DNA Repair in Primary Cultures of Rat Hepatocytes

James D. Yager, Jr., and Joseph A. Miller, Jr.

Department of Pathology, Dartmouth Medical School, Hanover, New Hampshire 03755

ABSTRACT

This report describes a precise quantitative analysis of DNA repair in cultured rat hepatocytes following exposure to ultraviolet light, 2-acetylaminofluorene, and two of its more active derivatives, N-hydroxy-2-acetylaminofluorene and N-acetoxy-2-acetylaminofluorene. Hepatocytes were isolated from young adult male Wistar rats with the collagenase perfusion technique and maintained in short-term monolayer culture on collagen-coated plates in a serum-free modified Waymouth’s medium. The nuclear [3H]thymidine ([3H]dTThd)-labeling index of control cultures was less than 0.1%, but significant cytoplasmic labeling was evident in autoradiographs. Of the total acid-precipitable radioactivity present in control cultures following exposure to [3H]dTThd, 54% of the 3H was found in protein, demonstrating the ability of these nonreplicating hepatocytes to catabolize [3H]dTThd and reutilize the labeled metabolites. Ultraviolet light irradiation of the cultured hepatocytes resulted in a dose-dependent increase of [3H]dTThd incorporation. That this represented nuclear DNA repair synthesis was demonstrated by detecting nonsemiconservative DNA synthesis (repair replication) with NaCl isopycnic centrifugation and autoradiography. Hydroxyurea (10 and 100 mM) had only a small inhibitory effect, while both 1β-o-arabinofuranosylcytosine (25 and 100 μM) and ethidium bromide (at 25 μM) dramatically inhibited the ultraviolet light-induced increase in [3H]dTThd incorporation. Repair synthesis also occurred in response to treatment of the hepatocytes with 2-acetylaminofluorene, demonstrating their ability to metabolize this prohepatocarcinogen to a form capable of damaging DNA. N-Hydroxy-2-acetylaminofluorene and N-acetoxy-2-acetylaminofluorene were more effective in inducing a repair response. These results represent additional characterization of the primary hepatocyte culture system and demonstrate its potential for studies on mechanisms of carcinogenesis and as a potential screening system for environmental chemicals suspected of being capable of damaging DNA and causing cancer.

INTRODUCTION

Recently, a considerable amount of work in several different laboratories has been devoted to establishing and characterizing rat hepatocytes in primary monolayer culture (2, 3, 16, 17, 20, 29). This relatively new epithelial cell culture system has several unique properties that make it attractive for use in short-term studies aimed at exploring biochemical changes caused by carcinogenic agents. One such property is their ability to respond to a variety of hormonal stimuli through induction of various metabolic processes (2, 3, 13, 14, 20, 28, 41) including DNA synthesis (34) and cell replication (18). A second important property concerns the broad drug-metabolizing capabilities of the liver as manifested by the cultured hepatocytes. Some work with this cell system indicated that the drug-metabolizing capacity of the cultured hepatocytes, as represented principally by cytochrome P-450 content, declined quickly (2, 6). However, other recent studies indicate that alteration of culture conditions and/or inclusion of key hormones in the culture medium allow the maintenance and/or the actual induction of cytochrome P-450 (4, 21, 23, 24). Preliminary reports from this laboratory (46, 47, 49) and studies by Williams (43—45) and Michalopoulos et al. (22) also indicate that, at least during the first day or 2 of culture, rat hepatocytes can convert a number of procarcinogens to forms capable of damaging their DNA. This ability was revealed by measuring DNA repair as unscheduled DNA synthesis following treatment with the compounds with the use of autoradiography (43—45) or analytical (22, 46, 47, 49) techniques and by their use as a drug-metabolizing feeder layer for enhancing mutagenesis in established liver cell lines (35).

The ability of hepatocytes to metabolize prohepatocarcinogens to forms that damage DNA, thus stimulating its repair, can be considered to be a "bioassay" for drug-metabolizing capacity. Perhaps it may be more meaningful in terms of cell function than an in vitro enzymatic assay for drug-metabolizing enzymes. Although indirect, the induction of DNA repair represents a relatively reliable indicator of the prior occurrence of genetic damage caused by chemical carcinogens (37, 38). The recent report by Williams (45) demonstrates that repair is stimulated in the cultured hepatocytes by chemicals known to be carcinogenic but not by a weak carcinogen and several noncarcinogens. However, Williams (45) used autoradiography to detect unscheduled DNA synthesis. While this technique does allow quantitation when grain counting is used, it may not be sensitive enough to facilitate a precise quantitative comparison of the amount of repair stimulated by different chemicals. However, precise quantitative comparisons obtained by methods less tedious than grain counting are desirable if the hepatocyte culture system is to be used to screen agents for their potential to inflict genetic damage on mammalian cells.

Our laboratory is involved with developing and further characterizing the primary hepatocyte culture system for studies on various aspects of the mechanisms involved in carcinogenesis and for use as a screening system. One of our goals is to investigate the DNA repair capacity of the...
cultured hepatocyte following treatment with various agents using sensitive analytical techniques. This report presents a protocol for carrying out quantitative analyses of DNA repair in these cells. The uptake and incorporation into DNA of $^{3}H$TdR in control hepatocyte cultures and in those exposed to UV and several derivatives of the carcinogen AAF are described.

