ABSTRACT

The procarcinogen, 2-acetylaminofluorene, the direct-acting carcinogen, methyl methanesulfonate, and two other hepatocarcinogens, thioacetamide and urethan, were tested for their ability to elicit unscheduled DNA synthesis in adult rat hepatocytes maintained in primary culture on collagen gel-nylon mesh. The carcinogens, dissolved in dimethyl sulfoxide, were added to 6-hr or to 28-hr cultures along with [methyl-3H]thymidine (1 μCi/ml medium) in the presence of 10 mM hydroxyurea. Twelve hr later, the hepatocytes were harvested from the cultures with collagenase, and their DNA was purified on CsCl isopyknic gradients. Unscheduled DNA synthesis was measured as the increase in [methyl-3H]thymidine radioactivity incorporated per μg DNA of the carcinogen-treated cultures as compared with that of control cultures. Both 2-acetylaminofluorene and methyl methanesulfonate demonstrated a concentration-dependent stimulation of unscheduled DNA synthesis in the 6-hr hepatocyte cultures. However, the response of the 28-hr cultures to these two carcinogens was absent unless the hepatocytes were preincubated for 22 hr in culture medium supplemented with 10⁻⁵ M dexamethasone and 10⁻⁶ M glucagon or in a more complete hormone-supplemented medium. Thioacetamide and urethan, on the other hand, failed to elicit a concentration-dependent unscheduled DNA synthesis under these conditions. The results obtained with this culture system are similar to those of other short-term tests for chemical carcinogenicity and support the potential use of the collagen gel-nylon mesh-hepatocyte primary culture as an in vitro screen for chemical carcinogens. Furthermore, this study suggests the importance of specific hormones in maintaining the capability for repair of DNA damage produced by carcinogenic and mutagenic chemicals in cultured hepatocytes.

INTRODUCTION

Studies from several laboratories have suggested the potential value of rat hepatocyte primary culture as an in vitro screening system for detecting carcinogenic chemicals (21, 31, 34). In this respect, adult rat hepatocytes maintained in short-term primary culture have been shown to exhibit DNA repair, measured as UDS, following their exposure to a number of known direct-acting carcinogens and procarcinogens (8, 21, 31-34). The ability of these cultured cells to metabolize procarcinogens to forms which damage DNA and thereby stimulate UDS, together with recent data which suggest that the metabolism of procarcinogens by rat hepatocytes in primary culture may be more like that in vivo than that shown with liver microsomal preparations (12, 27), further support the usefulness of such culture systems as a method for studying procarcinogen metabolism and its biological effects under the conditions of a controlled environment.

The metabolism of drugs and procarcinogens by the hepatocyte is associated with a multicomponent microsomal enzyme system, an integral part of which is a group of inducible hemoproteins known collectively as cytochrome P-450 (15, 16). The cytochrome P-450 content of adult rat hepatocytes maintained in primary culture under similar conditions to those used in the above UDS studies was demonstrated to decline quickly to a low but still measurable level within the first 24 to 48 hr of culture (12, 16, 22, 24). Similarly, the activities of some drug-metabolizing enzymes were shown to be significantly lower in rat hepatocytes maintained in short-term primary culture than in those of intact liver and in freshly isolated cell suspension (16). Suspensions of freshly isolated hepatocytes were also found to be more active in procarcinogen metabolism, as measured indirectly by their UDS response, than were hepatocytes in primary culture (21). However, because hepatocyte suspension cultures are short lived, their effectiveness in determining potentially optimum conditions for procarcinogen metabolism and for DNA repair is markedly limited.

In order to establish conditions in which hepatocytes in primary culture would more closely reflect the drug-metabolic capacity of the hepatocyte in vivo, Decad et al. (12) have recently formulated a culture medium that included a mixture of several hormones and δ-aminolevulinic acid. Adult rat hepatocytes cultured in this medium were shown by these investigators (12) and later by Paine and Legg (24) to maintain a near in vivo concentration of cytochrome P-450 for at least the first 24 hr of primary culture. Supplementation of culture medium with L-ascorbic acid was also found to maintain the cytochrome P-450 content of rat hepatocytes in 24-hr primary culture at a level that was significantly higher than that measured in cultures maintained without this vitamin (4).

