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Effect of Culture Conditions, Cell Type, and Species of Origin on the Distribution of Acetylated and Deacetylated Deoxyguanosine C-8 Adducts of *N*-Acetoxy-2-Acetylaminofluorene

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SUMMARY

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Antibodies directed against guanosin-8-yl-acetylaminofluorene have been employed in a sensitive radioimmunoassay able to distinguish between the acetylated and deacetylated deoxyguanosine C-8 adducts of 2-acetylaminofluorene. Most cultured cells (primary BALB/c and Sencar epidermal and fibroblast cells and primary rat epidermal cells and fibroblasts) form ≥90% of the total C-8 adducts as the deacetylated adduct after a 1-h exposure to 10^{-5} M *N*-acetoxy-2-acetylaminofluorene. However, primary rat hepatocytes, exposed to either the activated derivative of the carcinogen for 1 h or the parent compound 2-acetylaminofluorene for 5–24 h, form ≥80% of the C-8 adducts as the acetylated adduct. Treatment conditions were varied in order to alter the levels of binding and proportion of acetylated and deacetylated C-8 adducts formed in primary BALB/c epidermal cells. Cells exposed in medium 199 with 1.2 mM Ca^{2+} in the presence of 10% fetal calf serum (standard culture conditions) bind 100–200 fmol/μg DNA and form 3% of the C-8 adducts as acetylated. Exposure to carcinogen in the absence of serum increased binding levels two- to fivefold with 8% of the C-8 adducts in the acetylated form. The addition of 10^{-5} M ethidium bromide (a DNA intercalating agent), 10^{-3} M sodium butyrate (a deacetylase inhibitor), or 10^{-5} M harman (which augments 2-acetylaminofluorene mutagenesis) failed to change the level or pattern of binding. Carcinogen exposure under culture conditions which select for the growing fraction of epidermal cells (0.09 mM Ca^{2+}) also failed to alter binding. However, a 20-min pretreatment with 10^{-5} M paraoxon under standard culture conditions resulted in the inhibition of 99% of the binding and formation of deacetylated adduct. The small amount of C-8 adduct formed was acetylated. These results indicate that the extent and pattern of 2-acetylaminofluorene DNA binding vary in different cell types and can be altered by variations in exposure conditions. By these means it should be possible to investigate the biological importance of individual adducts.

INTRODUCTION

If DNA damage is an essential component of the process by which a cell becomes neoplastic, it would seem appropriate to investigate the biological consequences of specific types of damage in order to form a hypothesis on requirements for initiation of carcinogenesis. Many chemical carcinogens form several types of DNA adducts and the proportions of each can vary in different biological systems (1). The experimental carcinogen, 2-acetylaminofluorene (2-AAF),[1] presents one of the less-complicated compounds in this regard since two DNA adducts [deoxyguanosin-8-yl-acetylaminofluorene (dG-8-AAF) and deoxyguanosin-8-yl-aminofluorene (dG-8-AF); structures in Ref. 2] comprise approximately 90% of all DNA-bound products *in vivo*, while a minor adduct, 3-deoxyguanosin-N^2-yl-acetylaminofluorene (dG-N^2-AAF; structure in Ref. 3) has been observed to form about 5–10% of all DNA-bound products (2–4).

[1] Abbreviations used: RIA, radioimmunoassay; 2-AAF, 2-acetylaminofluorene; N-Ac-AAF, *N*-acetoxy-2-acetylaminofluorene; dG-8-AAF, *N*-(deoxyguanosin-8-yl)-acetylaminofluorene; dG-8-AF, *N*-(deoxyguanosin-8-yl)-aminofluorene; dG-N^2-AAF, 3-(deoxyguanosin-N^2-yl)-acetylaminofluorene; DMSO, dimethylsulfoxide; FCS, fetal calf serum; ultrasensitive enzyme-linked RIA, USERIA.

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We have previously reported the production of antiserum recognizing dG-8-AAF and dG-8-AF, which does not cross-react with DNA, nucleosides, or the carcinogen alone (5, 6). When employed in a radioimmunoassay (RIA), as little as 10 fmol/μg DNA of each adduct can be readily measured in hydrolyzed DNA from cells or tissues exposed to either 2-AAF or one of its activated derivatives. This capability has allowed us to extend our observations to the study of the natural variability which may exist in the extent and pattern of binding among cells from specific tissues and varying species of origin. In addition, we sought conditions which could alter the extent and pattern of binding in a particular cell type in order to correlate these alterations to biological effects of the carcinogen.