**MATERIALS AND METHODS**

**Hepatocyte Isolation and Establishment of Primary Cultures.** Young (8 to 11 weeks old) male Wistar rats (200 to 300 g), maintained on an inverted lighting schedule (lights off from 8:30 a.m. to 8:30 p.m.) and fed a 30% protein diet (42) during the first 8 hr of each dark period [8 + 16 feeding schedule (31)] for at least 8 days, were used in all experiments. Hepatocyte cell suspensions were prepared from rats taken from their cages 1 to 2 hr after the beginning of the dark period.

Hepatocytes were isolated from intact rats by a modification (3) of the collagenase perfusion technique of Berry and Friend (1). The perfusion medium consisted of Ca$^{2+}$-free Hanks’ balanced salt solution supplemented with gentamicin, 50 μg/ml (Schering Corp., Kenilworth, N. J.), and was gassed continuously with 95% O$_2$:5% CO$_2$. A 10- to 15-min perfusion with this solution was followed by a 30- to 35-min perfusion with an identical solution containing type 1 collagenase, 130 units/ml (Sigma Chemical Co., St. Louis, Mo.). At the end of the perfusion, the cells were dispersed in a beaker by gentle stirring and pipetting with a large-bore pipet. The cells were then filtered through nylon mesh, 253 μm (Tetko, Inc., Elmsford, N. Y.), into an ice-cold plastic centrifuge tube and washed 2 to 3 times in Ca$^{2+}$-free Hanks’ by centrifugation in the cold at 50 x g for 3 to 4 min. Cell yields were routinely 500 to 800 x 10$^6$ with greater than 85% viability as judged by trypan dye exclusion.

Following the last wash the hepatocytes were resuspended in Hanks’ solution, counted in a hemocytometer, and then added to an ice-cold modified Waymouth’s MB752/1 medium (Grand Island Biological Co., Grand Island, N. Y.) at a concentration of 4 x 10$^6$ cells/4 ml medium. The hepatocyte suspension was then inoculated into collagen-coated (36) 6-cm plastic tissue culture dishes (Falcon Plastics Co., Oxnard, Calif.) at 4 ml of suspension per dish. The cells were allowed to come to room temperature and attach for 20 to 30 min before being moved to the incubator. The hepatocytes were then cultured at 37° in a 5% CO$_2$ air environment. Attachment efficiency, based on DNA content, varied from 60 to 65%.

**Carcinogen Treatment.** Hepatocyte monolayers were UV irradiated, following removal of the medium, with the use of a germicidal lamp producing a dose rate of 18 ergs/sec/sq mm. Following irradiation, fresh medium was immediately added back to the hepatocytes. AAF, N-OH-AAF, and N-Ac-AAF were dissolved in DMSO immediately preceding addition to the medium in μl aliquots. In all instances, controls were exposed to the same volume of DMSO (20 μl/4 ml culture).

**Measurement of $^{3}H$TdR Incorporation into DNA.** DNA synthesis and repair were measured with $^{3}H$TdR ([methyl-$^{3}H$]TdR, 20 Ci/mm, New England Nuclear, Boston, Mass.) incorporation into hepatocyte DNA. Where indicated, HU, ara-C, or EB were added 1 hr prior to $^{3}H$TdR addition. Exposure of the cells to $^{3}H$TdR (10 μCi/ml culture) was varied for various times beginning immediately after UV irradiation or during or after exposure to the various AAF derivatives. At the termination of $^{3}H$TdR exposure, the medium was removed, and the hepatocyte monolayers were rinsed 3 times with cold 0.85% NaCl solution containing 1 mM nonradioactive dTdT and subsequently were frozen and stored at −20°. In experiments where the amount of acid-soluble radioactivity in the cells was determined, the monolayers were rinsed 6 times. Control experiments indicated that the label appearing in the acid-soluble fraction decreased when between 0 and 3 0.85% NaCl:dTdT solution washes were used and then remained constant between 3 and 9 washes. The hepatocytes, frozen on the plates, were dissolved in 0.3 N KOH. Following RNA hydrolysis at 37° (26), the KOH solution was cooled, neutralized, made 5% with TCA, and centrifuged. The supernatant constituted the acid-soluble fraction. ($^{3}H$ from $^{3}H$TdR catabolism incorporated into RNA was negligible; see “Results”.) The remaining TCA precipitate was washed 3 times with 5% TCA, and the DNA was subsequently hydrolyzed in 5% TCA at 90° for 20 min (48).