We have recently described a versatile collagen gel-nylon mesh support system for maintaining adult rat hepatocytes in primary culture (29). In order to determine the feasibility of developing this primary culture system as an in vitro screen for chemical carcinogens, we have tested the procarcinogen, 2-acetylaminofluorene, the direct-acting carcinogen, methyl methanesulfonate, and 2 other hepatocarcinogens, urethan and thioacetamide, for their ability to elicit UDS in the cultured...
hepatocytes. We have also compared the effects of different medium conditions, i.e., those known to effect cytochrome P-450 content, on the sensitivity of this bioassay system and have detailed a simple analytical procedure for quantitating carcinogen-induced UDS in adult rat hepatocytes in primary culture.

MATERIALS AND METHODS

Chemicals. Insulin, testosterone, L-ascorbic acid, vitamin E acetate, linoleic acid, δ-aminolevulinic acid HCl, and hydroxyurea were purchased from Sigma Chemical Co., St. Louis, Mo. Dexamethasone phosphate was purchased from Organon, Inc., West Orange, N. J., glucagon was purchased from Eli Lilly and Co., Indianapolis, Ind., and cesium chloride (optical grade) was purchased from Schwarz/Mann, Orangeburg, N. Y. Methylmethanesulfonate and urethan were obtained from Aldrich Chemical Co., Milwaukee, Wis., and thioacetamide was purchased from Fisher Scientific Co., Fairlawn, N. J. 2-Acetylamino-fluorene was a generous gift from Dr. E. C. Miller of the McArdle Laboratory. [4,5-3H]Leucine (60 Ci/μmole) was purchased from New England Nuclear, Boston, Mass., and [methyl-3H]thymidine was from Amersham/Searle Corp., Arlington Heights, Ill.

Tissue Culture Medium. Leibovitz (L-15) tissue culture medium, penicillin, streptomycin, and fetal calf serum were purchased from Grand Island Biological Co., Grand Island, N. Y. Medium preparations included: (a) a standard medium consisting of L-15 tissue culture medium supplemented with 4(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (18 mM), albumin (2 mg/ml), penicillin (100 μg/ml), streptomycin (100 μg/ml), glucose (1.5 mg/ml), insulin (0.5 μg/ml), and 5% fetal calf serum; (b) Dex + Glu medium; and (c) a complete medium. The complete medium, which represented a slight modification of the medium devised by Decad et al. (12), was composed of standard medium supplemented with dexamethasone (10^-5 M), glucagon (10^-6 M), testosterone (10^-6 M), δ-aminolevulinic acid (2 × 10^-6 M), linoleic acid (5 μg/ml), vitamin E acetate (5 μg/ml), and L-ascorbic acid (30 μg/ml).

Hepatocyte Isolation and Culture Conditions. Hepatocytes having an initial viability of greater than 80% as measured by trypan blue dye exclusion were isolated from the livers of normal adult male albino Holtzman rats (200 to 230 g) by the collagenase perfusion method of Berry and Friend (3) as modified by Bonney et al. (5). The hepatocytes were then plated at a cell density of 14 to 15 × 10^6 cells in 7 ml of standard or complete medium onto 100-mm-diameter collagen gel-nylon meshes with attached cells were transferred to air in an incubator. The cultures were then maintained at 37° in the individual meshes with attached cells were transferred to an incubator.

Experimental Protocols. Two protocol designs were used. In the first protocol, designated as the 6-hr protocol, the carcinogen was added at different concentrations to the hepatocyte cultures in 100 μl DMSO (final DMSO concentration per culture, 1.4%) at 6 hr after cell plating and 2 hr after the 4-hr medium change. Immediately following this addition, [methyl-3H]thymidine was added to each culture at a final concentration of 1 μCi/ml medium, and the cultures were then maintained for an additional 12 hr before the hepatocytes were harvested for biochemical analysis. Hydroxyurea (10 mM) was present in the culture medium for the entire period of culture. In the second protocol, designated as the 28-hr protocol, a fresh medium change with either standard, Dex + Glu, or complete medium was made at 4 and 20 hr after cell plating. At 26 hr, the medium was removed, and 7 ml of standard medium containing 10 mM hydroxyurea were added to each culture. The carcinogen and [methyl-3H]thymidine were added as above to the cultures 2 hr after the hydroxyurea addition, and the hepatocyte cultures were then incubated for an additional 12 hr before cell harvest. Appropriate DMSO control cultures were run for each of the chemical carcinogens tested.

