively.

Antipyrine hydroxylation. Figure 5 shows the effect of incubation time on the rate of antipyrine hydroxylation by hepatocytes in the presence of 2 mM antipyrine. The rate of antipyrine hydroxylation appeared to be linear for at least 90 min at all cell concentrations studied. In addition, the rate of hydroxylation was found to be proportional to the hepatocyte concentration at all incubation times studied (Fig. 6).

Hepatocytes from control and phenobarbital-pretreated rats were plated at a concentration of 8.3×10^5 cells/plate and incubated in the presence of 0.5 to 10 mM antipyrine. The results of the antipyrine kinetic studies are shown in Fig. 7. Unlike aminopyrine, antipyrine kinetics displayed typical linear Lineweaver–Burk plots. The apparent K_m and V_{max} values obtained from hepatocytes prepared from control rats were 4.55 ± 0.29 mM and $3.07 \pm 0.04 \times 10^{-9}$ m/10^6 cells per min respectively. The apparent K_m and V_{max} values derived from hepatocytes prepared from phenobarbital-pretreated rats were 5.0 ± 0.16 mM and $7.84 \pm 0.15 \times 10^{-9}$ M/10^6 cells per min respectively.

Effect of the time of hepatocyte culturing on aminopyrine N-*demethylase activity.* The rate of aminopyrine *N*-demethylase activity immediately following plating was 0.75 ± 0.08 nmole/10^6 cells per

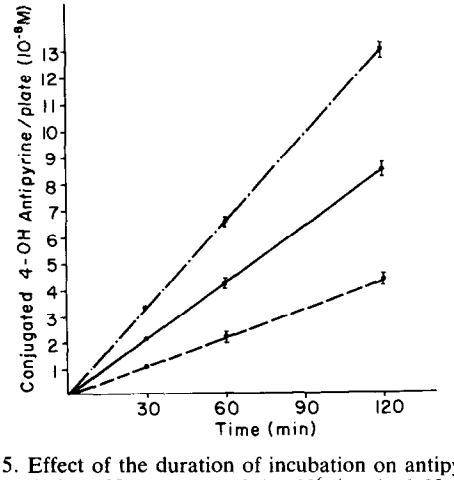

Fig. 5. Effect of the duration of incubation on antipyrine hydroxylation. Hepatocytes, 0.4×10^6 (---), 0.83×10^6 (——), 1.25×10^6 (—·—) cells, were cultured on collagen gels and incubated in the presence of 2 mM [^{14}C-methyl]antipyrine. The amount of antipyrine hydroxylated/plate was determined at 30, 60, and 120 min, as described in Materials and Methods. The data are expressed as the mean of three experiments ± S.E.

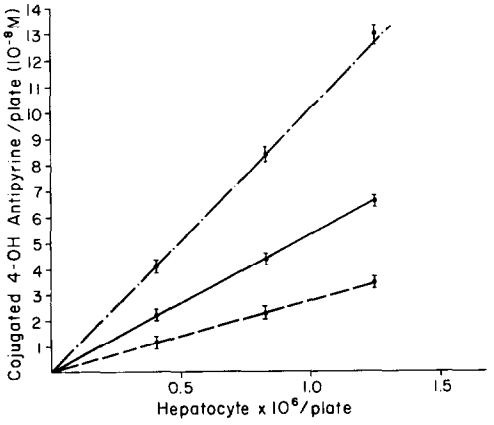

Fig. 6. Effect of hepatocyte concentration on antipyrine hydroxylation. Hepatocytes, 0.41 to 1.25×10^6 cells/plate, were incubated in the presence of antipyrine for 30 (---), 60 (——), and 120 (—·—) min. The rate of antipyrine hydroxylation/plate was determined as described in Materials and Methods. The data are expressed as the mean of three experiments ± S.E.