MATERIALS AND METHODS

Cell culture. Sencar mice (bred for sensitivity to skin carcinogenesis by R. K. Boutwell from STS Rockwell × CD1 Charles River mice) (7) were obtained from Dr. Thomas Slaga of the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tenn. Keratinocytes from BALB/c and Sencar mice and Fischer rats were prepared from newborn animals as described previously for BALB/c epidermal cells (8). Cultures of dermal fibroblasts were established from the dermal layer of the same newborn animals as described (9). Primary cultures of rat hepatocytes, previously shown to activate 2-AAF to a DNA damaging metabolite (10), were prepared and grown according to procedures established in the laboratory of Dr. G. M. Williams (11).

Exposure to carcinogen and other agents. Nearly confluent cells grown in 150-mm dishes (Falcon Plastics, Oxnard, Calif.) were exposed to 10^{-5} M 2-AAF or N-Ac-AAF (obtained from the National Cancer Institute Chemical Repository, IIT Research Institute, Chicago, Ill.) in 0.4% DMSO (packed under nitrogen, Pierce Chemical Co., Rockford, Ill.) for 1 h (N-Ac-AAF) or 5–24 h (2-AAF) in a medium containing 10% fetal calf serum (FCS). Epidermal cells and dermal fibroblasts were routinely cultured in medium 199 containing 1.2 mM Ca^{2+}; low-calcium medium, when utilized, contained 2% Chelex serum and 0.09 mM Ca^{2+} (12). Exposure to other chemical agents began 20 min before 10^{-5} M N-Ac-AAF and continued throughout the exposure to carcinogen. These included 10^{-5} M paraoxon (diethyl-*p*-nitrophenyl phosphate) and 10^{-5} M harman (both from Aldrich Chemical Co., Milwaukee, Wis.) and 10^{-5} M ethidium bromide and 10^{-3} M sodium butyrate (both from Sigma Chemical Co., St. Louis, Mo.). Harman and norharman (Aldrich Chemical CO) were administered to primary rat hepatocytes at a concentration of 10^{-4} M simultaneously with 10^{-5} M 2-AAF for a period of 5 h. DNA from harvested cells was prepared on CsCl gradients (13), dialyzed against deionized water, and hydrolyzed with Sl nuclease (14) before assay by RIA.

Radioimmunoassay. The specificity of the G-8-AAF antiserum as well as the preparation of labeled and unlabeled standard compounds for RIA have been described previously (6, 15). Assays have all been performed using sequential saturation procedures or nonequilibrium conditions (16). Procedures for the use of linear regression analysis to determine the percentage acetylation in mixtures containing >90% of the C-8 adducts in the deacetylated form have been described in detail (17). Ultrasensitive enzyme-linked RIA (USERIA; 18) has been employed to measure C-8 adducts in BALB/c epidermal cells exposed to paraoxon and N-Ac-AAF. The details of this procedure have been submitted for publication.[2]

Paraoxon toxicity in BALB/c epidermal cells. Semiconfluent BALB/c epidermal cells were exposed to either DMSO alone, 10^{-5} M paraoxon, 10^{-5} M N-Ac-AAF, or 10^{-5} M paraoxon and 10^{-5} M N-Ac-AAF. The medium was removed at 1 h and fresh medium containing either 2 μCi/ml of [3,4,5-(N)^{3}H]leucine (New England Nuclear, Boston, Mass.; 85 Ci/mmol) or 4 μCi/ml of [5-^{3}H]uridine (New England Nuclear; 28 Ci/mmol) was added. After incubation for 3 h, cell monolayers were washed four times with cold 0.2 N perchloroacetic acid and dissolved in 1 N NaOH. Separate aliquots were taken for radioactivity and protein determinations (19). Incorporation is expressed as counts per minute per microgram protein.