Measurement of Incorporated [methyl-3H]Thymidine into DNA. The hepatocytes were harvested by a brief treatment of the mesh cultures with a dilute collagenase solution (29) containing soybean trypsin inhibitor (0.1 mg/ml; Sigma). Isolated hepatocytes pooled from duplicate cultures were then solubilized at room temperature in 0.5 ml 0.01 M Tris-HCl buffer, pH 8.0, containing 1.0% sodium dodecyl sulfate and 1.0 mM EDTA. To this were added about 1.2 ml CsCl-Tris-HCl-1.0 mM EDTA (pH 8.0) to give a final CsCl density of approximately 1.64 g/ml. The resulting suspensions were then centrifuged at 23-25° for 30 min at 9.5 × 10^13 rpm with a SS-34 rotor in a Sorvall RC-5 centrifuge. This step served to separate the DNA from much of the hepatocyte protein and insoluble cell material. Following centrifugation, the supernatants containing the DNA were collected from layered in centrifuge tubes over 1.5 ml CsCl-Tris-HCl-1.0 mM EDTA (pH 8.0) with a density of 1.76 g/ml. Paraffin oil was used to fill the remainder of the centrifuge tube. The tubes were then centrifuged at 25° for 17 to 23 hr at 34 × 10^13 rpm with a SW-56 rotor in a Beckman L2-65B ultracentrifuge. The DNA was subsequently collected by fractionation of the gradient.

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and fixed for 2 hr in 3.0% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. Serial sections of the hepatocytes were then made from cell pellets embedded in paraffin. The sections were transferred to glass slides, processed, and coated with Kodak NTB-3 emulsion (Eastman Kodak Co., Rochester, N. Y.). After 2 weeks of exposure, the autoradiographs were developed and stained with hematoxylin and eosin. UDS was quantitated by counting nuclear grains in 50 consecutive cells chosen at random according to the method of Williams (32). A total of 200 nuclei were scored on each slide.

RESULTS

The hepatocytes attached to the collagen gel-nylon mesh substratum as single and small clusters of cells during the first 4 hr after cell plating, and then the cells flattened and came together to form a confluent monolayer within the following 20 to 30 hr of culture (Figs. 1 and 2). These cultures were found to be essentially free of contamination by other cell types during the first 48 hr after cell plating (29).

Analytical purification of DNA from rat hepatocyte primary culture

Adult rat hepatocytes were cultured according to the 6-hr protocol in standard medium without hydroxyurea. DNA was purified from duplicate cultures as described in "Materials and Methods." The DNA in each fraction was determined by the diphenylamine reaction, and incorporated [methyl-3H]thymidine radioactivity was measured according to the procedure described in "Materials and Methods." Each value represents the mean of duplicate measurements.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>DNA (μg)</th>
<th>% of recovery</th>
<th>[3H]Thymidine (dpm x 10^3)</th>
<th>% of recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell homogenate</td>
<td>205.34</td>
<td>100</td>
<td>126.80</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant (9.5 x 10^4 rpm)</td>
<td>117.16</td>
<td>57</td>
<td>67.20</td>
<td>53</td>
</tr>
<tr>
<td>CsCl gradient DNA fraction</td>
<td>93.86</td>
<td>46</td>
<td>63.46</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 1

UDS in Cultured Hepatocytes Exposed to Methyl Methanesulfonate or 2-Acetylaminofluorene According to the 6-hr Protocol. Dose-response curves for methyl methanesulfonate and for 2-acetylaminofluorene are shown in Chart 2. Under these conditions, each of the carcinogens was found to stimulate a concentration-dependent increase in UDS in the cultured hepatocytes when compared with respective DMSO control cultures. An optimum UDS response was produced with methyl methanesulfonate at a concentration of 10^{-4} to 10^{-3} M, whereas that elicited by 2-acetylaminofluorene was highest at a concentration of 10^{-3} M.

