REFERENCES

1. E. Costa and S. Garattini (Eds.), *International Symposium on Amphetamines and Related Compounds*, pp. 1–962. Raven Press, New York (1970).
2. S. S. Parmar, *Biochem. Pharmac.* **15**, 1497 (1966).
3. T. J. Mantle, K. F. Tipton and N. J. Garrett, *Biochem. Pharmac.* **25**, 2073 (1976).
4. H. H. Miller and D. E. Clarke, *Commun. Psychopharmac.* **2**, 319 (1978).
5. A. L. Green and M. A. S. el Hait, *J. Pharm. Pharmac.* **30**, 262 (1978).
6. I. Ota, *No-Kenkyu* **4**, 53 (1949).
7. H. Hada, *Psychiatria Neurol. jap.* **60**, 1259 (1958).
8. J. P. Johnston, *Biochem. Pharmac.* **17**, 1285 (1968).
9. H-Y. T. Yang and N. H. Neff, *J. Pharmac. exp. Ther.* **187**, 365 (1973).
10. H-Y. T. Yang and N. H. Neff, *J. Pharmac. exp. Ther.* **189**, 733 (1974).
11. O. Suzuki, Y. Katsumata, M. Oya and T. Matsumoto, *Biochem. Pharmac.* **28**, 2327 (1979).
12. J. Knoll and K. Magyar, in *Advances in Biochemical Psychopharmacology* (Eds. E. Costa and M. Sandler), Vol. 5, p. 393. Raven Press, New York (1972).
13. H. Köchli and J. P. von Wartburg, *Analyt. Biochem.* **84**, 127 (1978).
14. O. Suzuki, H. Hattori, M. Oya and Y. Katsumata, *Biochem. Pharmac.* **29**, 603 (1980).
15. G. G. Guilbault, P. J. Brignac, Jr. and M. Juneau, *Analyt. Chem.* **40**, 1256 (1968).
16. S. H. Snyder and E. D. Hendley, *J. Pharmac. exp. Ther.* **163**, 386 (1968).
17. O. Suzuki, E. Noguchi and K. Yagi, *Brain Res.* **135**, 305 (1977).
18. J. A. Roth and C. N. Gillis, *J. Pharmac. exp. Ther.* **194**, 537 (1975).
19. W. C. Prozialeck and W. H. Vogel, *Life Sci.* **22**, 561 (1978).
20. P. C. Waldmeier, A. Delini-Stula and L. Maitre, *Naunyn-Schmiedeberg's Archs Pharmac.* **292**, 9 (1976).

Biochemical Pharmacology, Vol. 29, pp. 2073–2076.

0006-2952/80/0715–2073 $02.00/0

Effect of drug vehicles on *N*-demethylase activity in isolated hepatocytes

(*Received* 5 *December* 1979; *accepted* 20 *February* 1980)

The use of isolated hepatocytes to study *in vitro* metabolism of drugs and other xenobiotics has become an important technique during the past 10 years. A problem often encountered in such studies is preparation of concentrated stock solutions of poorly soluble substrates for subsequent addition to incubation mixtures. It is often necessary to dissolve substrates in an organic solvent and subsequently add small amounts of this solution directly to hepatocyte incubations or to dry an aliquot of solution in the incubation vessel prior to cell addition. If the solvent is added directly to hepatocyte suspensions, it may, by several mechanisms, modify drug metabolism in the preparation. On the other hand, when poorly water soluble substrates are dried onto glass incubation vials, the rate of dissolution in the incubation mixture may be variable, resulting in poor reproducibility and unreliable estimates of actual substrate concentrations. Typical vehicles used in drug metabolism studies with hepatocytes include: ethanol [1–3], acetone [3–6] and dimethylsulfoxide [1, 7, 8].