RESULTS

Comparison of acetylated and deacetylated C-8 adducts in DNA of primary hepatocytes and epidermal cells. In earlier experiments we demonstrated antibodies against both dG-8-AF and dG-8-AAF in serum from rabbits immunized against G-8-AAF. These two antibody activities produce similar RIA standard curves in assays which compete [^{3}H]G-8-AAF vs dG-8-AAF or [^{3}H]G-8-AF vs dG-8-AF (Fig. 1A, ○——○ and ●——●, and Ref. 5). In addition, previous data have shown that the percentage of inhibition of a mixture of dG-8-AAF and dG-8-AF will be the sum of the percentages of inhibition of each component in the mixture (5). An initial indication of the proportion of acetylated and deacetylated C-8 adducts in an unknown 2-AAF-modified DNA can then be obtained by assaying the same amount of the unknown in assays which utilize [^{3}H]G-8-AAF and [^{3}H]G-8-AF as tracer. Data from several of these types of experiments are shown in Table 1. In all cases DNA from N-Ac-AAF-exposed BALB/c cells gave much higher percentages of inhibition in the assay with [^{3}H]G-8-AF, while DNA from AAF-exposed hepatocytes gave no inhibition in this assay. The last column in Table 1 lists the difference (Δ) percentage inhibition obtained when the percentage of inhibition observed in the [^{3}H]G-8-AAF assay was subtracted from that observed in the [^{3}H]G-8-AF assay. For a standard mixture of 5% dG-8-AAF:95% dG-8-AF, the Δ% inhibition observed in several experiments was 25 ± 5% with 2–4 pmol of C-8 adduct. Table 1 shows that the observed Δ% inhibition values for BALB/c DNA were 26–35%, indicating that these DNAs contained approximately 5% acetylated and 95% deacetylated C-8 adducts.

Further information concerning the nature of the adducts on DNA can be obtained from experiments in which *increasing concentrations* of hydrolyzed DNA are assayed with both acetylated and deacetylated radioactive tracers (Fig. 1B). The profile observed with BALB/c DNA assayed against [^{3}H]G-8-AF was a complete in-

[2] I. C. Hsu, M. C. Poirier, S. H. Yuspa, R. H. Yolken and C. C. Harris, submitted for publication.

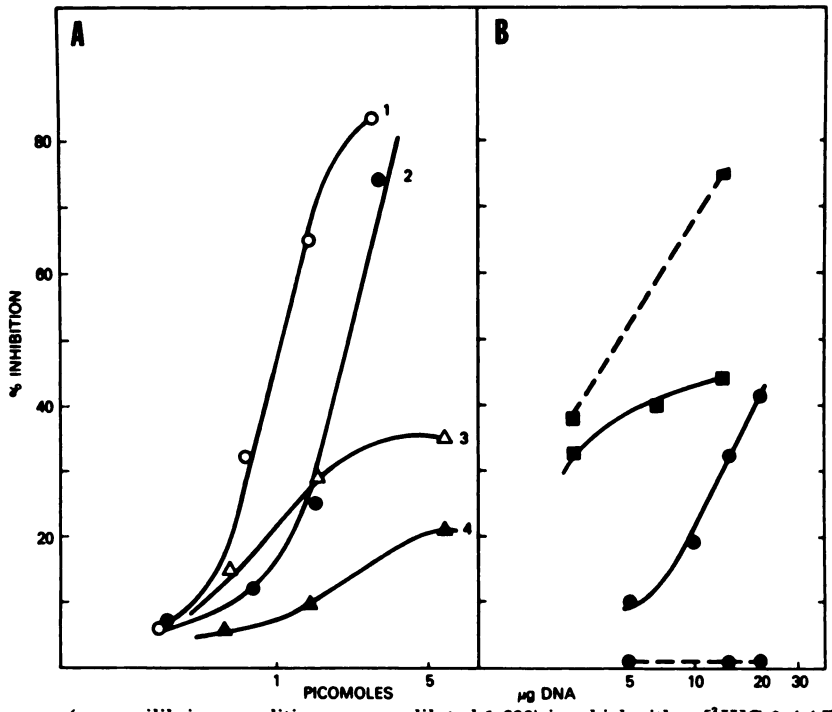

FIG. 1A. *RIA standard curves (nonequilibrium conditions, serum diluted 1:600) in which either [^{3}H]G-8-AAF (open circles and triangles) or [^{3}H]G-8-AF (closed circles and triangles) was competed as trace against dG-8-AAF (curves 1 and 4) or dG-8-AF (curves 2 and 3) as inhibitor*

Curve 1 is [^{3}H]G-8-AAF and dG-8-AAF. Curve 2 is [^{3}H]G-8-AF and dG-8-AF. Curve 3 is [^{3}H]G-8-AAF and dG-8-AF. Curve 4 is [^{3}H]G-8-AF and dG-8-AAF.