DNA content was determined from the TCA hydrolysate with the use of either the diphenylamine reagent (9) or a modification of the fluorometric technique of Kissane and Robbins (12) and Hinegardner (8). This modification involved incubation of 0.2 ml of TCA hydrolysate with 0.2 ml of the fluorometric reagent (diaminobenzoic acid dihydrochloride, Aldrich Chemical Co., Milwaukee, Wis.) at 65° for 30 min. Calf thymus DNA (Sigma) hydrolyzed in 5% TCA was used as a standard for both DNA determination methods. The DNA content of a single sample was the same when determined by both methods with the appropriate standard curves. Radioactivity in the acid-soluble and hydrolyzed DNA fractions was determined using Biofluor (New England Nuclear) and a Nuclear Chicago liquid scintillation spectrometer. In one experiment (Table 2), the cells were scraped off the plates in cold 5% TCA to obtain the acid-soluble fraction. This allowed the additional separation of approximately 7.2 upon equilibration with 5% CO$_2$:air. In addition, insulin, 10 milliuunits/ml (Eli Lilly Co., Indianapolis, Ind.), and dexamethasone, 1 μM (Decadron; Merck, Sharp and Dohme, West Point, Pa.), were present throughout the culture period. Day 1 of culture is the day that culture is initiated. The medium was changed approximately 18 to 20 hr after inoculation (Day 2) and daily thereafter. All cultures received 4 ml of medium at each change.

The abbreviations used are: dThd, thymidine; AAF, 2-acetylaminofluorene; N-OH-AAF, N-hydroxy-2-acetylaminofluorene; N-Ac-AAF, N-acetoxy-2-acetylaminofluorene; DMSO, dimethyl sulfoxide; HU, hydroxyurea; ara-C, 1-β-D-arabinofuranosylcytosine·HCl; EB, ethidium bromide; TCA, trichloroacetic acid; BrdUrd, bromodeoxyuridine; FdUrd, fluoro-deoxyuridine.
the RNA, DNA, and protein fractions and the determination of the amount of $^3$H from [3H]dThd in each.

**Measurement of DNA Repair Replication.** DNA repair replication was measured by the Nal isopycnic centrifugation technique of Lohman et al. (19). Hepatocyte monolayers in 10-cm culture dishes inoculated with 10 to 10 x $10^6$ cells were exposed to medium containing BrdUrd (10$^{-5}$ M) and FdUrd (5 x 10$^{-6}$ M) for 1 hr prior to carcinogen treatment. In cultures irradiated with UV, the medium was removed, the cells were irradiated, and fresh medium containing BrdUrd and FdUrd was added. In cultures treated with chemical carcinogens, fresh medium was added containing BrdUrd and FdUrd followed by carcinogen in DMSO. All cultures received [3H]dThd (20 µCi), and culture was continued for an additional 6 hr. Upon harvest, the monolayers were rinsed 3 times with 0.85% NaCl:dThd solution and frozen. The cells from duplicate cultures were pooled, and the DNA was semipurified as described by Lohman et al. (19) with the addition of a pronase (Sigma) digestion technique of Lohman et al. (19). Hepatocyte monolayers were exposed to a 3-hr pulse of [3H]dThd at various times after the medium change on Day 2 of culture. Acid-soluble radioactivity which may, at least initially, reflect the amount of [3H]dThd available for incorporation increased up to 60 min and then began a continuous decline (Chart 1). Incorporation of [3H]dThd into DNA was linear for the first 3 hr after the medium change (Chart 1). The rate of accumulation of [3H]dThd into DNA then decreased.

An attempt was made to determine whether the change in rate of [3H]dThd incorporation actually did decrease with time after the Day 2 medium change or whether the pattern seen in Chart 1 was an artifact due to prolonged exposure of the cells to [3H]dThd and exhaustion of labeled precursor available for incorporation. Hepatocyte cultures were subjected to a 3-hr pulse of [3H]dThd at various times after the Day 2 medium change and the amount of radioactivity in the acid-soluble fraction and in DNA determined (Table 1). Since accumulation of [3H]dThd into DNA is linear for 3 hr, the 3-hr pulse reflects the rate of incorporation. The data in Table 1 indicate that the rate of [3H]dThd incorporation into DNA decreases with time after the medium change reaching a minimum after 9 hr. No difference in the amount of acid-soluble radioactivity taken up during the 3-hr pulses was seen between 0 and 3 hr. However, a small but significant increase was detected at 9 hr and again at 24 hr (data not shown). Thus, the results seen in Chart 1 and Table 1 suggest that [3H]dThd incorporation into DNA is stimulated upon changing the medium and that the rate of incorporation subsequently declines. Refeeding the cells 6 hr or 24

**RESULTS**

**[3H]dThd Incorporation into DNA in Control Cultures.** Chart 1 shows the time course of [3H]dThd uptake and incorporation into DNA in control hepatocyte cultures. The cells were exposed continuously to [3H]dThd for the times indicated after the medium change on Day 2 of culture. Acid-soluble radioactivity which may, at least initially, reflect the amount of [3H]dThd available for incorporation increased up to 60 min and then began a continuous decline (Chart 1). Incorporation of [3H]dThd into DNA was linear for the first 3 hr after the medium change (Chart 1). The rate of accumulation of [3H]dThd into DNA then decreased.