Although *N*-demethylation can be quantitated in microsomal suspensions by monitoring formaldehyde formation from water soluble substrates such as ethylmorphine HCl, similar studies in hepatocyte suspensions are virtually impossible. When formaldehyde is formed in hepatocyte suspensions, it is further metabolized to formate and ultimately CO_2, making quantitation difficult [9]. Moreover, after dealkylation of many substrates, the dealkylated metabolite is further conjugated with sulfate or glucuronic acid in the hepatocyte system, again making quantitation difficult [5]. In order to overcome these problems, we have recently studied several substrates for *N*-demethylation that can be utilized in isolated hepatocyte suspensions. We have found that *N*,*N*′-dimethylphenobarbital (DMPB) is an excellent substrate since it is metabolized almost exclusively to *N*-methylphenobarbital (MPB) with little subsequent formation of phenobarbital or other metabolites.* Moreover, use of [^{14}C]DMPB allows one to accurately monitor formation of [^{14}C]MPB and not have to rely on formaldehyde measurements. Since DMPB is only slightly soluble in water, it was necessary to make concentrated stock solutions in organic solvents for subsequent addition to hepatocyte suspensions. Although organic solvents are often used to add substrates to microsomal suspensions and their effects on metabolism have been reported, it became apparent that extrapolation of microsomal data to the isolated hepatocyte system was not valid. We found during the development of the DMPB *N*-demethylase assay that the rate of the reaction in hepatocytes was highly dependent on the solvent used to dissolve DMPB. This report summarizes our initial observations and describes a subsequent study designed to quantitate vehicle effects on *N*-demethylase activity in isolated hepatocytes.

Male Wistar rats (225–300 g), obtained from Hilltop Labs, Scottdale, PA, were maintained on Purina Lab Chow and water *ad lib.* and were used throughout the experiments. Animals were fasted for 24 hr before being killed. Hepatocytes were isolated essentially as described by Berry and Friend [10], incorporating modifications introduced by Seglen [11]. After cannulation of the portal vein, the liver was perfused with calcium-free Krebs–Henseleit bicarbonate buffer (pH 7.5) for approximately 10 min. After hepatectomy, the liver was placed in a perfusion apparatus (MRA Corp., Boston, MA) and perfused for another 10–15 min with a recirculating solution of Sigma Type IV collagenase (90 units/ml) in Krebs–Henseleit buffer. After disruption of the liver capsule, cells were suspended in ice-cold Krebs–Henseleit buffer, successively filtered through gauze and weighted silk, and washed twice. Hepatocytes were finally resuspended to a final concentration of approximately 40 mg cells/ml in 'fortified' Krebs–Henseleit buffer containing 5.5 mM glucose, 2% bovine serum albumin, and

* R. Abraham, M. Sauers, M. Zemaitis and J. Alvin, manuscript submitted for publication.

Table 1. Effect of drug vehicle on dimethylphenobarbital (DMPB) *N*-demethylation in isolated hepatocytes*

Solvent	MPB formed (μmoles/g dry wt/15 min)
Acetone†	8.03
Acetone	6.92
Acetonitrile	6.45
Dimethylsulfoxide	1.74
Ethanol‡	1.78

* Twenty microliters of 75 mM DMPB was added to 2.0-ml incubation mixtures as described in the text. Incubations were carried out for 15 min (in triplicate for each solvent) and the formation of methylphenobarbital was subsequently determined.

† Cells were added to 1 ml of buffer containing 20 μl of DMPB after a 20-min preincubation to allow for evaporation of acetone from the solution.

‡ Fifty microliters of a 30 mM solution of DMPB in ethanol was used due to limited solubility of DMPB in ethanol.

Eagle's Minimum Essential Medium. Cell viability, as determined by Nigrosin exclusion, was consistently greater than 80 per cent.

Incubations were carried out by mixing 1 ml of hepatocyte suspension and 1 ml of 'fortified' Krebs–Henseleit buffer to which had been added 20 μl of 75 mM [^{14}C]DMPB (0.027 mCi/mmole) dissolved in acetone, dimethylsulfoxide or acetonitrile. Since the solubility of DMPB in ethanol is less than in the other solvents tested, 50 μl of a 30 mM solution of DMPB in ethanol was used. In addition, during certain experiments with acetone as the drug vehicle, 20 μl of 75 mM DMPB was added to 1 ml of 'fortified' Krebs–Henseleit buffer, and the mixture was preincubated at 37° for 20 min to evaporate most of the solvent, prior to cell addition. The final DMPB concentration in the incubation was 0.75 mM, and incubations were carried out at 37° for 15 min in a 95% O_2/5% CO_2 atmosphere. The reaction was stopped by removing 1.0 ml of the incubation mixture and immediately adding it to 4.0 ml of ice-cold isooctane. All incubations were carried out in triplicate and 'zero time' blanks were prepared by extraction with isooctane immediately after addition of cells to the incubation.

