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GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC STUDY OF DEACETYLATION AND OXIDATION OF 2-ACETYLAMINOFLUORENE BY RAT LIVER EPITHELIAL CELL LINES UPON COCARCINOGEN INDUCTION

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SUMMARY

Cell line cultures from postnatal and adult rats were incubated with 5–100 μmol/l [9-^{14}C]-2-acetylaminofluorene. On incubation of 10 μmol/l, ring-hydroxylated metabolites, expressed as nmol hydroxy-2-acetylaminofluorene (OH-2-AAF)/mg cell protein/24 h, were 9-OH- 1.28 ± 0.37, 7-OH- 1.08 ± 0.28 and 5-OH- 0.30 ± 0.08, and deacetylated 2-AAF (2-AF) 1.20 ± 0.18. For 5, 10, 50 and 100 μmol/l 2-AAF, the total production of OH-2-AAF (same units) and 2-AF (%) were, respectively, 0.86 (0%), 3.86 (35%), 17.8 (60%) and 35.03 (89%). On preincubation with phenobarbital (BP) or 3-methylcholanthrene (3-MC) and then incubation of 10 μmol/l 2-AAF, the total synthesis of OH-2-AAF increased 1.9-fold (PB) and 2.5-fold (3-MC). In addition, four other OH-2-AAF (1-OH-, 3-OH- and two unknown OH-2-AAF) were produced and glucuronidation of all metabolites was induced and amounted to 57% of the total after PB and 75% after 3-MC preincubation. Metyrapone or α-naphthoflavone inhibition of BP or 3-MC, respectively, markedly affected the production of free and conjugated metabolites and, almost completely, the deacetylation of 2-AAF.

INTRODUCTION

The procarcinogen 2-acetylaminofluorene (2-AAF) has often been studied for its carcinogenic and mutagenic properties [1]. This procarcinogen is submitted to activation steps mediated by cytochrome P-450-dependent monooxygenase enzyme systems [2], giving rise to the proximate carcinogen N-hydroxy-2-acetylaminofluorene (N-OH-2-AAF), also submitted to further activation steps, leading to the ultimate reactive forms. On the other hand, 2-AAF is also metabolized into ring-hydroxylated compounds. Ring hydroxylation, conjugation and elimination have been considered to be detoxification processes [3,4]. The formation of active and inactive metabolites in various proportions may constitute

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a factor modifying the carcinogenic activity. Therefore, quantification of metabolites appears to be extremely crucial. Trypsin dissociation of liver tissue allows epithelial cells to be selected, which are considered as prehepatocytes or stem cells [5–7]. They demonstrate a cytoskeleton of epithelial cells containing keratin, which reacts with liver keratin antibodies without any immunological response to vimentin as found in endothelial cells, cultured hepatocytes and hepatoma cells [8]. Primary proliferative cultures and cell lines were obtained in serum-supplemented medium (SSM) or in serum-free medium (SFM) according to Chessebeuf and Padieu [9]. These lines express liver markers: aldolase B in early passages [10], synthesis of albumin, transferrin, α-fetoprotein [11–13] and C_3 component of complement [14], induction of L-tyrosine aminotransferase activity by glucocorticoid incubation [15], epoxide metabolic activation of allylic carcinogens [16], biosynthesis of free and conjugated bile acids [9,17,18] and hepatic metabolism of progesterone [9,19].

We report here investigations on the metabolism of 2-AAF into hydroxylated compounds (OH-2-AAF) and glucuronides, and its deacetylation into 2-aminofluorene (2-AF) in rat liver epithelial cell lines. Assessments were made by gas chromatography–mass spectrometry (GC–MS) and mass fragmentography (MF) using a new method of derivatization of 2-AAF and its metabolites into *tert.*-butyldimethylsilyl (tBDMS) compounds [20,21].

EXPERIMENTAL

Reagents and materials

The labelled procarcinogen [9-^{14}C]-2-acetylaminofluorene ([9-^{14}C]-2-AAF) was purchased from NEN (DuPont de Nemours, Paris, France), 2-AAF and 2-AF from Aldrich-Chimie (Strasbourg, France). N-OH-, N-acetoxy-, 1-OH-, 3-OH-, 5-OH-, 7-OH- and 9-OH-2-AAF were given by the IIT Research Institute (Chicago, IL, U.S.A.). Solvents were obtained from Merck (Darmstadt, F.R.G.) and N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MtBSTFA) and dimethylformamide from Pierce Europe (Oud-Beijerland, The Netherlands). Sodium fluoride, diethyl *p*-nitrophenylphosphate (paraoxon), 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), α-naphthol flavone (α-NF), 3-methylcholanthrene (3-MC), phenobarbital (PB), β-glucoronidase type B-3 and sulphatase type V were purchased from Sigma (St. Louis, MO, U.S.A.). Standards were purified by thin-layer chromatography and controlled by GC–MS. Cell culture reagents were Ham's F10 and WME basal mediums (Gibco BRL, Cergy-Pontoise, France), fetal bovine serum (Biosys, Compiègne, France), dextran T2000 (Pharmacia, Les Ulis, France), bovine serum albumin (Sigma), free fatty acids (Nu-Check-Prep, Elysian, MN, U.S.A.) and gentamicin (Unicet, Levallois-Perret, France).