FIG. 1B. *RIA profiles of increasing concentrations of hydrolyzed DNA from N-Ac-AAF-exposed BALB/c epidermal and rat hepatocyte cells assayed with both [^{3}H]G-8-AAF* (——) *and [^{3}H]G-8-AF* (- - -) *under the same experimental conditions as in Fig. 1A*

Curves are as follows: BALB/c DNA with [^{3}H]G-8-AF (■- -■) and [^{3}H]G-8-AAF (■——■); hepatocyte DNA with [^{3}H]G-8-AAF (●——●) and [^{3}H]G-8-AF (●- -●).

hibition curve (Fig. 1B, ■- -■, similar to Fig. 1A, curve 2). In the [^{3}H]G-8-AAF assay, however, the same sample of DNA saturated at about 45% inhibition (Fig. 1B, ■——■, similar to Fig. 1A, curve 3). Thus the profiles for hydrolyzed BALB/c DNA behave as if most of the C-8 modification on the BALB/c DNA were dG-8-AF (5). Profiles for increasing concentrations of hepatocyte DNA were very different from profiles of epidermal DNA. Quantities of between 5 and 19 μg of DNA did not inhibit in the [^{3}H]G-8-AF assay (Fig. 1B, ●---●), but a linearly increasing profile (up to 80% inhibition in some experiments) was observed with [^{3}H]G-8-AAF (Fig. 1B, ●——●). Calculating from the amount of hepatocyte DNA assayed with [^{3}H]G-8-AF (19 μg) and the dG-8-AF standard curve in which 0.3 pmol of dG-8-AF standard gave 7.5% inhibition while the hepatocyte DNA gave no inhibition, at least 80% of the C-8 adduct would appear to be acetylated.

Quantitation of percentage of acetylation in DNAs in which >90% of the C-8 adducts are deacetylated. A more precise method for determining the residual percentage of acetylated C-8 adduct in DNAs >90% deacetylated has been described (17) and will be outlined here. Previous experiments have shown that standard dG-8-AF assayed in competition with [^{3}H]G-8-AAF at a serum dilution of 1:600 saturates at about 40% inhibition (Fig. 1A, curve 3). The plateau at the maximum percentage of inhibition can be increased if a small amount of dG-8-AAF is added when the total amount of C-8 adduct remains the same (in this case, 6 pmol; Fig. 2A). Presumably this is due to the recognition of dG-8-AAF by a cross-reacting population of antibodies. When a total of 6 pmol of adduct composed of mixtures of 5 and 8.7% dG-8-AAF (containing 95 and 91.3% of dG-8-AF, respectively) was assayed on 10 different occasions, the increase in percentage of inhibition for each mixture was a constant increment higher than the dG-8-AF saturation in the same experiment (Fig. 2A, compare ○——○, ▲——▲, and △——△ at 6 pmol). The line drawn in Fig. 2B is a single regression line representing a composite of the 10 experiments. An equation (see Fig. 2B legend) defining the regression line (20) can be employed to calculate the percentage dG-8-AAF in an unknown sample providing one determines the percentage of inhibition at 6 pmol of modification for the unknown sample, the dG-8-AF standard, and standard 5 and 8.7% dG-8-AAF mixtures simultaneously. Values for percentage of acetylation determined in four separate experiments with different preparations of BALB/c epidermal cells exposed to N-Ac-AAF were 3.24, 3.63, 3.20, and 3.06. The mean ± confidence limits (20) for this DNA was 3.3 ± 0.7%.

Species and tissue differences in formation of acetylated and deacetylated C-8 adducts after exposure of cultured cells to N-Ac-AAF. The differences in relative proportions of acetylated and deacetylated C-8 adducts in the DNA of mouse epidermal cells and rat hepatocytes led to an investigation of other cell types in an attempt

TABLE 1

Percentage of inhibition values for DNAs from N-Ac-AAF-exposed BALB/c epidermal cells and rat hepatocytes assayed by RIA with [^{3}H]G-8-AAF and [^{3}H]G-8-AF

Cells	μg DNA in RIA	Total pmol C-8 adduct	% Inhibition [^{3}H]G-8-AAF	% Inhibition [^{3}H]G-8-AF	Δ% Inhibition[a]
BALB/c epidermal	6.6	1.6	34	60	26
	12.0	2.4	45	74	29
	13.3	4.0	42	77	35
	(Predicted for 5% acetylated)[b]	(4.0)	(45)	(76)	(31)
Rat hepatocytes	13.6	2.5	30	0	−30
	17.0	1.7	20	0	−20
	14.2	0.5	27	0	−27
	(Predicted for 80% acetylated)	(0.5)	(24%)	(0%)	(−24)

[a] Δ% Inhibition = (% inhibition, [^{3}H]G-8-AF assay) − (% inhibition, [^{3}H]G-8-AAF assay), where the assays were performed simultaneously on the same quantity of hydrolyzed DNA. A mixture of standard compounds containing 5% dG-8-AAF and 95% dG-8-AF, assays with a Δ% inhibition of 25 ± 5% for amounts of total C-8 adduct ≥2 pmol.

