Binding of Carcinogenic Aromatic Amine to Rat Liver Nuclear Acidic Proteins *in vivo*

By PRABHAKAR D. LOTLIKAR and WOON K. PAIK

*Fels Research Institute and the Department of Biochemistry, Temple University School of Medicine,
Philadelphia, Pa. 19140, U.S.A.*

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Administration of chemical carcinogens to various mammalian species causes covalent binding of these parent compounds or their metabolites to susceptible tissue macromolecules such as proteins and nucleic acids (Miller, 1970; Miller & Miller, 1966). There is a direct relation between the degree of carcinogenicity of these chemicals and their macromolecular binding (Miller & Miller, 1966). However, there is no definitive evidence that would indicate that any particular macromolecular binding is involved in any way in the initiation of cancer.

The versatile hepatocarcinogen AAF* and its active metabolite *N*-hydroxy-AAF, when administered to rats, bind to liver cytosol basic proteins (Barry & Gutmann, 1966; Sorof, Young & Fetterman, 1960; Seal & Gutmann, 1961). Nuclear histones have been shown to be involved in the binding of AAF and its *N*-hydroxy metabolite (Barry, Ovechka & Gutmann, 1968; Jungmann & Schweppe, 1971). In view of the suggestion that non-histone nuclear proteins such as acidic proteins may play a role in the regulation of gene expression and function in mammalian cells (Gilmour & Paul, 1969; Stellwagen & Cole, 1969), it seemed important to investigate whether these acidic proteins are involved in AAF binding *in vivo*.

Adult male rats (180 g) of the Sprague-Dawley strain were used in these studies. *N*-Hydroxy-[9-¹⁴C]AAF (12.7 mCi/mmol) was purchased from Tracerlab, Waltham, Mass., U.S.A. Animals were injected intraperitoneally with 50 μ Ci, corresponding to about 0.94 mg of *N*-hydroxy-AAF (12.7 mCi/mmol), dissolved in 0.5 ml of ethanol-sesame oil (1:4, v/v) at 0 h and 24 h; they were given food and water *ad libitum*. At 24 h after the second injection the animals were killed by decapitation and their livers were immediately perfused *in situ* with 40-50 ml of ice-cold 0.9% sodium chloride solution. A 10% (w/v) liver homogenate prepared in 0.25 M-sucrose was passed through two layers of cheesecloth. A part of the homogenate was centrifuged at 105 000 g for 1 h to yield a supernatant fraction, designated the cytosol fraction. Liver nuclei were isolated essentially by the procedure of Allfrey, Mirsky & Osawa (1957). First, the homogenate was centrifuged at 700 g for 10 min. The

residue was suspended in 2.4 M-sucrose containing 1 mM-magnesium chloride and centrifuged at 50 000 g for 1 h according to the procedure of Widnell & Tata (1964). The residue suspended in 0.25 M-sucrose was the final nuclear preparation.

Fractionation of liver proteins was carried out by two different procedures.

Procedure A (Paik & Kim, 1967) consisted in adding 0.6 ml of 1 M-sulphuric acid to 5 ml of liver cell fraction. The mixture was then centrifuged at 10 000 g for 10 min and the residue was washed twice with 0.1 M-sulphuric acid to yield a sulphuric acid-insoluble fraction. The washings and the supernatant were combined and the combined solution was designated the sulphuric acid-soluble fraction. Then 2.5 vol. of ice-cold 95% (v/v) ethanol was added to the sulphuric acid-soluble fraction and the suspension was left for 2 h at -10°C. The suspension was centrifuged and the precipitate was subsequently washed several times with ice-cold 95% (v/v) ethanol to yield an ethanol-soluble fraction. The residue was then extracted with water and centrifuged at 10 000 g for 10 min. The residue was washed with cold water several times; washings and the supernatant contained sulphated histones. The residue was designated the water-insoluble fraction.

Procedure B (Friedmann, Shull & Farber, 1969) of nuclear protein fractionation was as follows. The nuclear suspension was centrifuged at 700 g for 10 min. The nuclear pellet was extracted several times with ice-cold 0.14 M-sodium chloride, followed by centrifugation at 8700 g for 20 min. The supernatant fraction is designated the sodium chloride-soluble fraction. The residue was extracted with 0.25 M-hydrochloric acid at 4°C for 1 h. The supernatant of this extract was centrifuged at 22 000 g for 10 min to yield the histone fraction, the residue representing the acid-insoluble fraction.

All protein fractions were treated twice with 15% (w/v) trichloroacetic acid at room temperature, once with 15% (w/v) trichloroacetic acid at 90°C for 15 min, once with ethanol at 80°C for 10 min and once with chloroform-ethanol-ethyl ether (1:2:2, by vol.) at 35°C for 10 min to remove the trichloroacetic acid-soluble fraction, nucleic acids and phospholipids (Allfrey *et al.* 1957). Protein pellets were suspended in 0.2 ml of 1.5 M-ammonia and

* Abbreviation: AAF, 2-acetamidofluorene,

Table 1. *Binding of [9-¹⁴C]fluorene residues to liver proteins after administration of N-hydroxy-[9-¹⁴C]AAF to rats*