Incubation conditions

Rat liver cell lines were initiated and cultured as described previously [9,10,20,22] and culture incubations were carried out in serum-free and protein-free medium (SPFM) according to Chessebeuf-Padieu et al. [22]. Incubations were carried out with 2-AAF which was an equal mixture of cold and ^{14}C-labelled

2-AAF, [9-^{14}C]/[9-^{12}C]-2-AAF (50:50, w/w), in 2–4 μl of ethanol per 5 ml of culture medium. Cells were always preincubated for 10 min in precursor-free SPFM. Extraction was done with diethyl ether. The remaining aqueous phases were treated with glucuronidase and sulphatase and then extracted with diethyl ether. Extracts were evaporated, derivatized into tBDMS compounds and analysed by GC–MS according to Diez Ibañez and co-workers [20,21]. Statistical analysis was performed using Student's *t*-test.

Gas chromatography and gas chromatographic–mass spectrometric analysis

Derivatization conditions and the GC method [20,21] were established with a Packard Model 427 gas chromatograph (Packard-Becker, Delft, The Netherlands) equipped with a fused-silica capillary column (25 m × 0.35 mm I.D.) coated with OV-1 stationary phase of 0.22 μm thickness (Spiral, Couternon-Dijon, France). The carrier gas was dry nitrogen at a flow-rate of 20 ml/min and the derivatized sample was injected by means of an all-glass solid injector [20] and monitored with a flame ionization detector. Levels of 2-AAF and metabolites in the extracts of incubated culture media were assessed using a Ribermag Model GC-MS R10-10C quadrupole mass spectrometer with a Sidar 150 computer system (Delsi-Nermag, Rueil-Malmaison, France) coupled to a Varian Model 3700 gas chromatograph (Varian, Orsay, France) equipped with the same capillary column under a 20 ml/min flow of helium. Derivatives were tBDMS compounds obtained as mono-tBDMS and di-tBDMS when using MtBSTFA [21]. This reagent permitted the derivatization of hydroxy groups, arylhydroxy groups and hydroxamic acid, resulting from the action of cytochrome P-450 monooxygenase either on the fluorene ring or on the amide nitrogen, and the remaining amino group, the primary amine of 2-AF or the secondary amine of 2-AAF and arylhydroxy metabolites.

RESULTS AND DISCUSSION

Basal production of metabolites and GC–MS assessment

Incubations in Fischer rat liver epithelial cell cultures, F♀12FR15, was done at confluency in SPFM, which consisted of Ham's F10, 50 mg/l T2000-dextran, 0.76 μmol/l of six free fatty acids and 50 mg/l gentamicin [22]. Using such an SPFM, which sustains normal cell proliferation and growth, the extraction yield was 94%. Fig. 1 shows the gas chromatogram of mono-tBDMS derivatives of standard compounds using 5β-pregnan-3α-ol-20-one as internal standard and Fig. 2 a similar chromatogram but for di-tBDMS derivatives using 4,4′-dihydroxybiphenyl as internal standard. The sensitivity of tBDMS derivatization was 10 and 100 times higher for mono-tBDMS and di-tBDMS, respectively, than that of TMS derivatization [20] when using electron-impact (EI) GC–MS. Fig. 3 shows a gas chromatogram of mono-tBDMS derivatives of 2-AAF and 2-AAF metabolites after a 48-h incubation of 50 μmol/l [9-^{14}C]/[9-^{12}C]-2-AAF without any cocarcinogen induction (see Table I, third row). The ^{14}C/^{12}C isotopic enrichment (50:50) monitored by GC–MS allowed the identification of [9-^{14}C]-2-AAF metabolites by the presence in the mass spectra of m/z and $(m+2)/z$ ions for all