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**Statistical Analysis.** Where indicated, statistical analysis was carried out on the data. The statistical tests used were one- or 2-way analyses of variance or the Newman-Kuhls test for multiple comparisons. The $p$ values for the data are presented where appropriate. Although the actual amount of [3H]dThd incorporation detected in control cultures can vary by a factor of up to 2 among different cell preparations, the overall patterns of incorporation described throughout the paper are highly reproducible. Each experiment presented is representative of at least several identical experiments.

**RESULTS**

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**Abbreviations.** All abbreviations are explained in the manuscript.

![Chart 1.](chart1.png)
hr later again stimulates incorporation to approximately the same extent (data not shown). The 3-hr pulse experiments indicate that the hepatocytes do not appear to decline in their ability to take up \([^{3}H]dThd\) with time after the medium change. The decline in acid-soluble radioactivity with time seen upon continuous exposure to \([^{3}H]dThd\) (Chart 1) may reflect, among other possibilities, an actual decrease in uptake and/or may be due to catabolism of the labeled dThd and its exhaustion from the medium. Evidence demonstrating the ability of these cells to catabolize \([^{3}H]dThd\) and incorporate \(^{3}H\) into macromolecules other than DNA will be presented below.

The pattern of \([^{3}H]dThd\) incorporation into DNA was intriguing in itself. In addition, the continual change occurring in controls represented a potential complication for conducting studies on DNA repair as measured by quantifying unscheduled DNA synthesis. Thus, we have conducted a more in-depth analysis of dThd incorporation in controls.

Autoradiography was carried out on cultured hepatocytes exposed to \([^{3}H]dThd\) for 24 hr following the Day 2 medium change. The nuclear labeling index of such control cultures revealed that less than 0.1% of the hepatocytes contained labeled nuclei. These results supported the findings of others (3, 17, 45) and demonstrated that the primary hepatocyte cultures were essentially nonreplicating. However, considerable cytoplasmic labeling was seen in the autoradiographs. These results suggested that either the incorporation detected analytically in extracted DNA was due to DNA synthesis in a few labeled cells, cytoplasmic (mitochondrial) DNA synthesis, or alternatively, was an artifact possibly due to incorporation of \(^{3}H\) from \([^{3}H]dThd\) into RNA not removed by the KOH hydrolysis but appearing in the hydrolyzed DNA fraction or to incorporation of \(^{3}H\) from \([^{3}H]dThd\) into protein hydrolyzed along with the DNA in the hot TCA extraction step.

Several lines of evidence suggested that the incorporation we see in the DNA fraction extracted by hydrolysis does in fact represent \(^{3}H\) incorporation into DNA: (a) use of CsCl and Nal isopycnic centrifugation suggested that a small amount of semiconservative DNA synthesis occurred in these cultures (see later). (b) Determination of the amount of \(^{3}H\) from \([^{3}H]dThd\) in the acid-soluble, protein, RNA, and DNA fractions revealed that a considerable amount of \(^{3}H\) from \([^{3}H]dThd\) appeared in the protein fraction (Table 2). However, we determined that the hydrolyzed DNA fraction contained only a small amount of contaminating protein indicating that following a 6-hr exposure to \([^{3}H]dThd\) the radioactivity in this fraction was almost entirely (90 to 95%) accounted for by \(^{3}H\) in DNA. In addition, UV irradiation, which caused large increases in labeled DNA as a result of repair (see below) had little effect on incorporation of \(^{3}H\) from \([^{3}H]dThd\) into the RNA or protein fractions.

**DNA Repair in Cultured Hepatocytes Irradiated with UV.** In our attempt to define the DNA excision repair capacity of the cultured hepatocytes we have used UV and AAF, including some of its more active derivatives, for damaging DNA. UV was chosen for determination of repair capacity since it represented a way of inflicting a relatively uniform, reproducible amount of DNA damage and avoided potential problems of uptake and metabolism associated with the use of chemicals. The response of the cultured hepatocytes to UV irradiation is shown in Chart 2. The data in Chart 2 are expressed as the increase in \([^{3}H]dThd\) incorporation over controls caused by UV irradiation at 100, 250, and 750 ergs/sq mm. The pattern of incorporation of \([^{3}H]dThd\) in controls was similar to that seen in Chart 1, and the control values for the experiment are presented in the legend to Chart 2. Continuous exposure to \([^{3}H]dThd\) for periods following UV irradiation revealed a clear dose response after 12 hr, with no additional incorporation taking place between 12 and 24 hr. Other experiments following incorporation for 6 and 12 hr indicated that the repair capacity of the cells saturated at between 250 and 500 ergs/sq mm. In addition, autoradiography indicated that 93 ± 3% (S.D.) of the hepatocyte nuclei were labeled following UV irradiation (250 ergs/sq mm).

