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# Metabolism of drugs by isolated hepatocytes

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THE STUDY of the mechanisms involved in the control of the drug-metabolizing enzymes of the hepatocyte would be greatly facilitated by the development of a system *in vitro* which could be maintained for long periods and at the same time permit manipulation of the cellular environment. Juchau *et al.*[1] reported both the metabolism of 3,4-benzo(α)-pyrene in the isolated perfused liver and the induction of increased metabolism by polycyclic hydrocarbons. Unfortunately, this method is so cumbersome that it is difficult to maintain simultaneously multiple incubations which receive different treatments. Further, although Juchau *et al.*[1] were able to achieve induction with 3,4-benzo(α)-pyrene, this preparation is not viable for a sufficient length of time to study the induction by other agents such as phenobarbital.

More recently Henderson and DeWaide[2] have demonstrated the ability of citrate-disassociated cells to metabolize a number of substrates. Several workers have shown, however, that this method of cell isolation yields cells which are inferior in metabolic activity and morphological integrity[3,4] when compared with those prepared by the enzymatic method of Howard and Pesch.[5] The latter procedure, which utilizes perfusion and subsequent incubation with a mixture of collagenase and hyaluronidase, gives a lower cell yield than the citrate method; but the cells incorporate both acetate and amino acids at a faster rate, are metabolically active for a longer period, and require no additions of cofactors for lipid synthesis.[4] In view of these observations, we have examined the ability of freshly prepared hepatocytes separated by this method to metabolize drugs.

All animals used in these experiments were 200–300 g, fed, male, Sprague–Dawley rats obtained from Charles River, Inc. In experiments where animals were induced with 3-methylcholanthrene, they received the drug (40 mg/kg in corn oil, i.p.) at 72 and 48 hr before sacrifice. Animals induced with phenobarbital received the drug in their drinking water (1 g/l.) for 4 days.

Hepatocyte suspensions were prepared by the method of Howard and Pesch.[5] The animals were anesthetized with ether and the livers were perfused *in situ* with 20 ml of cold $Ca^{2+}$-free Hanks' solution which contained collagenase (120 units/ml) and hyaluronidase (1 mg/ml). The liver was removed, minced with scissors, and incubated in a water-bath shaker with 30 ml of the above enzyme solution for 45 min at 37° under $O_2$–$CO_2$ (95:5). The dispersed cells were centrifuged at 50 *g* for 5 min at 5°, resuspended in standard Hanks' solution and recentrifuged. The cells were then suspended in standard Hanks' solution (7·65 ml). The incubation mixture consisted of 7·15 ml of the cell suspension, 0·64 ml

of a solution of sodium penicillin G (80 μg/ml); (Upjohn Company, Kalamazoo, Mich.) and streptomycin sulfate (1·25 μg/ml); (Pfizer Company, New York City), 18·7 ml of medium 199 and 6·6 ml of calf serum (Flow Laboratories, Rockville, Md.) to give a final volume of 33·1 ml. Cell counts indicated $5 \times 10^5$ to $10^6$ cells per 2 ml and 40–60 per cent of the cells did not stain with trypan blue. No other energy source or pyridine nucleotides were added.

The substrate (28 μl of a 0·15 M solution of ethylmorphine or aniline, or 40 μl of a 0·1 M 3,4-benzo(α)-pyrene solution in acetone*) was placed in a 25-ml Erlenmeyer flask and 2 ml of the incubation mixture were added. The flasks were placed in a shaking water-bath at 37°, gassed for 5 min with $O_2$–$CO_2$ (95:5), and then stoppered until the incubation was completed. Phenol red in the medium 199 showed no color change with incubation. Measurements of the pH confirmed that the preparation had adequate buffering. Formaldehyde, *p*-aminophenol, and 8-hydroxy-3,4-benzpyrene were assayed by previously described methods.[6–8] Some difficulty was observed with the formaldehyde determination due to the presence of phenol red, but significant absorbancy was consistently obtained. In some experiments the cells were incubated without drug, the incubate was cooled, and the cells were

TABLE 1. EFFECT OF TIME AND CALF SERUM ON THE 3,4-BENZO(α)-PYRENE HYDROXYLASE ACTIVITY OF ISOLATED HEPATOCYTES FROM 3-METHYLCHOLANTHRENE-TREATED MALE RATS*

| Medium | Preincubation time of hepatocytes at 37° (min) | 3-Hydroxy-3,4-benzo(α)-pyrene formed (pmoles/min/ml incubate) |
|---|---|---|
| Hanks' solution | 0 | 0·044† |
| | 60 | 0 |
| 20% Calf serum | 0 | 4·50 |
| | 60 | 0 |

* Preparations and assays are given in the text, except that the substrate was added to the incubation without removal of the acetone. Incubation time was 30 min.

† Values are average of duplicate incubations. The blank values were taken at zero time.

TABLE 2. EFFECT OF PREINCUBATION OF HEPATOCYTES ON THE 3,4-BENZO(α)-PYRENE HYDROXYLASE ACTIVITY OF WHOLE HEPATOCYTES AND 9000 *g* SUPERNATANT PREPARED FROM THE HEPATOCYTES OF 3-METHYLCHOLANTHRENE-TREATED MALE RATS*

| Preparation | Preincubation time of hepatocytes at 37° (min) | 3-Hydroxy-3,4-benzo(α)-pyrene formed (pmoles/min/ml incubate) |
|---|---|---|
| Hepatocytes | 0 | 1·16†,‡ |
| | 60 | 0† |
| 9000 *g* supernatant | 0 | 7·38§ |
| | 60 | 3·47§ |

* Preparations and assays are given in the text.