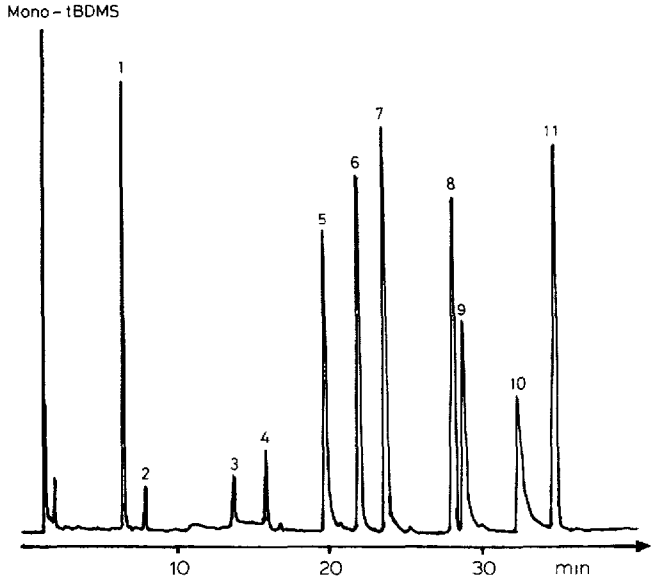

Fig. 1. Gas chromatogram of mono-tBDMS derivatives of 2-AAF and of the seven known metabolites from a standard solution. Pregnanolone (5β-pregnan-3α-ol-20-one) is the internal standard. Peaks: 1=C_{22}; 2=2-AF; 3=2-AAF; 4=N-OH-2-AAF; 5=9-OH-2-AAF; 6=3-OH-2-AAF; 7=1-OH-2-AAF; 8=pregnanolone; 9=5-OH-2-AAF; 10=7-OH-2-AAF; 11=C_{30}.

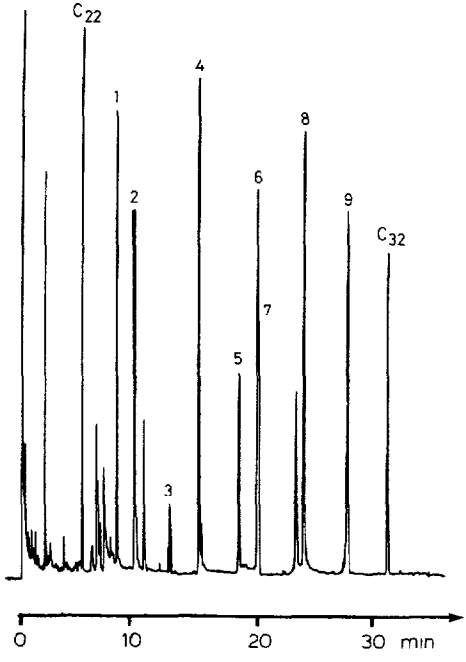

Fig. 2. Gas chromatogram of di-tBDMS derivatives of 2-AAF and metabolites. Peaks: 1=2-AF; 2=2-AAF; 3=N-OH-2-AAF; 4=3,4′-dihydroxybiphenyl (internal standard); 5=9-OH-2-AAF; 6=1-OH-2-AAF; 7=unknown; 8=5-OH-2-AAF; 9=7-OH-2-AAF.

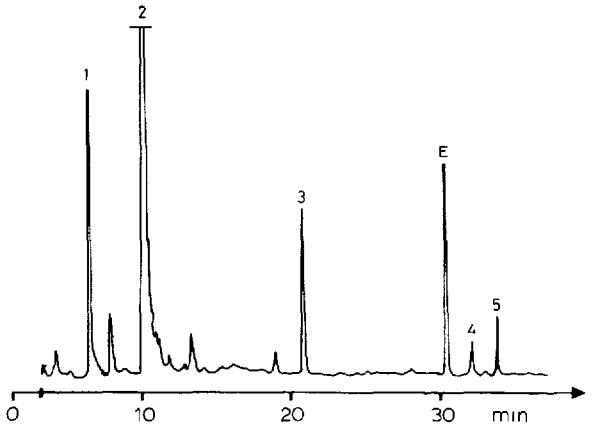

Fig. 3. Gas chromatogram of mono-tBDMS derivatives of 2-AAF and the four metabolites, 2-AF, 9-OH-2-AAF, 5-OH-2-AAF and 7-OH-2-AAF, synthesized by the liver epithelial cell line, WA♀7FR8 from Wistar rat, in conditions of basal production. Peaks: 1=2-AF; 2=2-AAF; 3=9-OH-2-AAF; E=pregnanolone (internal standard); 4=5-OH-2-AAF; 5=7-OH-2-AAF.