Separation of cultured hepatocyte DNA and protein on CsCl isopycnic gradients. In A, hepatocytes were harvested from culture at 40 hr after cell plating. Prior to cell harvest, the cells were pulse labeled in standard medium with [methyl-3H]thymidine (1 μCi/ml medium) for 12 hr or in standard medium minus L-leucine with L-(4,5-3H)leucine (5 μCi/ml medium) for 1.5 hr. In B, hepatocytes were harvested from culture at 18 hr after cell plating and at 12 hr after pulse labeling with [methyl-3H]thymidine in standard medium. DNA measured by diphenylamine reaction; O, incorporated [methyl-3H]thymidine radioactivity.

Concentration-dependent UDS stimulated in primary hepatocyte culture by methyl methanesulfonate and 2-acetylaminofluorene. Hepatocytes were maintained in primary culture with complete medium followed by Dex + Glu medium according to the 6-hr protocol. The carcinogens were added at the indicated concentrations with [methyl-3H]thymidine (1 μCi/ml medium) in the presence of 10 mM hydroxyurea at 12 hr before cell harvest. In Chart 2, duplicate measurements of carcinogen-induced UDS. Relative activity, dpm/μg DNA of the carcinogen-treated sample relative to that of the DMSO control.
when this procarcinogen was added to the medium at a concentration of \(10^{-3}\) M.

Table 2 further demonstrates the UDS response of the cultured hepatocytes to \(10^{-3}\) M 2-acetylaminofluorene when these cells were cultured for 18 hr under various supplemented medium conditions. In each case, 2-acetylaminofluorene stimulated a significant UDS in the cultured hepatocytes, although the amount of UDS elicited by this procarcinogen varied with the experiment and specific medium condition used to maintain the hepatocytes in primary culture. Furthermore, with this protocol, the amount of inhibition by hydroxyurea of incorporated "background" radioactivity from \([\text{methyl-}^3\text{H}]\text{thymidine}\) into the control DNA appeared to be dependent upon the medium condition under which the hepatocytes were cultured. In this respect, hydroxyurea (10 mM) inhibited the "background" radioactivity of control hepatocytes cultured in standard medium by 85%, but it showed little effect in further decreasing the already low "background" radioactivity of the control cultures maintained with complete medium followed by Dex + Glu medium.

Carcinogen-induced UDS was also demonstrated by autoradiography in hepatocytes cultured according to the 6-hr protocol in standard medium containing \(10^{-3}\) M 2-acetylaminofluorene, 10 mM hydroxyurea, and \([\text{methyl-}^3\text{H}]\text{thymidine}\) (Figs. 3 and 4). The average percentage of hepatocytes with a nuclear labeling of 6 grains or more was 50.4 for the 2-acetylaminofluorene-treated cultures as compared with 1.0 for the DMSO control cultures. Also, the mean number of grains per nucleus for the 2-acetylaminofluorene-treated cultures was 6.44 ± 1.1 (S.D.) compared with a mean nuclear grain count of 0.6 ± 0.31 for the DMSO-controls. In comparison, the mean numbers of grains per cytoplasm of the 2-acetylaminofluorene-treated and DMSO control hepatocytes were 2.70 ± 0.23 and 3.63 ± 0.23, respectively. There was no evidence of replicative DNA synthesis in either the carcinogen-treated or control cultures as evidenced by nuclei blackened with grains too numerous to count. However, in a separate experiment, the \([\text{methyl-}^3\text{H}]\text{thymidine}\) labeling index of replicative DNA synthesis was found to be less than 1.0% for hepatocytes cultured for 24 to 48 hr in standard medium without hydroxyurea and the carcinogen.