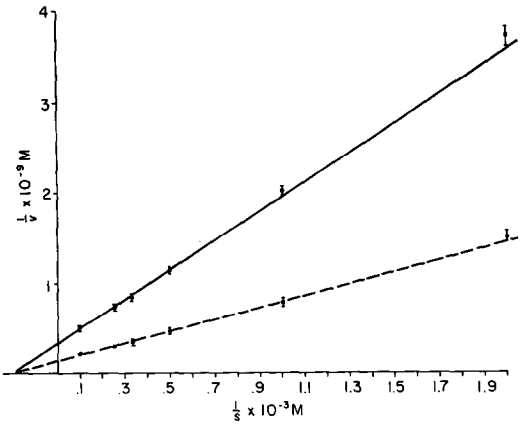

Fig. 7. Lineweaver–Burk plot of antipyrine hydroxylation by isolated hepatocytes. Cultured hepatocytes, 8.3×10^5 cells/plate, prepared from control (——) and phenobarbital-pretreated (– – –) rats were incubated in the presence of 0.5 to 10 mM antipyrine for 60 min. The rate of antipyrine hydroxylation/10^6 cells per min was determined as described in Materials and Methods.

min. With increased culturing time, demethylase activity slowly increased to a maximum of 0.85 ± 0.05 nmole/10^6 cells per min at 4 hr. This level of activity was maintained for an additional 3–4 hr, at which time aminopyrine *N*-demethylase activity slowly began to decline to a low of 0.35 ± 0.07 nmole/10^6 cells per min at 24 hr.

DISCUSSION

The biotransformation of drugs by isolated hepatocytes has been investigated using a wide variety of substrates [1–7]. These studies utilized freshly prepared hepatocytes, and incubations were performed using a cell suspension system. The use of cell suspensions for the study of drug metabolism suffers from a number of disadvantages. The most serious of these problems relates to their lack of stability. Long-term cultures of suspensions are characterized by a rapid decline in activity and viability [14, 15]. Monolayer cultures are more stable for longer periods of time and, therefore, are more suitable for many chronic studies such as studies of induction of enzyme activity, toxicity, and effects of hormones on drug metabolism. Bissell and Guzelian [16] have reported using a primary hepatocyte monolayer to study the effect of long-term culturing on *p*-nitroanisole *O*-demethylation activity. Unfortunately, the metabolizing activities were determined on homogenates prepared from the hepatocytes and not on the cultured hepatocytes themselves. The use of monolayer cultures of hepatocytes to study the biotransformation of xenobiotics has been limited by the plating efficiency, the sensitivity of assays available, and the physical characteristics of the culture system, which often necessitate extensive enzymatic or physical manipulation of the cells prior to assay.

We overcame some of these limitations by developing a method by which all incubations could be done in the original culture plate. The characteristics of isolated primary hepatocyte monolayers prepared on a floating collagen gel matrix made it difficult to develop a simple method for studying the biotransformation of aminopyrine and antipyrine. The cells in this system could not be transferred to a normal incubation system without changing the activity of the preparation. Furthermore, maintenance of an accurate volume during transfer was extremely difficult. In addition, hepatocyte cultures had to be incubated under an atmosphere of 95% O_2/5% CO_2 to obtain reproducible rates of drug biotransformation. These problems were eliminated by placing the 35-mm culture dish in a gas tight container with two resealable openings on the lid to provide access to the contents. Metabolic studies could then be performed in the original culture plate under a 95% O_2/5% CO_2 atmosphere. This method eliminates the need for physical manipulation of the cultured hepatocytes. We have found that any extensive manipulation of the cells affects drug-metabolizing activity and creates severe problems in obtaining reproducible results.