TABLE I

PRODUCTION OF FREE METABOLITES OF 2-AAF FROM DIFFERENT AMOUNTS OF 2-AAF INCUBATED FOR 48 h IN THE FISCHER RAT LIVER EPITHELIAL CELL LINE F♀12FR IN CONFLUENT CULTURES AT THE FIFTEENTH PASSAGE

2-AAF[a] (μmol/l)	Metabolite (nmol/mg cell protein/24 h)					2-AF/total (%)
	2-AF	9-OH-2-AAF	5-OH-2-AAF	7-OH-2-AAF	Total	
5	-	0.45±0.12	0.05±0.04	0.36±0.11	0.86±0.10	-
10	1.20±0.18	1.28±0.37	0.30±0.08	1.08±0.28	3.86±0.23	35
50	10.77±0.50	3.89±0.43	0.39±0.19	2.76±0.11	17.81±0.31	60
100	31.32±2.52	0.44±0.31	0.19±0.07	3.08±0.13	35.03±0.76	89

[a]Two confluent cell cultures in 25-cm^2-area flasks were incubated per point ($n=3$).

metabolites and the finding of the original isotopic abundance ratio, except for 9-OH-2-AAF (see below). Fig. 4 shows the mass fragmentogram of di-tBDMS derivatives of 2-AAF metabolites from freed glucuro conjugates after a 48-h incubation of 10 μmol/l [9-^{14}C]-[9-^{12}C]-2-AAF which was preceded by a 6-day preincubation of 1 μmol/l 3-MC using the WA♀7FR13 rat liver epithelial liver cell culture. Selected ions were m/z 410=$(M-57)^+$ and $(m+2)/z$ 412=$(M+2-57)^+$ from the ^{14}C-labelled incubated 2-AAF. Figs. 5–9 show mass spectra of identified metabolites with the twin m/z and $(m+2)/z$ ions, the latter arising from the [9-^{14}C]fluorene ring: 2-AF mono-tBDMS (Fig. 5), 3-OH-2-AAF di-tBMDS (Fig. 6), the two unknown x-OH-2-AAF di-tBDMS (Fig. 7), 7-OH-2-AAF di-tBDMS (Fig. 8) and 9-OH-2-AAF di-tBDMS (Fig. 9). The kinetics of OH-2-AAF production were measured by GC–MS after incubation for 1–96 h of 5–100 μmol/l [9-^{14}C]-2-AAF in the cultures at 37°C (Table I). Up to 100 μmol/l, 2-AAF was not cytotoxic. In addition to hydroxylation, 2-AAF, when incubated

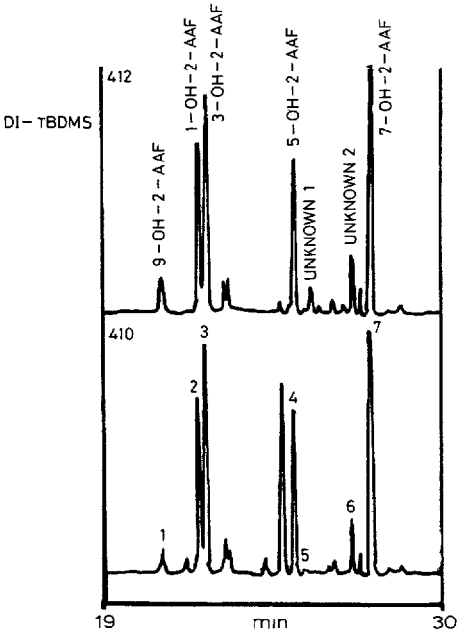

Fig. 4. EI mass fragmentogram of di-tBDMS derivatives of OH-2-AAF compounds synthesized in the WA♀7FR13 culture after incubation of 10 μmol/l [9-^{14}C]/[9-^{12}C]-2-AAF for 24 h which was preceded by a 6-day preincubation of 1 μmol/l co carcinogen 3-MC to induce hydroxylation and glucuronidation. Compounds resulted from the freed glucuronidated fraction of metabolites (see text for origin of MF ions *m/z* 410 and 412).

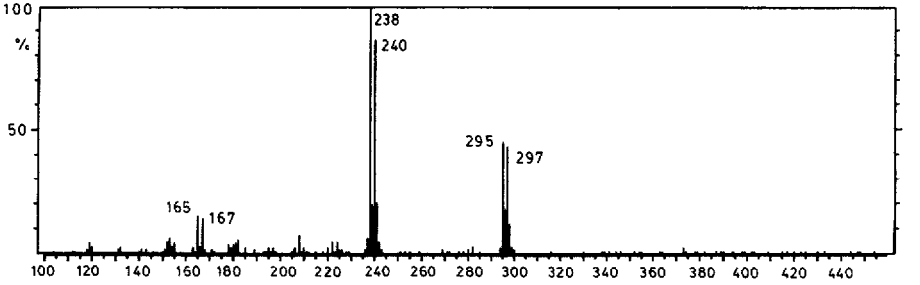

Fig. 5. EI mass spectrum of 2-AF as mono-tBDMS derivative after basal incubations of 10 μmol/l [9-^{14}C]/[9-^{12}C]-2-AAF (see text and Table I for incubation conditions). Fragments as $(m+2)/z$ ions are 297 = $(M+2)^+$, 240 = $(M+2-57)^+$, 182 = $(M+2-115)^+$ and 167 = $(M+2-115-15)^+ = [(\text{fluorene}+2)-H]^+$.