**Stimulation of UDS by Carcinogens Tested According to the 28-hr Protocol.** The design of the 28-hr experimental protocol was based in part upon a consideration of conditions which would minimize the incorporation of "background" \([\text{methyl-}^3\text{H}]\text{thymidine}\) radioactivity into the DNA of hepatocytes cultured in standard medium. This, in turn, necessitated determinations of (a) the amount of incorporated "background" radioactivity as a function of time of medium change prior to isotope administration, (b) the optimum inhibitory concentration of hydroxyurea under these conditions, and (c) the time of administration of hydroxyurea prior to the addition of the isotope which would produce an optimum inhibition of "background" radioactivity (Chart 3). In these experiments, the protocol called for a fresh medium change with standard medium containing 10 mM hydroxyurea at 2 hr prior to the addition of the carcinogen and the isotope. These latter additions were made in standard medium in order to permit a uniform testing condition.

Chart 4 shows the UDS response of the rat hepatocytes to \(10^{-3}\) M 2-acetylaminofluorene and to \(5 \times 10^{-4}\) M methyl methanesulfonate when these cells were cultured according to the 28-hr protocol under several different hormone-supplemented medium conditions. As demonstrated, both of these carcinogens were essentially ineffective in stimulating UDS in hepatocytes which were maintained with standard medium during their entire time in primary culture. However, hepatocytes cultured in either Dex + Glu medium or complete medium for 22 hr prior to the medium change with hydroxyurea exhibited a significant UDS response to each of these carcinogens when compared with that shown by the respective DMSO control cultures. In contrast, thioacetamide and urethan (when

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**Table 2**

Effect of different hormone-supplemented medium conditions on 2-acetylaminofluorene-induced UDS in rat hepatocytes in early primary culture

<table>
<thead>
<tr>
<th>Plating medium (0 hr)</th>
<th>Transfer medium (4 hr)</th>
<th>Carcinogen + isotope (6 hr)</th>
<th>DMSO control</th>
<th>2-Acetylaminofluorene-treated</th>
<th>Mean difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>Standard</td>
<td>2-Acetylaminofluorene + [methyl-(^3\text{H})]thymidine(^b)</td>
<td>597.1 ± 124</td>
<td>ND(^d)</td>
<td>ND</td>
</tr>
<tr>
<td>Standard + hydroxyurea(^a)</td>
<td>Standard + hydroxyurea(^d)</td>
<td>2-Acetylaminofluorene + [methyl-(^3\text{H})]thymidine(^d)</td>
<td>87.3 ± 16</td>
<td>289.8 ± 28</td>
<td>202.5</td>
</tr>
<tr>
<td>Standard + hydroxyurea(^d)</td>
<td>Dex + Glu + hydroxyurea(^d)</td>
<td>2-Acetylaminofluorene + [methyl-(^3\text{H})]thymidine(^d)</td>
<td>108.4 ± 15</td>
<td>238.8 ± 12</td>
<td>130.4</td>
</tr>
<tr>
<td>Complete</td>
<td>Dex + Glu</td>
<td>2-Acetylaminofluorene + [methyl-(^3\text{H})]thymidine(^d)</td>
<td>98.1 ± 24</td>
<td>246.4 ± 25</td>
<td>148.3</td>
</tr>
<tr>
<td>Complete + hydroxyurea(^d)</td>
<td>Dex + Glu + hydroxyurea(^d)</td>
<td>2-Acetylaminofluorene + [methyl-(^3\text{H})]thymidine(^d)</td>
<td>148.8 ± 14</td>
<td>356.3 ± 77</td>
<td>207.5</td>
</tr>
<tr>
<td>Complete + hydroxyurea(^d)</td>
<td>Standard + hydroxyurea(^d)</td>
<td>2-Acetylaminofluorene + [methyl-(^3\text{H})]thymidine(^d)</td>
<td>174.1 ± 7</td>
<td>368.1 ± 28</td>
<td>194.0</td>
</tr>
<tr>
<td>Complete + hydroxyurea(^d)</td>
<td>Complete + hydroxyurea(^d)</td>
<td>2-Acetylaminofluorene + [methyl-(^3\text{H})]thymidine(^d)</td>
<td>93.6 ± 18</td>
<td>169.5 ± 9</td>
<td>75.9</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± S.D. of triplicate determinations.