Aminopyrine *N*-demethylation, determined by the rate of production of $^{14}CO_2$, was found to be linear for at least 60 min. The rate of $^{14}CO_2$ production, however, increased proportionately only between 0.41 and 1.25×10^6 cells/plate. At hepatocyte concentrations of 1.67×10^6 cells/plate, a rate of $^{14}CO_2$ production that was slight, but significantly greater than expected, occurred. The cause of this increased rate of metabolism is not yet understood. One possibility is that denser plating of hepatocytes resulted in increased cell contact. In this regard, Michalopoulos and Pitot [9] observed an increase in the rate of collagen gel contraction as the number of cells plated was increased, suggesting a general enhancement of activity. It is possible that increased contact may stimulate drug-metabolizing activity by another mechanism. Although the rate of $^{14}CO_2$ production increased linearly with time, a line drawn through the data points obtained intersects at 7×10^4 cells/plate instead of at zero, as would be expected. Two possible explanations for this observation are (1) a minimum amount of formaldehyde must be formed before $^{14}CO_2$ can be detected, and (2) the amount of $^{14}CO_2$ that could be detected was limited by the assay, and at least 7×10^4 M hepatocytes were

required to produce the minimal detectable quantity. It should be noted, however, that the rate of shaking of the cells during the incubation period did not affect the rate of aminopyrine metabolism under the present conditions.

The kinetics of AP *N*-demethylation in hepatocyte preparations derived from control and from phenobarbital-pretreated animals were markedly different. Hepatocytes derived from control animals displayed biphasic Lineweaver–Burk plots qualitatively similar to those found for microsomal preparations in which the rate of formaldehyde was used as a measure of the rate of aminopyrine *N*-demethylation. The quantitative differences observed between the kinetic profiles of hepatocyte and microsomal preparations indicate that they are not equivalent. Such quantitative differences between hepatocyte and microsomal preparations were observed previously by Erickson and Holtzman [17] for ethylmorphine *N*-demethylation kinetics undertaken with hepatocyte suspensions and microsomal preparations. The differences in the variables monitored as an index of the rate of aminopyrine *N*-demethylation, $^{14}CO_2$ for hepatocytes and formaldehyde for microsome preparations, may explain the differences (observed) in the kinetic values observed in these two systems. However, Erickson and Holtzman utilized a similar variable (^3H_2O) in their study of ethylmorphine *N*-demethylation and still reported different kinetic constants.

Hepatocytes prepared from animals pretreated with phenobarbital gave classical Lineweaver–Burk plots that differed slightly from those obtained from microsomes derived from similarly treated animals. Although no indication of a high apparent K_m system was observed for hepatocyte preparations, microsomal preparations displayed both a high and a low apparent K_m. The low apparent K_m was similar to that observed with control microsomes, but the high K_m value observed with phenobarbital microsomes was 35 per cent lower than that observed for control microsomes. These differences cannot be easily explained. It is possible that the low K_m value reflects a single enzyme system, whereas the high K_m value resulted from a composite of two enzyme systems with the absolute value being dependent upon the proportion of high and low K_m systems in the preparation. If this is the case, an increase in the proportion of the low K_m system by phenobarbital induction should result in a shift in the high K_m value towards the low K_m value. The lack of a high K_m system observed with hepatocyte preparations derived from phenobarbital-pretreated rats may reflect the lack of expression of the enzyme system due to a functioning repressor which is removed or deactivated upon homogenization. In addition, the *N*-demethylation assay used with the hepatocyte preparation may not be sensitive enough to detect the contribution of the high K_m system to the overall demethylation of aminopyrine in phenobarbital-pretreated preparations.

Recently, Stewart and Inaba [18] reported using the rate of $^{14}CO_2$ production from the *N*-demethylation of AP as a measure of oxidative drug metabolism in isolated hepatocyte suspensions. Although similar non-linear kinetics for AP *N*-demethylation in control preparations were observed, their apparent K_m values of 32 μM and 710 μM were significantly different from the high K_m values in this paper. Our low apparent K_m value, however, was similar to their high K_m value. A number of factors may be responsible for the differences observed between the reported values: (1) differences in the strain of rats used (Wistar versus Sprague–Dawley), (2) differences in the hepatocyte preparations and media used (suspension versus cultured monolayer system and Ringer-phosphate versus serum supplemented L-15 media), and (3) differences in the range of AP concentrations used (25 μM to 2 mM versus 83 μM to 10 mM).