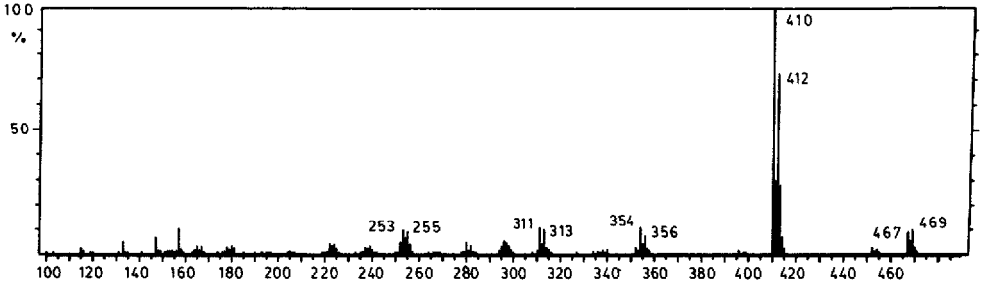

Fig. 6. EI mass spectrum of di-tBDMS derivative of 3-OH-2-AAF synthesized during the incubation according to Fig. 4. Fragments as $(m+2)/z$ ions are $469=(M+2)^+$, $412=(M+2-57)^+$, $356=[M+2-57-(57-H)]^+$, $313=(M+2-\text{tBDMSi-OC}=CH_2+H)^+$, $255=[313-(57+H)]^+$.

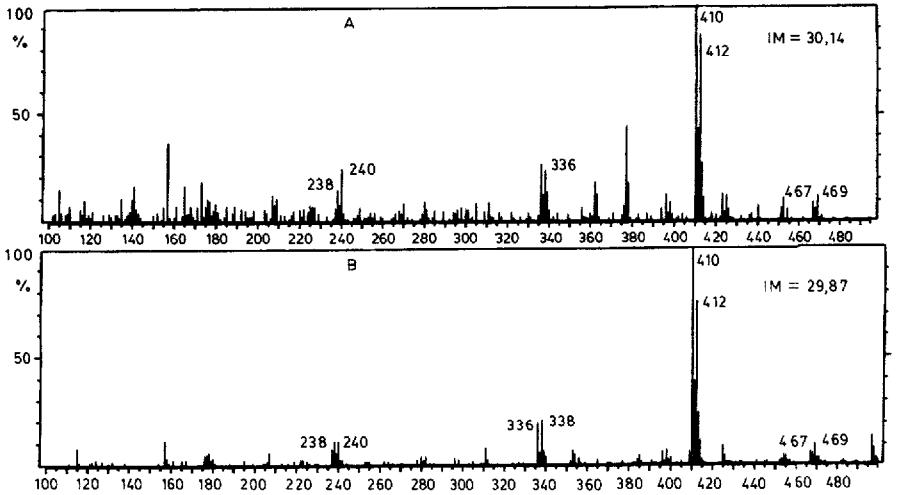

Fig. 7. EI mass spectra of the two unknown *x*-OH-2-AAF as di-tBDMS derivatives found in the cell extract after incubation according to Fig. 4. Fragments of lower mass spectrum as $(m+2)/z$ ions are $469=(M+2)^+$, $412=(M+2-57)^+$, $338=(M+2-131)^+$, $282=[M+2-13-(57-H)]^+$, $240=(M+2-115-(115-H)]^+$.

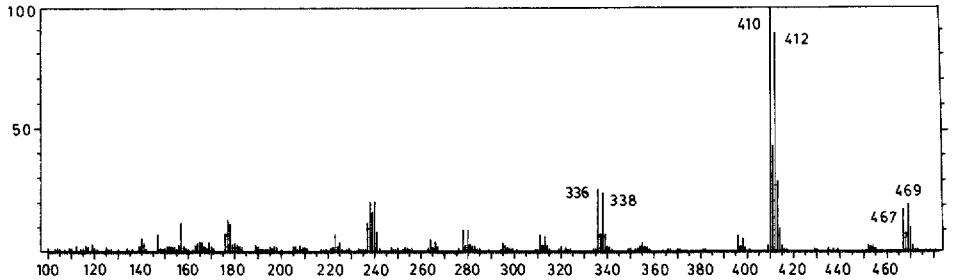

Fig. 8. EI mass spectrum of 7-OH-2-AAF as the di-tBDMS derivative found in the cell extract after incubation according to Fig. 4. Fragments as $(m+2)/z$ ions are $469=(M+2)^+$, $412=(M+2-57)^+$, $338=(M+2-131)^+$, $313=(M+2-\text{tBDMSi-OCH}=CH_2+H)^+$, $280=[M+2-131-(57+H)]^+$, $240=[M+2-115-(115-H)]^+$, $223=(M+2-131-115)$.

above 10 μmol/l, was deacetylated to 2-AF (Table I). On incubating 5 μmol/l 2-AAF in a Wistar rat liver epithelial cell line, WÖ95FR8, the time and order of metabolite appearance and amount (in nmol/mg cell protein) were (i) 9-OH at 6 h, 0.12; (ii) 7-OH at 12 h, 0.21; and (iii) 5-OH-2-AAF at 36 h, 0.03. Conjugates, 2-AF and N-OH-2-AAF, the ultimate carcinogen, were not found (Table I).