Liver fraction	Extraction procedure	Protein fraction	Total protein (mg)	Sp. radioactivity (c.p.m./mg of protein)	Total radioactivity (c.p.m.)	Distribution of radioactivity (%)
Whole homogenate	A	H ₂ SO ₄ -insoluble	30.5	1532	46 726	45.4
		H ₂ SO ₄ -soluble:				
		Sulphated histones	4.26	988	4209	4.1
		Ethanol-soluble	2.59	212	549	0.5
		Water-insoluble	18.26	2817	51 438	50.0
Cytosol	A	H ₂ SO ₄ -insoluble	0.2	1325	265	0.6
		H ₂ SO ₄ -soluble:				
		Sulphated histones	1.34	2852	3822	9.1
		Ethanol-soluble	1.82	286	521	0.2
		Water-insoluble	12.48	3013	37 602	89.1
Nuclear	A	H ₂ SO ₄ -insoluble	1.0	2045	2045	58.8
		H ₂ SO ₄ -soluble:				
		Sulphated histones	2.75	500	1375	39.5
		Ethanol-soluble	0.18	583	58	1.7
		Water-insoluble	0	0	0	0
Nuclear	B	HCl-insoluble	0.77	7844	6040	78.8
		HCl-soluble (histones)	2.34	133	311	4.1
		0.14 M-NaCl-soluble	1.35	974	1315	17.2

radioactivity measurements were made by using 10 ml of Bray's (1960) solution. Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as the standard.

Several investigators have shown that administration of *N*-hydroxy-AAF to rats produced two- to three-fold more binding to liver macromolecules than that produced by ingestion of an equimolar dose of AAF (Barry, Malejka-Giganti & Gutmann, 1969-70; Irving, Veazey & Williard, 1967; Marroquin & Farber, 1965). Therefore, in the present studies, *N*-hydroxy-AAF was employed instead of AAF in order to obtain larger amounts of protein binding. In preliminary experiments it was observed that the protein-carcinogen binding could be doubled when two equal doses (50 μ Ci of *N*-hydroxy-[9-¹⁴C]AAF corresponding to about 0.94 mg of the compound/dose) were administered over a period of 24 h instead of a single dose of 50 μ Ci given at 0 h. In the present studies two equal doses of 50 μ Ci each of *N*-hydroxy-[9-¹⁴C]-AAF were given over a period of 24 h (Table 1). When proteins were fractionated from the whole

liver homogenate it was found that the total radioactivity was about equally distributed between the sulphuric acid-insoluble and -soluble fractions. The highest specific radioactivity was associated with the water-insoluble protein fraction. The amount of fluorene residues bound to total liver protein was about 1.0% of the *N*-hydroxy-AAF administered. In the cytosol fraction most of the protein-bound radioactivity was associated with the water-insoluble fraction of the sulphuric acid-soluble material. These results are in agreement with those of other investigators (Barry & Gutmann, 1966; Barry *et al.* 1969-70; Sorof *et al.* 1960; Seal & Gutmann, 1961), who reported that most of the [9-¹⁴C]AAF or *N*-hydroxy-[9-¹⁴C]AAF was associated with the acid-soluble basic protein fraction in the rat liver cytosol.

In the present studies, when liver nuclear proteins were fractionated by procedure A (Paik & Kim, 1967) it was found that the specific radioactivity of the sulphuric acid-insoluble fraction was about four times that of the sulphuric acid-soluble fraction. The proportions of radioactivity in the acid-insoluble and acid-soluble fractions were

about 60% and 40% respectively of the total nuclear radioactivity. Radioactivity associated with the nuclear fraction could not be entirely due to contamination with the cytosol fraction in view of the large differences in the distribution of radioactivity between these two cell fractions. There are reports indicating that [9-¹⁴C]fluorene residues are bound to nuclear proteins (Barry *et al.* 1968; Jungmann & Schweppe, 1971). After administration of [9-¹⁴C]AAF or its *N*-hydroxy metabolite to rats, arginine-rich histones in liver nuclei were found to have higher specific radioactivities than those of the other acid-soluble nuclear proteins (Barry *et al.* 1968; Jungmann & Schweppe, 1971). In the present experiments the nuclear acid-soluble fraction (sulphuric acid- or hydrochloric acid-soluble fraction) would contain these arginine-rich histone fractions (Friedmann *et al.* 1969; Paik & Kim, 1967).

Liver nuclear acid-insoluble proteins would be mostly composed of acidic proteins. In the present experiments (Table 1) the specific radioactivity and total radioactivity in the acid-insoluble fraction were much higher than those of the acid-soluble fraction, even though the nuclear proteins were fractionated by two different procedures (Friedmann *et al.* 1969; Paik & Kim, 1967). Jungmann & Schweppe (1971) have also shown that, after administration of *N*-hydroxy-[9-¹⁴C]AAF to rats, radioactivity was associated with the liver nuclear acidic proteins.

It was suggested that arylation of histones by AAF or its metabolite might result in modified histones no longer capable of inhibiting DNA-dependent DNA or RNA synthesis (Barry *et al.* 1968). However, in the present studies the fluorene residues were bound to nuclear acidic proteins much more than to histones. In view of these results and the suggestions that acidic proteins may be involved in the regulation of gene expression in mammalian cells (Stellwagen & Cole, 1969), it appears that modification of these acidic proteins

by arylation with fluorene residues might alter their regulatory properties of gene expression and function. It is possible that such modifications of acidic proteins may play a role in the initiation of liver cancer by aromatic amines such as AAF and its *N*-hydroxy metabolite.

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