\(^b\) 2-Acetylaminofluorene (10\(^{-3}\) M) and [methyl-\(^3\text{H}\)]thymidine (1 μCi/ml medium) added to treated dishes.

\(^c\) ND, not determined.

\(^d\) Hydroxyurea (10 mM) was included in the perfusion medium and in the hepatocyte culture medium.
tested at concentrations ranging from $10^{-5}$ to $10^{-2}$ M) both failed to elicit a demonstrable UDS in hepatocytes cultured in either complete or standard medium according to the 28-hr protocol (data not shown).

**DISCUSSION**

Adult rat hepatocytes maintained on collagen gel-nylon meshes for more than 3 days in primary culture were shown to express a number of phenotopic properties characteristic of the fetal liver parenchymal cell in vivo, as well as to exhibit an increase in DNA synthesis with an increasing age of culture (29). Thus, we have confined the present study to the first 40 hr of primary culture in order that the cultured hepatocytes would more closely resemble in their function, morphology, and replicative activity those of the intact adult rat liver.

Williams (31, 32) has used autoradiography to first demonstrate carcinogen-induced UDS in adult rat hepatocytes in short-term primary culture. However, while autoradiography can provide for the quantitation of UDS by nuclear grain counting, it is desirable in the context of the present study to develop less tedious, more rapid and precise analytical procedures for evaluating carcinogen-induced UDS in hepatocyte primary culture. This regard, autoradiographs often require more than 1 week before they can be developed; grain counting is generally tedious, particularly when many cultures are to be evaluated; and quantitative measurements can be affected if care is not taken to ensure an emulsion coating of even thickness.

The procedure used in this study to isolate DNA from the cultured hepatocytes represents a relatively simple but precise quantitative approach to the measurement of carcinogen-induced UDS. In this respect, the gradient profiles obtained after CsCl isopyknic centrifugation (Chart 1) clearly reflect the measurement of incorporated [methyl-3H]thymidine radioactivity into hepatocyte DNA and not into protein. This distinction is quite important in view of the findings of Morley and Kingdon (23) which demonstrated that a significant amount of radioactivity becomes bound nonspecifically to the protein of freshly isolated hepatocytes incubated with [methyl-3H]thymidine. Furthermore, carcinogen-induced UDS could not be demonstrated with methods involving the direct TCA precipitation of cultured hepatocytes after their incubation with [methyl-3H]thymidine because of a high control background radioactivity that probably resulted from the incorporation of label into cellular macromolecules other than DNA (17, 21). In comparison, the control background radioactivity obtained in our study was generally much lower than that reported by others for hepatocyte-DMSO control cultures (6, 21, 34).

Although the amount of DNA replication in 1- and 2-day-old adult rat hepatocyte primary cultures is very low, it is still sufficiently high to interfere with measurements of carcinogen-induced UDS. Hydroxyurea, frequently used as an inhibitor of semiconservative DNA synthesis (30), was found, under conditions reported herein, to inhibit the incorporation of [methyl-3H]thymidine into the DNA of control cultures by 60 to 85% when the hepatocytes were maintained with standard medium. Michalopoulos et al. (21) have also shown a lower but still significant amount of inhibition of DNA synthesis by 5 to 25 mM hydroxyurea when administered simultaneously with [methyl-3H]thymidine to control adult rat hepatocytes maintained in primary culture for 6 days on floating collagen membranes. However, Yager and Miller (34) were unable to show a significant inhibition by 10 and 100 mM hydroxyurea of [methyl-3H]thymidine incorporation into the DNA of control rat hepatocytes in primary culture for 2 days on collagen-coated plastic surfaces. It is likely that the differences between our results with hydroxyurea on control DNA synthesis and those of these other investigators are due to differences in culture conditions, experimental design, DNA isolation procedures, and possibly to the activity of the respective hydroxyurea preparations.