Deacetylation of 2-acetylaminofluorene

Deacetylation of 2-AAF is an important metabolic process in the detoxification pathway by the liver and was found in the liver epithelial cell lines (Table I). When the liver cell culture was preincubated for 48 h with a blank incubation solution without 2-AAF, and then incubated for 48 h with 5 μmol/l 2-AAF after careful elimination of the preincubation solution, 2-AF was produced when the preincubated blank solution contained dimethyl sulphoxide (DMSO), which was

TABLE II

PERCENTAGE OF 2-AF METABOLIZED FROM INCUBATED 2-AAF IN THE RAT LIVER EPITHELIAL CELL CULTURE W♀FRS22 VERSUS SEVERAL CONCENTRATIONS OF PREINCUBATED DMSO AND EFFECT OF THE DEACETYLASE INHIBITORS SODIUM FLUORIDE AND PARAOXON

DMSO[a] (%)	2-AF (%)		
	2-AAF[b]	2-AAF + NaF	2-AAF + paraoxon
0.2	16	12	2
1.2	37	20	5
2.5	41	30	10
5.0	37	33	7
Inhibition ratio (%) (±S.E.M.)[c]	-	27.2 (±14.4)	87.7 (±5.5)

[a]DMSO (%, v/v, in the blank preincubation solution) was preincubated for 48 h before 2-AAF incubation.
[b]2-AAF was incubated for 48 h after careful elimination of DMSO from the extracellular space of the culture.
[c]Standard error of the mean.

used as the solvent for 2-AAF, although at this level of 2-AAF deacetylation should not occur (Table II). Therefore, DMSO, which is an inducer of deacetylases in liver cell culture, as shown in Table I, was no longer used to dissolve 2-AAF. In addition, incubation of known potent deacetylase inhibitors, starting 15 min before 2-AAF incubation, sodium fluoride [23,24] and paraoxon [23,25], elicited different effects. In these cultured cells the inhibiting action of sodium fluoride was low, 27% (±14.4% S.E.M.), despite its being a strong inhibitor in vivo, whereas paraoxon provoked a high inhibition, 87.7% (±5.5% S.E.M.) (Table II). Not many studies on the pharmacology of DMSO have been made in culture systems [26], but we can postulate that DMSO stimulated the deacetylation of 2-AAF. Deacetylation of 2-AAF and of N-OH-2-AAF constitutes an important pathway of carcinogenic activation of these molecules by the liver [23,27]. Therefore, its expression in the rat liver epithelial cell lines constitutes a notable participation to the biotransformation of 2-AAF.

Isotopic effect of [9-^{14}C]-2-acetylaminofluorene on its hydroxylation

The use of GC–MS for the determination of OH-2-AAF arising from [9-^{14}C]/[9-^{12}C]-2-AAF allowed the recording of mass specrtra with an altered isotopic peak ratio in the case of ions arising from the fluorene ring of 9-OH-2-AAF, as already shown by the mass spectrum (Fig. 9). This metabolite resulted from the oxidation of C-9, which was also labelled with ^{14}C. Isotopic ratios were measured by MF on extracts of cell culture incubations using three different solutions of [9-^{14}C]-2-AAF diluted with [9-^{12}C]-2-AAF in order to obtain an initial isotopic ratio between 0.8 and 1.2. During the course of the work, each 2-AAF solution was utilized for fifteen culture incubations. Table III shows the initial isotopic ratio of the labelled 2-AAF and the measurements of isotopic ratios of 2-AF, 7-OH-2-AAF and 9-OH-2-AAF, the three major metabolites (Tables I and IV) after