In order to quantitate further vehicle effects on DMPB metabolism, a second study was done in which 1.2 ml of 75 mM DMPB in acetone was slowly added to 60 ml of 'fortified' Krebs–Henseleit buffer with constant stirring. The solution was then incubated at 37° for 30 min to evaporate as much acetone as possible. The volume of the buffer (now containing 1.5 mM DMPB) was measured and brought back to a volume of 60 ml with distilled water. To 1.0 ml of buffer containing 1.5 mM DMPB was added either 2, 5, 10 or 15 μl of a specific vehicle and 1.0 ml of hepatocyte suspension. Incubations were then carried out as described previously. Since the effects of 20 μl of acetone on DMPB metabolism were minimal (Table 1), smaller additions of acetone were not included in the second study. Although propylene glycol is not a good solvent for DMPB, it is a commonly used solvent for other compounds and, therefore, this vehicle was included in the second study. Conversion of DMPB to *N*-methylphenobarbital was analyzed

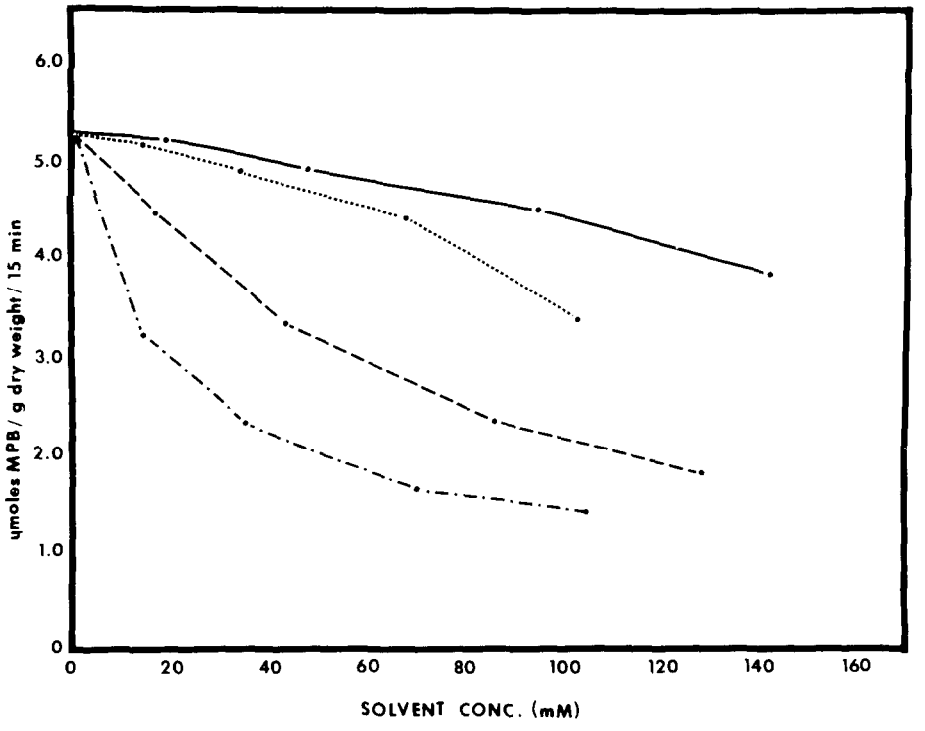

Fig. 1. Effect of selected drug vehicles on dimethylphenobarbital (DMPB) *N*-demethylation by isolated hepatocytes. Various amounts of each vehicle were added to hepatocyte suspensions (containing 0.75 mM DMPB) and the metabolism of DMPB was measured after a 15-min incubation. Key: acetonitrile (———); propylene glycol (· · · · · ·); ethanol (– – – –); and dimethylsulfoxide (– · – · –). Each point represents the mean of triplicate incubations.