There have been several conflicting reports on whether...
hydroxyurea can inhibit, enhance, or have no effect at all on UV- or carcinogen-induced UDS in cultured mammalian cells (9, 10, 18, 30). Andrae and Greim (2) have also recently demonstrated a DNA repair replication induced in human lymphoblastoid cells following their incubation with hydroxyurea in the presence of hepatic microsomes and NADPH. Since the hydroxyurea-induced DNA repair in these cells could be prevented with catalase, it was suggested that hydrogen peroxide derived from hydroxyurea in the presence of the metabolic activation system was involved in the production of DNA damage. However, even though the use of hydroxyurea may have limitations in the study of carcinogen-induced UDS, it did not under the present conditions interfere with the demonstration of carcinogen-induced UDS in the hepatocyte primary cultures. For example, hydroxyurea at a concentration of 10 mM produced only a marginal increase in the incorporation of \([\text{methyl-}^3\text{H}]\text{thymidine}\) into the DNA of control rat hepatocytes cultured for 18 hr in complete medium followed by Dex + Glu medium. The inhibition did not prevent nor markedly enhance the 2-acetylaminofluorene-induced UDS in these cells under these medium conditions (Table 1). Furthermore, the low background radioactivity obtained with the control hepatocyte cultures maintained for 18 hr without hydroxyurea in the complete medium followed by Dex + Glu medium may be related in part to the continued presence of a high concentration of dexamethasone since corticosteroid hormones have been shown to inhibit DNA synthesis in rat hepatocytes in vivo (13), as well as in those maintained in primary culture on collagen gel-nylon meshes (29).

Brandt et al. (6) have demonstrated in HeLa cells maintained in the presence of low concentrations of hydroxyurea (1 and 5 mM) that certain N-hydroxy compounds but not methyl methanesulfonate gave false-positive results of UDS by relieving the inhibition of hydroxyurea on replicative DNA synthesis. However, our results obtained with autoradiography confirmed the induction of UDS and not the restoration of replicative DNA synthesis by 2-acetylaminofluorene in short-term hepatocyte primary culture. Brandt et al. also suggested in their report that the problem of false-positive results of UDS is most likely to occur in rapidly dividing cells but is not likely to be the case in cells such as adult rat hepatocytes in 1- and 2-day-old primary culture in which the percentage of cells exhibiting replicative DNA synthesis is very low (less than 1%). In addition, Brandt et al. showed that the false-positive results obtained in HeLa cells were significantly reduced when these cells were maintained in 10 mM hydroxyurea, the same concentration used in our studies.

Of the carcinogens tested, methyl methanesulfonate and 2-acetylaminofluorene were found to stimulate a significant UDS in the cultured hepatocytes. In addition, the ability to demonstrate this response with the 28-hr protocol was dependent upon the specific medium conditions used to maintain the adult rat hepatocytes in primary culture. On the other hand, thioacetamide and urethan were both negative in this system when tested according to the 28-hr protocol under medium conditions in which methyl methanesulfonate and 2-acetylaminofluorene were positive. In this respect, there is a good correlation between the present results and those obtained for these respective carcinogens in the Ames’ Salmonella-rat liver microsome test (1, 20). The dose-response curves obtained with the 18-hr cultures for methyl methanesulfonate and for 2-acetyl-

aminofluorene (Chart 2) are also consistent with autoradiographic measurements of the amounts of UDS induced by these carcinogens in 1-day-old adult rat hepatocyte primary cultures (31, 32). Furthermore, the negative response obtained with thioacetamide is in agreement with the inability of this carcinogen to stimulate DNA repair replication in rat liver in vivo, as well as with the lack of evidence for its reaction with DNA (11). However, urethan when incubated in the presence of a postmitochondrial supernatant from phenobarbitone-treated rats was found to stimulate a small but significant UDS in HeLa cells, but the amount of UDS elicited by urethan in these cells was much lower than that induced by methyl methanesulfonate (19).