[b] An estimate of the proportion of acetylated and deacetylated C-8 adduct in the epidermal DNA can be obtained by calculating the percentages of inhibition predicted with a particular total modification and a particular set of four standard curves run simultaneously (as in Fig. 1A). For example, for a total of 4 pmol of modification, if 1 pmol were dG-8-AAF and 3 pmol were dG-8-AF (25% dG-8-AAF), the standard curves in Fig. 1A predict values of 82% inhibition in the [^{3}H]G-8-AAF assay and 75% inhibition in the [^{3}H]G-8-AF assay. If, however, the mixture was 5% dG-8-AAF:95% dG-8-AF and the total modification 4 pmol, one would predict 45% inhibition in the [^{3}H]G-8-AAF assay and 76% in the [^{3}H]G-8-AF assay. This is close to observed values.

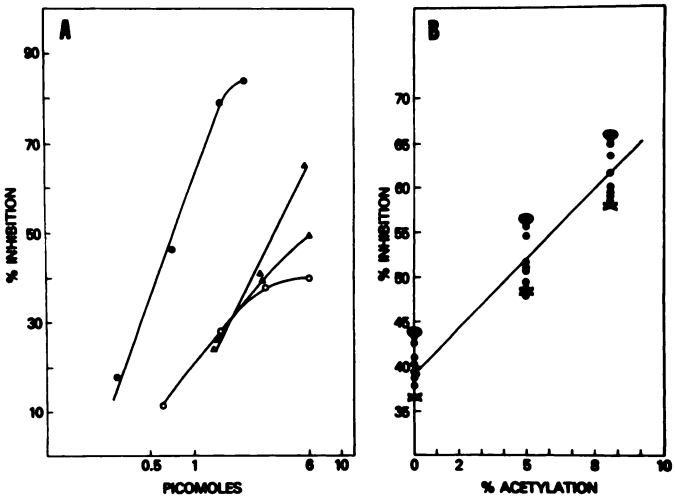

FIG. 2A. *RIA standard curves (nonequilibrium conditions, serum diluted 1:600) in a competition assay with [^{3}H]G-8-AAF and a series of mixtures of nonradioactive dG-8-AAF and/or dG-8-AF*

The curves are as follows: 100% dG-8-AAF, ●——●; 100% dG-8-AF, ○——○; 8.7% dG-8-AAF:91.3% dG-8-AF, Δ——Δ; 5% dG-8-AAF:95% dG-8-AF, ▲——▲.

FIG. 2B. *Percentage inhibition (ordinate) observed in the RIA at 6 pmol of total adduct against percentage acetylation (abscissa) in standard mixtures of 0, 5, and 8.7% dG-8-AAF*

The trace was [^{3}H]G-8-AAF. The regression line is for the average of 10 experiments. The points with the open circles are from the experiment with the highest observed percentages inhibition. The points with the Xs are from the experiment with the lowest observed percentage inhibition. This variability is due to slight differences in the dilution of the antiserum as well as RIA interassay variability. One can estimate the unknown percentage dG-8-AAF (acetylation) (19) using $Z = \bar{X} + (Y - \bar{Y})/b$, where Z is the estimate of the percentage dG-8-AAF in the unknown mixture, Y is the percentage inhibition for the unknown mixture at 6 pmol total modification, $\bar{Y}$ is the average percentage inhibition for 6 pmol of the three standard mixtures assayed simultaneously with the unknown mixture, $\bar{X}$ is the average percentage dG-8-AAF of the standard mixtures, 0, 5, and 8.7%, used in the assays (4.56), and b is the common slope, 2.46.

to determine whether or not the differences were species-specific or tissue specific. Cultured cells were exposed to 10^{-5} M N-Ac-AAF for 1 h, DNAs prepared on CsCl gradients, and RIAs performed as described. DNA was isolated from six different cell types after carcinogen exposure and was assayed at the same concentration in both [^{3}H]G-8-AAF and [^{3}H]G-8-AF RIAs. The results are presented in Table 2. DNA from keratinocytes of BALB/c and Sencar mice and Fisher rats, as well as dermal fibroblast DNA from BALB/c mice and Fisher rats, give a percentage of inhibition considerably higher in the [^{3}H]G-8-AF RIA than the [^{3}H]G-8-AAF RIA, indicating that most of the C-8 adducts formed were deacetylated.