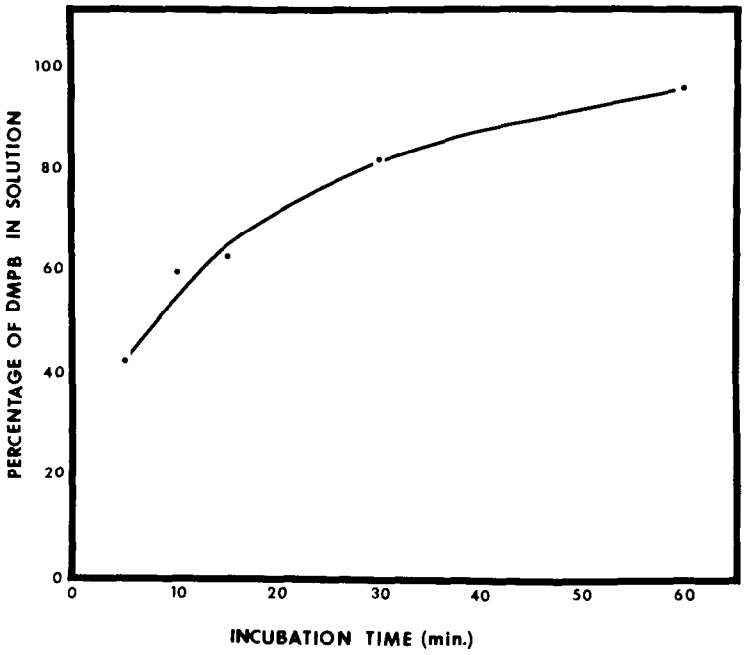

Fig. 2. Rate of dimethylphenobarbital (DMPB) dissolution in incubation buffer. Twenty microliters of 75 mM [^{14}C]DMPB (0.027 mCi/mmole) in acetone was dried onto the inside of a series of incubation vials. Two milliliters of 'fortified' Krebs–Henseleit buffer was then added to each flask and incubated for various time periods at 37° with constant shaking. At each time point the percentage of total counts in the buffer was calculated. Values graphed are the means of triplicate incubations.

by differential extraction and liquid scintillation counting as described previously.*

Although direct vehicle effects on hepatocyte metabolism can be avoided by drying an aliquot of substrate solution onto incubation vials prior to addition of cells, the dissolution of poorly soluble substrates into the incubation mixture may be incomplete, especially during short incubations. To evaluate this possibility with DMPB, 20 μl of 75 mM [^{14}C]DMPB (0.027 mCi/mmole) in acetone was dried in a series of incubation vials. To each vial was added 2.0 ml of 'fortified' Krebs–Henseleit buffer, and the mixture was incubated for various time periods. At the end of each period 1.0-ml samples were removed from three vials, and each sample was added to 10 ml of ACS-Aqueous Counting Scintillant (Amersham/Searle Corp., Arlington Heights, IL). Triplicate 'control' samples were prepared by adding 1.0 ml of buffer and 10 μl of 75 mM [^{14}C]DMPB directly to 10 ml of ACS. The samples were counted, and the mean value for each time point was compared to the mean control value to determine what percentage of substrate had actually dissolved in the buffer.

Table 1 summarizes a typical study using the same batch of hepatocytes to which DMPB was added in different vehicles. The fastest rate of metabolism occurred in incubations to which DMPB was added in acetone followed by a 20-min preincubation to evaporate the vehicle; direct addition of DMPB in acetone without preincubation resulted in a 14 per cent decrease in DMPB *N*-demethylation. The rate of DMPB demethylation in the presence of acetonitrile was similar to activity in the presence of acetone. However, addition of DMPB in 20 μl of dimethylsulfoxide or 50 μl of ethanol decreased metabolism by about 80 per cent in each case.

To characterize further the effects of drug vehicles on *N*-demethylase activity in isolated hepatocytes, various amounts of acetonitrile, propylene glycol, ethanol or dimethylsulfoxide were added to hepatocyte suspensions containing 0.75 mM DMPB (previously added in acetone and preincubated). Figure 1 summarizes the effect of each vehicle on DMPB metabolism. The volume of each vehicle added per 2-ml incubation was 2, 5, 10 or 15 μl; these additions are converted to the final mM concentration of each vehicle on the *x*-axis of Fig. 1. Consistent with the data in Table 1, acetonitrile had the least effect on DMPB *N*-demethylation; below a 50 mM final concentration, inhibition was less than 5 per cent. Below this concentration, propylene glycol also produced only minimal inhibition, but above 50 mM it was more inhibitory than equal concentrations of acetonitrile. In contrast to acetonitrile and propylene glycol, ethanol and dimethylsulfoxide were potent inhibitors of DMPB metabolism even at concentrations below 50 mM.