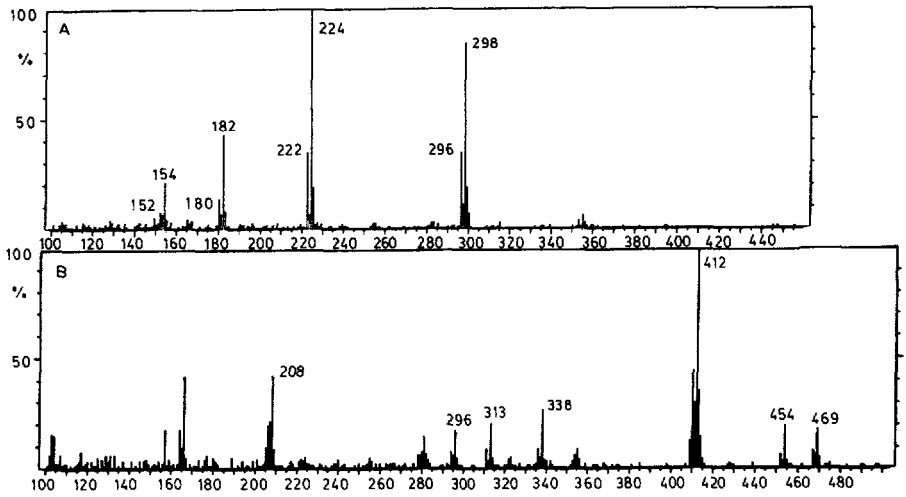

Fig. 9. EI mass spectrum of [9-^{14}C]/[9-^{12}C]-OH-2-AAF after 48-h incubation of [9-^{14}C]/[9-^{12}C]-2-AAF (50:50, w/w) for 48 h in a rat liver epithelial cell culture. (A) Main $(m+2)/z$ ions of mono-tBDMS derivatives [20] are $355 = M^+$, $298 = (M-57)^+$, $224 = (M-131)^+$, $182 = (M-131-42)^+$. (B) Main $(m+2)/z$ ions of di-tBDMS derivatives [20] are $469 = M^+$, $454 = (M-15)^+$, $412 = (M-57)^+$, $354 = (M-115)^+$, $338 = (M-131)^+$.

TABLE III

^{14}C/^{12}C ISOTOPIC RATIO OF 2-AAF METABOLITES AFTER INCUBATION FOR 48 h OF [9-^{14}C]/[9-^{12}C]-2-AAF IN RAT LIVER EPITHELIAL CELL CULTURES

Compound	Isotopic ratio		
2-AAF[a]	1.17 ± 0.05^a	1.14 ± 0.015^a	0.78 ± 0.04^a
2-AF	1.20 ± 0.24	1.19 ± 0.09	0.75 ± 0.07
7-OH-2-AAF	1.25 ± 0.11	1.20 ± 0.13	0.81 ± 0.05
9-OH-2-AAF	2.61 ± 0.07^b	2.45 ± 0.12^b	1.78 ± 0.21^c

[a]Three standard solution of [9-^{12}C]-2-AAF and [9-^{14}C]-2-AAF were prepared in order to obtain an isotopic ratio between 0.8 and 1.2. Fifteen incubations of 10 μmol/l [9-^{14}C]/[9-^{12}C]-2-AAF were carried out for each standard solution. Isotopic ratios were assayed by MF.
[b]$p < 0.01$.
[c]$p < 0.05$.

48-h incubation of 2-AAF at 5 or 10 μmol/l. The variations of these ratios are not significant in MF assays of 2-AF and 7-OH-2-AAF but they are highly significant ($p < 0.05$ and $p < 0.01$) for 9-OH-2-AAF. It appears that the labelled C-9 is preferentially hydroxylated by cytochrome P-450 monooxygenase. The effect on a metabolic reaction of a labelled carbon at the site or near the site of a biochemical reaction [28] is well known [28–31], but it seldom occurs. However, it has been reported that some cytochrome P-450 monooxygenase biotransformations exhibit a measurable isotopic effect [32,33]. In the C-9 hydroxylation of 2-AAF the enzymatic system does not follow Michaelis–Menten kinetics [34,35], in contrast to similar oxidations at the other sites of the fluorene ring. We have observed

TABLE IV

PRODUCTION OF 2-ACETYLAMINOFLUORENE FREE METABOLITES AND GLUCURONIDES FROM A 48-h INCUBATION OF 10 μmol/l 2-AAF IN A WISTAR RAT LIVER EPITHELIAL CELL LINE, WA♀7FR, AT THE THIRTEENTH CONFLUENT PASSAGE