The stimulation of UDS in the culture hepatocytes following their exposure to 2-acetylaminofluorene provides an indirect measurement of the ability of these cells to metabolize this procarcinogen to its active form. With the 6-hr protocol, the hepatocytes cultured in standard medium alone and in the presence of hydroxyurea were found to exhibit a UDS response to 2-acetylaminofluorene which was comparable to that shown by hepatocytes maintained in primary culture with hydroxyurea-supplemented complete medium followed by Dex + Glu medium or standard medium (Table 1). However, in terms of cytochrome P-450, this is not too surprising since rat hepatocytes in 4- to 8-hr primary culture under basal medium conditions were shown to possess a cytochrome P-450 content which was about 70% higher than that of 24-hr cultures (4, 24). It is also interesting that the UDS response to 2-acetylaminofluorene was lower in hepatocytes cultured according to the 6-hr protocol in complete medium only as compared with that shown for these other medium conditions. This may be due in part to the continued presence of testosterone in the medium environment since this component is an effective competitive substrate inhibitor of hepatic cytochrome P-450-mediated drug metabolism (14, 25).

Michalopoulos et al. (21) have detected a measurable but low UDS response to a number of procarcinogens, including 2-acetylaminofluorene, by adult rat hepatocytes cultured for 6 days on floating collagen membranes in a medium that was almost identical to our standard medium. Yager and Miller (34) were also able to measure a low amount of 2-acetylaminofluorene-induced UDS in adult rat hepatocytes cultured for 2 and 4 days on collagen-coated plastic surfaces in a modified Waymouth’s medium supplemented with insulin and 1 mM dexamethasone. In comparison, we were unable with the 28-hr protocol to demonstrate a UDS response by the rat hepatocytes on collagen gel-nylon meshes to 2-acetylaminofluorene or to methyl methanesulfonate unless these cells were preincubated for 22 hr in either Dex + Glu or complete medium. Furthermore, the lack of a carcinogen-induced UDS response by hepatocytes maintained according to this protocol with standard medium alone was shown by trypan blue dye exclusion (data not shown), as well as by a number of functional and morphological criteria (29), not to be the result of a decrease in cell viability. It is therefore likely that the measurable UDS response to 2-acetylaminofluorene by the hepatocytes pretreated with the Dex + Glu medium or complete medium was related, at least in part, to the maintenance of their cytochrome P-450 content since media conditions similar to these (12, 22) have been shown to be effective in mitigating the rapid loss of cytochrome P-450 from rat hepatocytes during their first 24 hr in primary
culture. However, our results with the 28-hr protocol for the direct-acting carcinogen methyl methanesulfonate also suggest that the maintenance of the DNA repair response of the cultured hepatocytes may be dependent upon specific hormonal conditions independent of xenobiotic metabolism.

The hepatocyte primary culture system as presently devised is relatively simple and is capable of monitoring procarcinogens such as 2-acetylaminoﬂuorene without the addition of xenobiotic-metabolizing enzymes. Furthermore, as shown by Regan and Setlow (26), carcinogenic chemicals that damage DNA fall into 2 categories, the ﬁrst exempliﬁed by methyl methanesufonate which elicits a “short-patch” type of DNA repair and the second such as the active form of 2-acetylaminoﬂuorene which results in a “long-patch” type of DNA repair. Therefore, further studies are needed to deﬁne the biochemical effects of speciﬁc hormones like dexamethasone and glucagon on these 2 types of carcinogen-induced DNA repair in the rat hepatocyte in primary culture.

REFERENCES

Figs. 1 and 2. Primary culture of adult rat hepatocytes on collagen gel-nylon meshes. Phase-contrast micrographs of 4-hr culture (Fig. 1; x 40) and 40-hr culture (Fig. 2; x 100).
Figs. 3 and 4. Autoradiographs of DMSO control (Fig. 3) and 2-acetylaminofluorene-treated hepatocytes (Fig. 4; × 1000). Hepatocytes in primary culture were exposed to 10^{-3} M 2-acetylaminofluorene or DMSO in the presence of 10 mM hydroxyurea and [methyl-3H]thymidine (1 μCi/ml medium) in standard medium according to the 6-hr protocol. Note difference between the control and carcinogen-treated cells with respect to nuclear grains.
Use of Primary Cultures of Adult Rat Hepatocytes on Collagen Gel-Nylon Mesh to Evaluate Carcinogen-induced Unscheduled DNA Synthesis

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