Experiments were conducted to determine whether the incorporation stimulated by UV actually represented repair replication, i.e., nonsemiconservative DNA synthesis, with the use of Nal isopycnic centrifugation as described under "Materials and Methods." A typical sedimentation profile from such gradients is shown in Chart 3. In control cultures following the medium change on Day 2 of culture

<table>
<thead>
<tr>
<th>Time after medium change (hr)</th>
<th>DNA (dpm/µg)(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1631 ± 74</td>
</tr>
<tr>
<td>3</td>
<td>991 ± 160</td>
</tr>
<tr>
<td>9</td>
<td>287 ± 11</td>
</tr>
<tr>
<td>24</td>
<td>392 ± 53</td>
</tr>
</tbody>
</table>

\(^{a}\) The hepatocytes were exposed to \([^{3}H]dThd\) for a 3-hr period at the indicated times after the medium change.

\(^{b}\) Mean ± S.D. for triplicate cultures.

<table>
<thead>
<tr>
<th>Fraction(^{a})</th>
<th>Control</th>
<th>UV-irradiated (^{c})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid-soluble</td>
<td>261.5 ± 23.7</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>37.5 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>54.7 ± 4.1</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Upon harvest, the hepatocytes were rinsed 6 times in 0.85% NaCl:dThd solution should be standardized with form used previously and frozen. The cells were scraped from the culture dishes in cold 5% TCA and allowed to stand in the 5% TCA for 1 hr at 2-3°. Following centrifugation, the supernatant was saved as the acid-soluble fraction. RNA and DNA were obtained as described under "Materials and Methods"; TCA washes were conducted between extractions to ensure removal of all radioactivity freed by the particular extraction step. The final TCA precipitate remaining after DNA extraction was rinsed in TCA and solubilized in KOH. This was considered the protein fraction. The protein content of each fraction was determined by the method of Hartree with appropriate standard curves and blanks (75).

\(^{b}\) On Day 2 the medium was changed, and the hepatocytes were exposed to \([^{3}H]dThd\) for 6 hr.

\(^{c}\) After removal of the medium, the monolayer cultures were irradiated with 750 ergs/sq mm UV. Fresh medium plus \([^{3}H]dThd\) was added, and culture was continued for 6 hr.

\(^{d}\) Mean ± S.D. of triplicate cultures.
The hepatocytes were pretreated with BrdUrd and FdUrd 1 hr prior to treatment. The medium was removed, the monolayers were irradiated, fresh BrdUrd-FdUrd medium plus [3H]dThd (25 pCi) were added back, and culture was continued for 6 additional hr. UV dose (ergs/sq mm) DNA (cpm/ug) 

<table>
<thead>
<tr>
<th>UV dose (ergs/sq mm)</th>
<th>DNA (cpm/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30.7 ± 3.9</td>
</tr>
<tr>
<td>50</td>
<td>118.2 ± 16.9</td>
</tr>
<tr>
<td>100</td>
<td>173.4 ± 14.3</td>
</tr>
<tr>
<td>250</td>
<td>286.5 ± 6.9</td>
</tr>
<tr>
<td>500</td>
<td>399.6 ± 25.3</td>
</tr>
<tr>
<td>750</td>
<td>390.0 ± 26.5</td>
</tr>
</tbody>
</table>

The data represent the specific activity of the fraction containing the most DNA plus one fraction on either side of it. Mean ± S.D. of the specific activity in the 3 peak fractions.

The hepatocytes were treated with UV and immediately cultured in fresh medium, which, as seen above, stimulated [3H]dThd incorporation in control cultures. Several attempts were made to determine the effect on repair of altering this protocol. This was accomplished by adding inhibitors or allowing the cells to "condition" their medium prior to UV irradiation. Table 4 shows the effects of ara-C, HU, and EB on the increase in [3H]dThd incorporation into DNA in controls and caused by irradiation of the cultured hepatocytes with UV (750 ergs/sq mm). Both doses of ara-C and EB significantly inhibited incorporation in control cultures whereas HU treatment had no effect. The UV-induced increase in the inhibitor-treated cultures was significantly less than in the non-inhibitor-treated controls in all cases. However, a 2-way analysis of variance between non-UV- and UV-treated groups indicated that the inhibition of the UV response caused by the inhibitors did not parallel their inhibition of incorporation in the non-UV-treated controls (interaction; p < 0.001). This is obvious from the data in the UV-induced increase column in Table 4. The inhibition of UV-induced [3H]dThd incorporation...
Table 4

Effect of ara-C, HU, and EB on \( ^{3}H \)dTdR incorporation into DNA in control and UV-irradiated hepatocytes

<table>
<thead>
<tr>
<th>Inhibition(^a)</th>
<th>Control</th>
<th>UV (750 ergs/sq mm)</th>
<th>UV-induced increase(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1412 ± 169</td>
<td>4708 ± 121 (^c)</td>
<td>3296 ± 121</td>
</tr>
<tr>
<td>ara-C 25 µM</td>
<td>1022 ± 60 (^d)</td>
<td>2042 ± 33</td>
<td>1020 ± 33 (^d)</td>
</tr>
<tr>
<td>100 µM</td>
<td>619 ± 36 (^d)</td>
<td>1144 ± 83</td>
<td>525 ± 83 (^d)</td>
</tr>
<tr>
<td>HU 10 mm</td>
<td>1432 ± 40</td>
<td>4240 ± 79</td>
<td>2908 ± 79 (^d)</td>
</tr>
<tr>
<td>100 mm</td>
<td>1449 ± 77</td>
<td>4044 ± 276</td>
<td>2595 ± 276 (^d)</td>
</tr>
<tr>
<td>EB 2.5 µM</td>
<td>657 ± 64 (^d)</td>
<td>3043 ± 27</td>
<td>2386 ± 27 (^d)</td>
</tr>
<tr>
<td>25.0 µM</td>
<td>140 ± 2 (^d)</td>
<td>318 ± 11</td>
<td>178 ± 11 (^d)</td>
</tr>
</tbody>
</table>