In subsequent experiments increasing concentrations of DNA from these cell types were assayed against the [^{3}H]G-8-AAF tracer, and data from these profiles subjected to the linear regression analysis (17). Data from two or more experiments are presented in Table 2. By this method of analysis all of the highly deacetylated DNAs give values between 3.3 and 9.0% acetylated. While the BALB/c mouse fibroblasts were not subjected to linear regression analysis, the Δ% inhibition (Table 2) would suggest a 5% acetylation of C-8 adducts (see footnotes, Table 1). By a semiquantitative measurement previously described (5), we have reported that C-8 adducts formed in BALB/c and Sencar dermal fibroblasts and human dermal fibroblasts are at least 90% deacetylated. Therefore, of eight different primary cell types studied from three species, only rat hepatocytes form a high proportion of acetylated C-8 adducts. The high degree of acetylated C-8 adduct seen with rat hepatocytes does not appear to be a function of the medium since BALB/c epidermal cells grown in Williams E medium (hepatocyte medium) form the same adducts as in medium 199.

Formation of acetylated and deacetylated C-8 adducts in mouse epidermal cells exposed to N-Ac-AAF under differing treatment conditions. Several chemicals and treatment conditions were selected as likely to change the extent and type of binding observed in BALB/c epidermal cells. Under standard culture conditions (10^{-5} M N-Ac-AAF for 1 h in medium 199 with 10% FCS and 1.2 mM Ca^{2+}), binding was between 75 and 250 fmol/μg DNA, and the DNA was 3.3% acetylated (Table 3, line 1). When cells were exposed in the absence of FCS, a two- to fivefold increase in binding was observed and the percentage acetylation increased from 3.3 to 8% (Table 3). In the latter instance cells were washed two times with serum-free medium 45 min before exposure to carcinogen, whereas under standard conditions fresh medium is last applied 48 h prior to carcinogen exposure. To rule out an effect of fresh medium, cells were exposed in serum-free medium which was placed on the cultures either 48 h or 45 min before the time of carcinogen

TABLE 2

Formation of acetylated and deacetylated C-8 adducts in cells from different species and tissues

Cells	Δ% Inhibition[a]	% Acetylation[b]
BALB/c epidermal	35	3.3 ± 0.3
	29	
	26	
Sencar epidermal	14	8.0 ± 0.3
	20	
BALB/c fibroblast	26	—
	26	
Rat epidermal	19	9.0 ± 0.3
	11	
Rat fibroblast	30	5.4 ± 0.3
	33	
	34	
Rat hepatocyte	−20	≥80
	−27	
	−28	

[a] See footnote *a*, Table 1.

[b] Mean ± range of two or more experiments determined by linear regression analysis applies to all values except rat hepatocyte.

treatment. The value for percentage of acetylation did not change significantly, indicating that the serum, rather than an aging effect of the medium, was responsible for the difference in deacetylation. The pattern of binding was not altered when epidermal cells were cultured under low-calcium conditions (including 2% Chelex serum). Low-calcium conditions select for the proliferating keratinocyte population which is likely to represent the true basal cell population (12).

A dramatic decrease in total binding was observed when the cells were pretreated for 20 min with 10^{-5} M paraoxon (diethyl-*p*-nitrophenyl phosphate), a compound previously shown to inhibit the enzyme responsible for the deacetylation of N-Ac-AAF (21–24). In the presence of paraoxon, binding was generally less than 1% of the control values (Table 3) and the C-8 adducts appeared to be 100% acetylated. This determination was made by USERIA[2] (18), because the binding levels were below the limits of detectability for the conventional RIA. It was of interest to determine at what dose level paraoxon no longer exerted an inhibitory effect on adduct formation. In these studies the conventional RIA was employed to measure C-8 adducts, the lower limits of sensitivity being about 5 fmol/μg DNA. In the absence of paraoxon and the presence of 2×10^{-5} M N-Ac-AAF, the levels of C-8 adducts formed in three experiments on different occasions were almost identical (160–174 fmol/μg DNA). When cells were preincubated with 10^{-5} to 10^{-8} M paraoxon, no adducts were detectable by the conventional RIA, but binding was observed (108 fmol/μg DNA) when the carcinogen was administered in the presence of 10^{-9} M paraoxon. The possibility remained that a generalized toxicity was responsible for the loss of binding capacity, although BALB/c cells in the presence of 10^{-5} M paraoxon did not show any morphological changes during several days of observation. Experiments in which the incorporation of [^{3}H]leucine or [^{3}H]uridine into acid-insoluble material was followed for 3 h after exposure of BALB/c cells to either paraoxon or N-Ac-AAF or both showed that these indicators of cellular metabolism were unaffected by paraoxon (Table 4). There is, however, about a 25% decrease in macromolecular synthesis (i.e., toxicity) due to the carcinogen alone, which is not influenced by paraoxon.