† Values are the average of duplicate incubations.

‡ Incubation time was 30 min.

§ Incubation time was 10 min.

* The acetone was blown off before the cell suspension was added to the incubation vessel.

collected by centrifugation at 9000 *g* for 10 min. The pellet was resuspended in KCl–Tris (0·15 M–0·02 M, pH 7·4) and homogenized in a Teflon-glass homogenizer run at 2000 rev/min. The homogenate was centrifuged at 9000 *g* for 15 min and the pellet discarded. Ethylmorphone *N*-demethylase and aniline hydroxylase activities and the concentration of cytochrome P-450 were determined as previously described.[7] The protein was determined by the method of Sutherland *et al.*[9]

There was essentially no 3,4-benzo(α)-pyrene hydroxylase activity when Hanks' solution was substituted for calf serum. Inclusion in calf serum produced significant activity when the substrate was present from the beginning of the incubation. On the other hand, when the substrate was added after 60 min of preincubation, the cells were unable to form the hydroxylated product (Table 1). This loss of hydroxylase activity could have resulted from denaturation of the microsomal enzyme complex; however, even after preincubation, the enzymatic activity in the 9000 *g* supernatant remained intact (Table 2). These data suggest that the freshly prepared cells are capable of synthesizing the necessary cofactors involved in these reactions, but this ability is lost during incubation.

TABLE 3. METABOLISM OF ETHYLMORPHINE, ANILINE AND 3,4-BENZO(α)-PYRENE BY ISOLATED RAT HEPATOCYTES AND 9000 *g* SUPERNATANT PREPARED FROM ISOLATED RAT HEPATOCYTES OBTAINED FROM PHENOBARBITAL-TREATED MALE RATS*

| Preparation | Substrate | Preincubation time of hepatocytes (min) | Incubation time (min) | Product formed | Amount of product formed (pmoles/min) |
|---|---|---|---|---|---|
| Hepatocytes | Ethylmorphine | 0 | 30 | HCHO | 500†,‡,§ |
| | Aniline | 0 | 30 | *p*-Aminophenol | 24§ |
| | 3,4-Benzo(α)-pyrene | 0 | 30 | 3-Hydroxy 3,4-benzo(α)-pyrene | 7·57§ |
| 9000 *g* supernatant | Ethylmorphine | 0 | 10 | HCHO | 2500‖ |
| | | 60 | 10 | HCHO | 2860‖ |
| | Aniline | 0 | 10 | *p*-Aminophenol | 118‖ |
| | | 60 | 10 | *p*-Aminophenol | 343‖ |

* Preparations and assays are given in the text.

† Values are average of duplicate incubations.

‡ Recovery of added HCHO was 54 per cent. The values listed do not include a correction for this recovery.

§ Values are per ml of incubate.

‖ Values are per mg of protein in the 9000 *g* supernatant. In our experience, the microsomal protein is about one-fifth of the total protein of this fraction.

The isolated hepatocytes obtained from phenobarbital-induced animals and the 9000 *g* supernatant prepared from the isolated hepatocytes are active in the metabolism of aniline and ethylmorphine as well as 3,4-benzo(α)-pyrene. In fact, the activity per milligram of protein, when corrected for our usual observation that the microsomal protein represents only 20–25 per cent of the total protein from the 9000 *g* supernatant, was in the expected range for the usual microsomal preparation (Table 3).

Table 4 shows that significant quantities of cytochrome P-450 are present in the preparations. Further, there appears to be no loss of this important component of the mixed-function oxidases from the hepatocyte during preincubation of cells at 37°.

There are several advantages in the use of hepatocytes from adult animals as opposed to the use of hamster embryo liver cultures as described by Nebert and Gelboin.[10] Since the embryonic liver cells grow rapidly and are relatively undifferentiated with little endoplasmic reticulum, they may be a poor model for the study of the mechanisms involved in drug metabolism by the relatively stable adult hepatocyte.

TABLE 4. CONCENTRATION OF CYTOCHROME P-450 IN ISOLATED HEPATOCYTES AND 9000 *g* SUPERNATANT PREPARED FROM ISOLATED HEPATOCYTES OBTAINED FROM PHENOBARBITAL-TREATED MALE RATS*

| Preparation | Cytochrome P-450 | |
|---|---|---|
| | (nmoles/ml incubation mixture) | (nmoles/mg protein) |
| Hepatocytes | 8·5 | |
| 9000 *g* supernatant | 11·6 | 0·424 |
| 9000 *g* supernatant from cells incubated 60 min | 14·9 | 0·595 |

* Preparations and assays are given in the text.

A second advantage of the technique described in this paper is the ease of obtaining the adult tissues in almost unlimited quantities. Use of adult tissues eliminates problems associated with procuring or breeding animals so that embryos of the desired age are available.

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## Hydroxylation of phenylalanine by various areas of brain *in vitro**

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WE HAVE observed the formation of labeled tyrosine in several areas of brain *in vivo* following the injection of [$^{14}$C]phenylalanine into the lateral ventricle of rat brain.[1,2] The demonstration of the formation of tyrosine from phenylalanine in brain tissue *in vitro* is essential for a number of obvious

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