\(^a\) On Day 2 the hepatocytes were preincubated with the inhibitors for 1 hr and UV-irradiated, fresh medium plus inhibitor and \( ^{3}H \)dTdR were added, and culture was continued for 6 hr.

\(^b\) Calculated by subtracting the mean control value from each of the triplicate cultures of the UV-treated group and determining the mean ± S.D. of such calculated differences.

\(^c\) Mean ± S.D. of triplicate cultures.

\(^d\) Significantly different from controls, \( p < 0.05 \).

The duration of DNA repair following UV irradiation of the culture hepatocytes exposed to the higher UV dose. Twenty-four hr following treatment incorporation in the cultures irradiated with 100 ergs/sq mm UV light is at control levels, and that in cultures treated with the higher dose is only slightly, but significantly (\( p < 0.05 \)) greater. Correction of the incorporation data for the amount of acid-soluble radioactivity available for incorporation in each treatment group did not alter these results. These results suggest that repair synthesis persisted longer in cells subjected to a higher dose of UV but that by 24 hr it had assumed a very low level. This pattern basically supports that seen in Chart 2 where the cells were exposed to \( ^{3}H \)dTdR continuously following UV irradiation. However, while high levels of repair synthesis persisted only for several hr following the occurrence of DNA damage, cessation of detectable repair probably does not mean that all the damaged DNA had been repaired.

DNA Repair in Cultured Hepatocytes Exposed to AAF and Some of Its More Active Derivatives. The work of others and preliminary reports from this laboratory cited in "Introduction" have demonstrated that hepatocytes in primary culture retain the ability to metabolize procarcinogens to forms capable of damaging DNA, thus stimulating a repair response. As an extension of these results, we have conducted a study of repair following treatment of the cultured hepatocytes with AAF and 2 of its more active derivatives. Chart 5 shows the stimulation of \( ^{3}H \)dTdR incorporation into DNA as a result of exposure of hepatocytes to AAF at 2 concentrations on Day 2 of culture. Fresh medium was added to the cells immediately followed by AAF and \( ^{3}H \)dTdR; exposure was continuous for the indicated times. After an initial lag, incorporation was approximately linear for 12 hr in cultures treated with 10\(^{-4}\) M AAF but appeared to decrease between 6 and 12 hr in cultures exposed to 10\(^{-5}\) M. The actual increase over control values induced by AAF and UV varied to some extent among different batches of cells; with AAF this was especially noticeable after short exposures to \( ^{3}H \)dTdR. However, all
DNA Repair in Cultured Hepatocytes

Chart 5. DNA repair in hepatocytes exposed to AAF. On Day 2 of culture, fresh medium containing AAF at $10^{-8}$ or $10^{-6}$ M plus $[^{3}H]dThd$ ($[^{3}H]dThd$) was added, and culture was continued for the times indicated. Data are the mean ± S.D. of the difference between the average control value and each of the triplicate cultures of each treatment group. The control values for DNA (mean ± S.D. of triplicate cultures) for each time point are: 3 hr, 1145 ± 127; 6 hr, 1650 ± 78; 12 hr, 1164 ± 114 dpm/μg.

The increase in $[^{3}H]dThd$ incorporation occurring in response to AAF reflects repair replication. This was determined by Nal and CsCl isopycnic centrifugation carried out as described previously (data not shown). In addition, autoradiography indicated that 69 ± 4% of the cells were labeled following AAF treatment.

Experiments were carried out to determine the duration of DNA repair following exposure of the cultured hepatocytes to AAF. On Day 2 of culture, the cells were fed with fresh medium and exposed to AAF (to $10^{-8}$ and $5 \times 10^{-8}$ M) for 1 hr. The AAF medium was then removed and replaced with fresh medium, and the hepatocytes were subjected to 3-hr pulses of $[^{3}H]dThd$ at various times thereafter. The rate of $[^{3}H]dThd$ incorporation was elevated immediately following carcinogen treatment and declined with time thereafter with a pattern similar to that seen in Chart 4 following UV irradiation (data not shown). After 24 hr, incorporation was still significantly ($p < 0.05$) elevated in cultures previously treated with $10^{-6}$ M AAF suggesting that repair was still occurring. Incorporation in cultures treated with the lower concentration of AAF was not significantly different from control after 24 hr.