TABLE 3

Variations in acetylated and deacetylated C-8 deoxyguanosine adducts in BALB/c epidermal cells exposed to 10^{-5} M N-Ac-AAF under differing treatment conditions[a]

Exposure	Total binding	% Acetylated C-8 adducts[b]
	% of control	
N-Ac-AAF (10% FCS)	100	3.3 ± 0.3
N-Ac-AAF (no FCS)	350 ± 150	8.6 ± 0.2
N-Ac-AAF (0.09 mM Ca^{2+}, 2% Chelex serum)	160 ± 20	2.6 ± 0.8
N-Ac-AAF (1.2 mM Ca^{2+}, 2% Chelex serum)	125 ± 25	2.3 ± 0.4
10^{-5} M Paraoxon + N-Ac-AAF	1 ± 0.75	100
10^{-5} M Ethidium bromide + N-Ac-AAF	85 ± 30	2.8
10^{-3} M Butyrate + N-Ac-AAF	88 ± 15	3.5
10^{-5} M Harman + N-Ac-AAF	110 ± 5	1.8

[a] Mean ± range for two or more experiments.

[b] Determined by linear regression analysis.

Further experiments were performed using other agents with the anticipation that either the overall binding levels or the nature of the adducts bound might be altered relative to the controls. Ethidium bromide, a known intercalator of DNA (25), was chosen in an attempt to alter the structure of DNA at the time of exposure to N-Ac-AAF. Sodium butyrate, an inhibitor of histone deacetylation (26), was chosen to investigate the possibility that histone deacetylases might influence the form of bound carcinogen. Finally, harman and norharman were chosen because they enhance the mutagenicity of both direct-acting and metabolism-requiring 2-AAF derivatives in *Salmonella* (27). BALB/c epidermal cells were pretreated with either 10^{-5} M ethidium bromide, 10^{-3} M sodium butyrate, or 10^{-5} M harman for 20 min before exposure to 10^{-5} M N-Ac-AAF. These doses were chosen because preliminary experiments showed that they induced no morphological changes in the cells, yet were in the reported effective dose range. The results are shown in Table 3. The overall levels of binding and percentages acetylated C-8 adducts observed in the pres-

TABLE 4

Effect of paraoxon, N-Ac-AAF, or both on [^{3}H]uridine and [^{3}H]-leucine incorporation into macromolecules in BALB/c mouse keratinocytes

	[^{3}H]Uridine		[^{3}H]Leucine	
	cpm/μg protein	% Incorporation	cpm/μg protein	% Incorporation
No treatment	567	100	90	100
10^{-5} M Paraoxon	544	96	97	100
10^{-5} M N-Ac-AAF	435	77	66	73
10^{-5} M Paraoxon + 10^{-5} M N-Ac-AAF	456	80	58	65

ence of these agents were not significantly different from those observed in the controls.

Since rat liver microsomes have been the most commonly employed activating system in *Salmonella* mutagenesis studies in which harman and norharman have enhanced mutagenesis, we investigated binding of 2-AAF to DNA in primary rat hepatocytes exposed for 5 h to 10^{-4} M harman or norharman simultaneously with 10^{-5} M 2-AAF. There was no significant increase in the formation of C-8 adducts when 2-AAF exposure occurred in the presence of harman or norharman (data not shown).

DISCUSSION

Previous data (5, 6, 15, 17) have indicated that both acetylated and deacetylated C-8 deoxyguanosine adducts of 2-AAF can be detected by G-8-AAF antiserum, although activity directed against the deacetylated adduct forms a minor portion of the total antibodies. This serum contains no antibody activity against the minor adduct, dG-N^2-AAF. It is possible, by assaying increasing amounts of DNA with either [^{3}H]G-8-AAF or [^{3}H]G-8-AF as trace, to distinguish and quantitate the two adducts in an unknown mixture. Such assays, in combination with standard curves of known mixtures, yield differences in percentage of inhibition which can be used to estimate the relative portion of each adduct in the unknown mixture. The utility of these measurements is well demonstrated with AAF-substituted DNA from BALB/c keratinocytes and rat hepatocytes, since they represent both possible ends of the spectrum: The former is highly deacetylated (97%) and the latter is highly acetylated (≥80%). These results are confirmed by the data in Fig. 1B and Tables 1 and 2. A precise value for relative proportions can be obtained for DNAs >90% deacetylated by a linear regression equation (15) which takes advantage of the fact that the deacetylated adduct saturates at a low percentage of inhibition when assayed against [^{3}H]G-8-AAF. At the present time, since both antibody activities are in the same antiserum, the determination of acetylated and deacetylated adducts in an unknown mixture requires multiple assays with each labeled adduct. The data from each assay method confirm and add to the validity of the final result. However, these determinations would be facilitated by the production of monoclonal antibodies against each adduct which would provide a separate assay for each adduct in a mixture.