Inhibitor	Incubation mode[a]	Compound (nmol/mg cell protein/24 h)									
		2-AF	9-OH-2-AAF	1-OH-2-AAF	3-OH-2-AAF	5-OH-2-AAF	7-OH-2-AAF	Unknown I	Unknown II	Total free or glucuronides	Total free + glucuronides
-	Control (free)	1.03 ±0.09	1.95 ±0.15	-	-	0.21 ±0.11	1.37 ±0.33	-	-	4.55±0.17	-
PB	Free	-	0.37 ±0.11	0.54 ±0.13	1 12 ±0.19	0.55 ±0.01	1 12 ±0.30	-	-	3.70±0.15 FI/C=81%[b]	8.67±0.12 IR=1.90[c]
	Glucuronides	-	0.13 ±0.04	0.41 ±0.02	1.21 ±0.26	1.08 ±0.12	1.95 ±0.29	0.16 ±0.08	0.03 ±0.01	4.97±0.12	G/T=57%[d]
PB+ME	Free	0.39 ±0.05	0.24 ±0.03	0.04 ±0.02	0.14 ±0.04	0.26 ±0.08	0.93 ±0.12	-	-	1.90±0.06 InF=49%[e]	2.45±0.04 IR=0.54
	Glucuronides	-	0.02 ±0.01	0.08 ±0 01	0.15 ±0.01	0.12 ±0.03	0.18 ±0.04	0.03 ±0.02	-	0 55±0.22 InG=89%[f]	G/T=22% InT=73%[g]
3-MC	Free	-	0.25 ±0.04	0.40 ±0.03	0.81 ±0.01	0.54 ±0.02	0.84 ±0.18	-	-	2.84±0.07 FI/C=62%	11.24±0.08 IR=2.47
	Glucuronides	-	0.49 ±0.06	0.80 ±0.38	2.73 ±0.10	2.30 ±0.03	1.78 ±0 07	0.26 ±0.04	0.04 ±0.01	8 40±0 10	G/T=75%
3-MC+αNF	Free	-	0.10 ±0.02	0.02 ±0.01	0.25 ±0.03	0.15 ±0.01	0.45 ±0.07	-	-	0.97±0.03 InF=66%	2.5 ±0.03 IR=0.55
	Glucuronides	-	0.07 ±0.02	0.10 ±0.04	0.42 ±0.07	0.27 ±0.02	0.63 ±0.05	0.04 ±0.02	-	1.53±0.04 InG=82%	G/T=61% InT=78%

[a]Two confluent cell cultures in 25-cm^2-area flasks incubated per point assayed in duplicate ($n=4$).
[b]Relative free induction ratio FI/C=induced free/control (%).
[c]Induction ratio IR=total induced/total control.
[d]Relative glucuronidation ratio G/T=glucuronides/total induced (%).
[e-g]Relative inhibition ratio (In) of free (InF), glucuronides (InG) or total (InT)=(induced−inhibited)/induced (%).

the same isotopic effect in other rat liver cell cultures: hepatocyte primary cultures and co-cultures of primary hepatocytes and liver epithelial cells.

Glucuronide metabolites

Each inducer was preincubated for at least 5 days in confluent cultures, either 1 μmol/l 3-MC or 1 mmol/l PB, the medium being changed every 2 days. Then 10 μmol/l 2-AAF was incubated for 48 h at 37°C after washing out the effector carefully. The inhibitor, 100 μmol/l or 50 μmol/l αNF, was preincubated with its respective inducer PB or 3-MC. Table IV shows that two other free and glucuronidated OH-2-AAF were synthesized, following 3-MC or PB preincubation, especially the synthesis of 3-OH-2-AAF and 1-OH-2-AAF, which are significant metabolic markers of this metabolism in the liver. Glucuronides of the five OH-2-AAF were more abundant than free metabolites and three were the major products: 7-OH- > 3-OH- > 5-OH- after PB preincubation and 3-OH- > 5-OH- > 7-OH- after 3-MC preincubation. As shown by the induction ratio (IR), the increase in the production of these compounds was more pronounced after 3-MC preincubation (IR = 2.47) than after PB preincubation (IR = 1.90). However, the free compound levels were lower and represented 81% or 62% of the control after PB or 3-MC preincubation, respectively.

Induction inhibitors, either ME for PB or αNF for 3-MC, affected the synthesis of both free and hydroxylated metabolites such that the IR was lowered either to 0.54 (PB + ME) or to 0.55 (3-MC + αNF), compared with 1.90 (PB) and 2.47 (3-MC). When these cell lines were assayed at different passages, over 1 year, the same metabolic activity was maintained. On comparing two cell lines adapted to grow in the three different media, SSM, SFM and SPFM, all metabolites were found and the PB or 3-MC inducibility was similar. However, in SPFM, a depleted nutrient medium, the same overall metabolism was found but reduced to 30% of the SSM level. The ultimate carcinogen N-OH-2-AAF was not detected owing either to a lack of sensitivity of the method or to extinction of the metabolic step. However, N-OH-2-AAF should be produced, because in vivo it is unstable and isomerizes to 3-OH-2-AAF, the first or the second metabolite in importance, and to 1-OH-2-AAF, as shown by Weisburger and Weisburger [36] and Smith and Thorgeirsson [37]. In addition, N-OH-2-AAF has a low GC–MS response despite the fact that we greatly improved existing techniques of analysis of 2-AAF metabolism.

In conclusion, we were able to carry out the first complete study on 2-AAF metabolism by rat liver epithelial cell lines owing to new developments in serum-free cell culture and in the mass spectrometric analysis of 2-AAF and its metabolites.

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