A similar technique for monitoring AP *N*-demethylase activity in isolated hepatocyte suspensions was described by Weigl and Sies [19]. They reported that hepatocytes derived from phenobarbital-pretreated animals and incubated in the presence of 1 mM aminopyrine *N*-demethylated aminopyrine at a rate of 1.38×10^{-6} M/g dry weight per min. Based on the conversion of the number of hepatocytes to dry weight published by Zahlten and Stratman [8], we obtained a value of 2.6×10^{-6} M $^{14}CO_2$/g dry weight per min, which is close to the reported values of Weigl and Sies.

Whereas aminopyrine *N*-demethylation was linear for only 60 min, antipyrine hydroxylase activity was linear with time and hepatocyte concentration for at least 120 min. A number of possible explanations for these differences in behavior exist: (1) these biotransformation processes may be controlled by different cytochrome P-450s, (2) aminopyrine or its metabolites may be affecting the viability of the hepatocytes, resulting in a decrease in linearity after 60 min, (3) one of the secondary oxidation components responsible for the transformation of formaldehyde to CO_2 may be more labile than the step involving cytochrome P-450, and (4) a cofactor required for the conversion of formaldehyde to CO_2 may become rate limiting with time. At present we have no data to indicate which one of the proposed explanations may be responsible for the observed difference between the metabolism of aminopyrine and antipyrine.

The rate of *N*-demethylation of antipyrine as determined by the rate of $^{14}CO_2$ formation was found to be $94 \times 10^{-12} \pm 0.05$ M/10^6 cells per min at a concentration of 2 mM, and was found to account for approximately 10 per cent of the combined rates of hydroxylation and *N*-demethylation of antipyrine. This is within the range reported by Schuppel [20]. Hayes and Brendel [21], using [^{3}H-methyl] antipyrine as a substrate, reported that hepatocyte suspensions *N*-demethylated antipyrine at a rate of 3.2 μM/10^9 cells per 60 min. At a similar [^{14}C-methyl]antipyrine concentration we obtained a rate of 1.2 μM/10^9 cells per 60 min.* This difference between the two values may reflect differences between the hepatocyte systems and/or the assays used to determine the rate of antipyrine *N*-demethylation. Hayes and Brendel [21] used a hepatocyte suspension system (4.4 to 5.9 $\times$ 10^6 cells/ml), and the

* A. N. Kotake, unpublished data.

rate of antipyrine *N*-demethylation was determined by measuring the amount of tritium that failed to bind to a cationic exchange resin. We used plated hepatocytes at 0.83×10^6 cells/plate (volume = 1.6 ml), and the rate of antipyrine *N*-demethylation was determined by measuring the amount of $^{14}CO_2$ produced.

Time studies following hepatocyte plating demonstrated that AP *N*-demethylase activity is higher and less variable at 4 hr than immediately following plating. This finding suggests that a minimum length of time of culturing is required for hepatocyte. AP *N*-demethylase activity to stabilize. The maximum rate observed 4 hr after plating was maintained for 3–4 additional hr and slowly declined to 40 per cent of the maximum rate by 24 hr.* This finding is in agreement with the findings of Guzelian and Barwick [22] and Guzelian *et al.* [23], who reported a dramatic decline in cytochrome P-450 content and drug-metabolizing capabilities after 24 hr of culturing.

In summary, we have described a method for monitoring the drug-metabolizing capabilities of cultured primary hepatocyte monolayer cultures. The radiometric assays used to study aminopyrine *N*-demethylase and antipyrine hydroxylase activities are sensitive and simple. Furthermore, the sensitivity of the assay can be improved by increasing the specific activity of the substrate or by increasing the time of incubation or the amount of cells plated. Finally, the method requires minimal manipulation of hepatocytes, and thus preserves hepatocyte drug-metabolizing capabilities.

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