Hepatocyte cultures were also exposed to 2 more active derivatives of AAF, namely, N-OH-AAF and N-Ac-AAF, and the amount of repair-induced incorporation of $[^{3}H]dThd$ was determined (Chart 6). A dose response to N-OH-AAF is evident. A comparison of the response caused by AAF (Chart 5) and N-OH-AAF (Chart 6) shows that the latter caused much higher levels of incorporation at lower doses. AAF must be metabolized by the microsomal mixed-function oxidase system, whereas the N-OH-AAF bypasses that step (25). Cytochrome P-450 is reduced in these cells on this compound actually entered the cells and reached the nucleus to damage DNA. The fact that the responses observed with these AAF derivatives reflected repair replication has also been demonstrated by Nal isopycnic centrifugation, and similar relationships between the extent of response produced were seen (data not shown).

Additional experiments were conducted to determine whether the repair capacity of the cultured hepatocytes changed with time in culture. Thus, the cells were exposed to either AAF, N-OH-AAF, or UV on either Day 2 or Day 4 of culture, and the amount of $[^{3}H]dThd$ incorporation induced over a 12-hr period was determined. The results are shown in Chart 7. These data indicate that, by Day 4, the repair response induced by AAF was somewhat reduced; however, in this experiment, the difference between the response to AAF on Days 2 and 4 was not significant; the response to N-OH-AAF and UV was not reduced. However, as seen from the control values presented in the legend to Chart 7, incorporation was higher on Day 4.

In order to correct for any cellular changes that occurred between Days 2 and 4, we normalized the repair response induced by the chemicals to that induced by UV since a given dose of UV should yield similar amounts of DNA damage on both days. Thus, on Day 2 the ratios of the N-OH-AAF- and AAF-induced responses to that seen with UV were 0.90 and 0.45, respectively; on Day 4, these ratios were 0.88 and 0.27, respectively. This suggests that, relative to the UV-induced repair response, no change occurred between Days 2 and 4 in response to N-OH-AAF treatment, whereas AAF induced less repair on Day 4 than on Day 2. This suggests that on Day 4, while the cultured hepatocytes can still metabolize AAF to a form capable of damaging...
by fresh medium; (b) DNA repair in response to UV; and (c) DNA repair in response to treatment with the prohepatocarcinogen AAF and its proximate and ultimate derivatives N-OH-AAF and N-Ac-AAF, respectively. The results and their implications for each of these characteristics will be discussed briefly.

The data obtained with control cultures suggest that the addition of fresh medium stimulated \(^{3}H\)dThd incorporation into DNA. The rate of incorporation subsequently declined reaching a minimum after 6 hr. This is supported both by experiments with continuous exposure to \(^{3}H\)dThd and short pulses of \(^{3}H\)dThd at various times after the medium change. When hepatocytes were pulse-labeled at various times after addition of fresh medium, \(^{3}H\)dThd incorporation into DNA declined with time but the amounts of acid-soluble radioactivity present after each 3-hr pulse did not. This suggests that the decline in incorporation is not due to the regulation of some dThd transport mechanism. However, upon continuous exposure of hepatocytes to \(^{3}H\)dThd, the level of acid-soluble radioactivity declined continuously after 1 hr. Our results suggest that this may be due to the degradation of dThd. Evidence demonstrating that the primary hepatocytes isolated from young adult rats catabolize \(^{3}H\)dThd comes from our studies on the distribution of \(^{3}H\) from \(^{3}H\)dThd in the acid-soluble fraction and the acid-precipitable RNA, DNA, and protein fractions. Most (71%) of the total cellular radioactivity was found in the acid-soluble fraction. Of the total acid-precipitable \(^{3}H\) from \(^{3}H\)dThd, 8.8% was found in the RNA, 37.1% in the DNA, and 54.1% in the protein fractions (Table 2). In UV-irradiated cultures, 53% of the total label was in the acid-soluble fraction. In the acid-precipitable fractions, 5.1% was in the RNA, 64.5% in the DNA, and 30.4% in the protein fractions. These results, together with those of Hopkins and Wakefield (10), indicate that, particularly with liver or hepatoma cells, care must be taken to demonstrate that radioactivity incorporated into TCA-precipitable material truly reflects incorporation of \(^{3}H\)dThd into DNA. Simply looking at acid-precipitable radioactivity without determining the macro-molecular fraction(s) in which it has occurred is unacceptable for liver and possibly other cell types (10, 39). This point is illustrated further in the recent paper by Michalopoulos et al. (22) where studies on unscheduled DNA synthesis induced in isolated rat hepatocytes by procarcinogen treatment were reported. These workers were able to demonstrate repair in DNA isolated on CsCl gradients but not in solubilized TCA precipitates containing both DNA and protein. The high background observed by Michalopoulos et al. (22) under these latter conditions was most probably due to \(^{3}H\) from \(^{3}H\)dThd catabolism incorporated into protein and masking the small carcinogen-induced increases in incorporation of \(^{3}H\)dThd into DNA.