It would be useful to determine the biological effects of specific adducts in studies concerned with DNA binding, repair, mutagenesis, and transformation. As a prelude to this we have followed two lines of investigation: First, we have simply compared the formation of adducts in several different types of cells under standard conditions of cell confluency, culture medium, serum, and method of carcinogen exposure; second, we have manipulated conditions to alter the extent of binding and the type of adduct formed in mouse keratinocytes.

By earlier observations using a semiquantitative technique (5), we established that about 97% of the C-8 deoxyguanosine adducts formed on the DNA of BALB/c epidermal cells exposed to 10^{-5} M N-Ac-AAF for 1 h in a medium with 10% FCS are deacetylated. This finding was subsequently confirmed by a study in which high-pressure liquid chromatography revealed only one major adduct, dG-8-AF, in DNA from BALB/c epidermal cells exposed to [^{14}C]-N-Ac-AAF.[3] We were somewhat surprised that the degree of deacetylation was so high since Kriek reported earlier that about 70% of the total 2-AAF adducts in rat liver DNA are dG-8-AF and 20% are dG-8-AAF (3). More-recently, Cerutti has found variable amounts of deacetylated C-8 adduct in the DNA of cultured cells but his values have not been as high as 90% (28). The high degree of deacetylation observed in the DNA of cultured cells in this laboratory does not appear to be a function of the medium since cells grown in three different media exhibit the same characteristic. The phenomenon is not species specific since the present studies confirm that both rat and mouse fibroblasts showed the same pattern of adduct formation and previous data (5) indicate the formation, primarily, of deacetylated C-8 adducts in human skin fibroblasts. Thus only the hepatocytes form a high degree of acetylated C-8 adducts. With the exception of rat liver (3), for which 2-AAF is carcinogenic (29), data on *in vivo* formation of adducts in corresponding tissues are not available. The primary rat hepatocytes however, do not resemble other cultured cells or rat liver *in vivo*, with respect to C-8 adduct formation. Preliminary evidence with these cells shows that they actively *N*-acetylate aromatic amines.[4]

Of the several different agents and culture conditions employed in these studies to alter the pattern of N-Ac-AAF binding to mouse keratinocytes, only two, serum and paraoxon, appear potentially useful. The fact that treatment in the absence of serum yields higher binding levels is not surprising since serum proteins provide nucleophilic targets which most certainly lower the effective carcinogen dose. In addition, our results indicate that serum factors are responsible for the formation of some deacetylated carcinogen.

The data obtained with paraoxon are the most remarkable and potentially useful for future studies. Pretreatment with 10^{-5}–10^{-8} M paraoxon almost completely inhibits formation of the deacetylated C-8 adduct and the small amount of adduct which does form (1%) is only acetylated. Preliminary results suggest that levels of binding measurable by RIA can be obtained by increasing the concentration of N-Ac-AAF in conjunction with the same dose of paraoxon; under these conditions the bound adduct is primarily acetylated. Thus, the formation of deacetylated C-8 adducts in BALB/c epidermal cells under standard culture conditions would seem to proceed primarily through the microsomal deacetylase enzyme which is inhibited by paraoxon (24). The inhibition of this enzyme by paraoxon has been previously shown to reduce mutagenesis in bacterial assays (23). This would infer that the same DNA-bound products important for mutagenesis are also the primary adducts observed upon exposure of BALB/c keratinocytes to N-Ac-AAF. The capability to manipulate the nature and extent of binding of 2-AAF to DNA could provide a way of determining the effect of each adduct individually on transformation in a quantitative *in vitro* assay. Such a model is currently

[3] These studies were in collaboration with F. A. Beland.

[4] C. A. McQueen and G. M. Williams, personal communication.

being developed in this laboratory (30). At the present time, by proper manipulation of experimental conditions, it is possible to obtain cells exposed to N-Ac-AAF which have few (<1 fmol/μg DNA) C-8 adducts on DNA or many (100–500 fmol/μg DNA) C-8 adducts, 97% of which are deacetylated or 100% of which are acetylated. The application of these procedures to assays for malignant transformation *in vitro* should provide insight into the biological effects of specific adducts.

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