In order to overcome vehicle effects on *in vitro* metabolism in hepatocyte suspensions or microsomal preparations, aliquots of drug solution are often dried onto incubation vessels prior to addition of other components. However, we found that this method had drawbacks in our system since it took at least 30 min for 80 per cent of [^{14}C]DMPB dried onto a vial to be recovered in the suspension buffer. The time course of dissolution of DMPB at several points during a 1-hr incubation is presented in Fig. 2.

The previous studies demonstrate that drug vehicles can have profound effects on drug metabolism reactions measured *in vitro* using isolated hepatocytes. At least for the *N*-demethylase reaction studied, addition of drug in acetone to an aliquot of buffer, followed by a preincubation prior to cell addition, is the best method to handle the poorly soluble substrate. Of the vehicles tested, acetone, acetonitrile and propylene glycol appear to have only slight effects on *N*-demethylation if the volume is kept below 5 μl vehicle/ml incubate. On the other hand, ethanol and dimethylsulfoxide significantly inhibited DMPB

* R. Abraham, M. Sauers, M. Zemaitis and J. Alvin, manuscript submitted for publication.

N-demethylation even at concentrations as low as 1.0–2.5 μl/ml incubate. Therefore, these solvents appear to be poor vehicles for metabolism studies with isolated hepatocytes despite their use in several studies [1–3, 7, 8]. It should be noted, however, that our results were obtained with only one substrate, and when other reactions are studied, the effects of various vehicles may differ. Nevertheless, our studies demonstrate that drug vehicles used during metabolism studies can be a critical variable that must be investigated before data obtained with isolated hepatocytes is reported. Our studies also indicate that simply drying substrates on the surface of incubation vials prior to addition of incubation components does not guarantee that poorly soluble substrates will rapidly dissolve in the medium. In most cases, the rate of dissolution can be easily determined and should be calculated before metabolism is measured in hepatocyte suspensions when this method of substrate addition is used. This is particularly critical during time course studies involving short incubation times or in kinetic studies where precise substrate concentrations are required.

With the increasing use of isolated hepatocytes to study *in vitro* drug metabolism, careful control of incubation variables, such as drug vehicles, is essential. Proper selection of appropriate vehicles and reporting of vehicle effects will serve to standardize many techniques and simplify comparison of data from one laboratory to another.

* To whom all correspondence should be addressed.

Department of Pharmacology, University of Pittsburgh, School of Pharmacy, Pittsburgh, PA 15261, *U.S.A.*

MARION E. SAUERS
ROBERT T. ABRAHAM
JOHN D. ALVIN
MICHAEL A. ZEMAITIS*

REFERENCES

1. K. W. Bock, G. Van Ackeren, F. Lorch and F. W. Birke, *Biochem. Pharmac.* **25**, 2351 (1976).
2. D. Hultmark, K. Sundh, C. A. Wachtmeister and E. Arrhenius, *Biochem. Pharmac.* **27**, 1129 (1978).
3. G. L. Lowery and W. R. Bidlack, *Biochem. biophys. Res. Commun.* **83**, 747 (1978).
4. J. L. Holtzman, V. Rothman and S. Margolis, *Biochem. Pharmac.* **21**, 581 (1972).
5. P. Moldéus, H. Vadi and M. Berggren, *Acta pharmac. tox.* **39**, 17 (1976).
6. P. Moldéus, B. Andersson and A. Norling, *Biochem. Pharmac.* **27**, 2583 (1978).
7. J. S. Hayes and K. Brendel, *J. med. Chem.* **19**, 1324 (1976).
8. H. Thor, P. Moldéus, A. Kristoferson, J. Högberg, R. J. Reed and S. Orrenius, *Archs Biochem. Biophys.* **188**, 114 (1978).
9. C. Waydhas, K. Weigl and H. Sies, *Eur. J. Biochem.* **89**, 143 (1978).
10. M. N. Perry and D. S. Friend, *J. Cell Biol.* **43**, 506 (1969).
11. P. O. Seglen, in *Methods of Cell Biology* (Ed. D. M. Prescott), Vol. 13, p. 29. Academic Press, New York (1976).