Several inhibitors of DNA synthesis were used in an attempt to reduce the control \(^{3}H\)dThd incorporation to facilitate detection of unscheduled DNA synthesis. To our surprise, we found that HU failed to inhibit \(^{3}H\)dThd incorporation in control cultures. In contrast, ara-C and EB did inhibit \(^{3}H\)dThd incorporation. However, all 3 inhibitors also reduced the amount of UV-induced incorporation (repair) to varying degrees. Thus, caution must be exercised in the use of inhibitors in DNA repair studies. However,
other results presented above suggest that no special precautions need to be taken to inhibit the “background” level of [3H]dThd incorporation occurring in order to study repair synthesis in the cultured hepatocytes. Since the rate of [3H]dThd incorporation in controls changed with time up to at least 6 hr after a medium change, each experiment must include controls at each time point, and presentation of the data is facilitated by subtraction of the control value. However, the inhibitor studies did suggest that something was unique about the [3H]dThd incorporation occurring in DNA. HU is a well-known, widely used inhibitor of semiconservative DNA synthesis that works by reducing the deoxyribonucleotide pools through its inhibition of ribonucleoside diphosphate reductase (33). It has been reported to result in the appearance of DNA strand breaks (40), and perhaps the repair of such damage is responsible for the elevated [3H]dThd incorporation occasionally observed in hepatocyte cultures exposed to high concentrations of it. ara-C is an arabinosyl analog of cytidine and, upon incorporation into DNA, stops chain growth (15). It is effective in both bacterial and animal cells and has recently been used to inhibit [3H]dThd incorporation in controls in the study of Karran et al. (11) on DNA repair in developing chick neural retina cells. However, these workers apparently did not detect an inhibition of repair by ara-C as was the case in the present study. This was most likely because 10 μM ara-C was sufficient to inhibit [3H]dThd incorporation by 98.9% in their chick retina cell system, whereas higher levels were required to inhibit incorporation in the hepatocytes. In addition, their cultures were exposed to the inhibitor for a shorter time. EB is an intercalating agent (15) capable of inhibiting DNA replication in bacterial and animal cells. At low doses, 1 μg/ml (2.5 μM), it appears to inhibit replication of closed circular supercoiled DNA such as found in mitochondria preferentially (27, 30, 32). In this study, EB was found to inhibit [3H]dThd incorporation in controls at the relatively low dose of 1 μg/ml; however, like ara-C, it too appeared to inhibit repair synthesis, particularly at the high dose. These inhibitor studies suggested to us that, possibly, the [3H]dThd incorporation into DNA in our control cultures was in fact not entirely nuclear, but rather also due to mitochondrial DNA synthesis. Preliminary cell fractionation studies have supported this hypothesis and also indicate that the UV-induced repair response reflects enhanced [3H]dThd incorporation only in the nuclear fraction. Additional work is under way to confirm these preliminary results.

The results obtained with UV indicate that the majority (93 ± 3%) of the cultured hepatocytes respond to the DNA damage it causes in a UV-dose-dependent manner. DNA repair can persist in the cells for up to 24 hr following irradiation, depending upon the dose used. Additional studies are required to determine the efficiency with which the hepatocytes remove the thymine dimers formed and how this correlates with the duration of the repair response.

The results obtained with AAF indicate that 69 ± 4% of the cultured hepatocytes respond to AAF treatment by carrying out repair synthesis. The repair synthesis that ensues following AAF treatment is dose dependent as is the duration of the repair response. These results suggest that the majority of the cultured hepatocytes retain the ability to metabolize this prohepatocarcinogen to forms capable of damaging DNA, thus eliciting a repair response. These findings are in basic agreement with the autoradiographic results reported by Williams (45). In contrast to the response with AAF, N-OH-AAF, one of its proximate derivatives is much more effective at stimulating repair synthesis. Doses of N-OH-AAF at one-tenth to one-hundredth those of AAF result in repair responses comparable to those seen with AAF. The ultimate carcinogenic derivative of AAF, namely, N-Ac-AAF, has also been shown to induce repair synthesis in these cells and appears to be as effective as the N-OH-AAF at equal doses.

It is probable that N-OH-AAF is so much more effective than AAF in this cell system because its metabolism to an ultimate carcinogenic form does not depend upon the microsomal mixed function oxidases and cytochrome P-450. It is known from our own unpublished studies and those of others (2, 6, 23) that P-450 decreases rapidly and dramatically with time of culture of the hepatocytes. However, alteration of the culture conditions (Refs. 4 and 24; J. D. Yager, Jr., and B. D. Roebuck, unpublished observations) can prevent the rapid decay in drug metabolism generally seen, and future work in our laboratory will explore the effect of such alterations on carcinogen-induced damage and repair in these cells.

The results described above demonstrate the potential of the primary hepatocyte culture system for use in studies on the mechanisms by which carcinogens interact with cells and possibly for use in screening for chemical agents capable of directly or indirectly damaging cellular DNA. Much additional work is necessary to expand the usefulness of this cell system. However, in the face of a careful analytical approach to the response of these cells to various environmental insults and alterations, the system is gradually becoming better characterized and may become extremely valuable in furthering our knowledge of mechanisms of carcinogenesis.

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REFERENCES

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James D. Yager, Jr. and Joseph A. Miller, Jr.


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