Increased Selectivity of Interaction between Fluorenylamine Carcinogens and Liver Proteins during Hepatocarcinogenesis

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SUMMARY

The continued ingestion of a fluorenylamine hepatocarcinogen by rats results in a marked change toward a highly selective interaction between the carcinogen and particular target liver proteins. Adult male rats were fed for 5 weeks a diet lacking (control) or containing the liver carcinogen *N*-2-fluorenylacetamide (2-acetylaminofluorene, FAA). They were then given single doses of *N*-2-fluorenylacetamide-9-^{14}C intragastrically and killed 48 hr later. The soluble liver proteins were resolved extensively by column electrophoresis. Control liver profiles displayed a diffuse distribution of fluorenyl-^{14}C-proteins. In contrast, the profiles from rats previously fed the carcinogen displayed a highly localized concentration of bound ^{14}C-labeled carcinogen at one or two weakly basic classes of proteins (fast h_2 and/or slow h_1). These regions contained about one-third of all the soluble fluorenyl-^{14}C-proteins of liver, and represented a 3–4-fold increase over that in control profiles. Each of the two conjugates displayed a degree of electrophoretic homogeneity resembling that of a single macromolecule. The relative proportion of the two species varied considerably. The more anionic one (slow h_1) was labile.

The possibility that the specificity of protein binding *in vivo* resided in the activation of FAA by *N*-hydroxylation was examined by administration of the proximate carcinogen *N*-hydroxy-FAA-9-^{14}C to rats likewise previously fed FAA. This premise was not supported by the finding of a similar *h* specificity of the distribution of radioactivity.

In contrast to the *h* specificity of the preneoplastic livers of FAA-fed rats, unperfused primary liver tumors induced by FAA contained mostly soluble fluorenyl-^{14}C-proteins which were weakly acidic (*A* proteins) and had a mobility similar to that of serum albumin.

INTRODUCTION

A fundamental problem in the mechanisms of chemical carcinogenesis is the search for the interactions between carcinogens and cellular macromolecules which may be essential for tumorigenesis. Metabolites of chemical carcinogens that have been studied in depth combine *in vivo* least in amount with DNA, more with RNA, and usually most extensively with proteins of the target preneoplastic organs (1–3). The functional relationship of any of these interactions to carcinogenesis has yet to be determined. Protein conjugates of the liver carcinogens (aminoazo dyes and fluorenylamines) and the skin carcinogens (polycyclic hydrocarbons) are isolated mainly in the soluble fraction [references in (4)]. These soluble conjugates belong principally to a small electrophoretic group of relatively basic proteins, desig-

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nated h_2 or h_2-like (4–7). In contrast, the subsequent primary tumors (4, 7, 8) and transplanted highly differentiated liver tumors (9) do not form h_2 protein conjugates with the carcinogens which originally induced the neoplasms.

This paper describes in detail the ability of fluorenylamines to form specific h_2 protein-carcinogen conjugates in preneoplastic liver. The level of target selectivity of protein binding was previously found to be low in the livers of rats given a single dose of the carcinogen (10). The present study revealed that prolonged ingestion of the carcinogen induces highly preferential formation of particular h_2 and/or h_1 fluorenyl-protein conjugates. The marked increase in the selectivity of interaction with liver proteins during the preneoplastic stage represents a newly recognized biochemical action of this type of hepatocarcinogen. Portions of this study have been summarized previously (6, 8, 11, 12).

METHODS

Rats, diets, and carcinogens. The details of 13 experiments are listed in Table 1. In the experiments (A–K) dealing with binding of fluorenyl-^{14}C ring metabolites to liver proteins, adult male rats (CFN strain, Carworth Farms) weighing 100–179 g were fed ad libitum a grain diet (13) without (control) or with 0.036% FAA[1] (Distillation Products Industries) for 5 weeks. The control rats gained considerably more weight than did the others. The control diet was then made available for 12–15 hr to lessen the animals' stores of fluorenyl metabolites. Thereafter, single intragastric doses of FAA-9-^{14}C (experiments A–I) or *N*-hydroxy-FAA-9-^{14}C (experiments J and K) in 1,2-propanediol were administered. The rats were maintained on the control diet for the following 48 hr and killed. Their weights at death are given in Table 1. Initially FAA-9-^{14}C was kindly donated by Drs. John H. and Elizabeth K. Weisburger of the National Cancer Institute. Samples were subsequently obtained from Tracerlab, as were those of *N*-hydroxy-FAA-9-^{14}C.

Two experiments (L and M) dealt with the binding of fluorenyl-^{14}C metabolites to proteins of FAA-induced liver tumors. Rats of the above description, weighing 98–122 g or 182–231 g, were fed the FAA diet for 13 or 15 weeks, respectively, followed by the control diet for 25–30 weeks, when large liver tumors were discernible. Under light ether anesthesia, the rats received single intraperitoneal doses of FAA-9-^{14}C in 1,2-propanediol (experiment L) or *N*-hydroxy-FAA-9-^{14}C suspended in 0.15 M NaCl containing 7% acacia (14) (experiment M). They were then maintained on the control diet for 48 hr and killed. Their weights at death are given in Table 1.

Isolation and column electrophoresis of the soluble proteins of liver and liver tumor. The soluble liver proteins were isolated at 2–4° as described previously (4, 15), and as specified in Table 1, experiments A–K. Livers were perfused with 0.08 M sodium phosphate buffer, pH 7.8, containing 0.075 M NaCl. They were then disrupted with a Potter-Elvehjem homogenizer in 1 ml of this buffer for each gram of liver weight. Homogenates were centrifuged at 105,000 $\times$ *g* for 1 hr, yielding clear extracts containing 4.3 (3.4–5.6) g of protein per 100 ml and 41% (38–44%) of the nitrogen of the whole liver homogenates.

In experiments L and M, unperfused tumors were quickly excised, chilled, cut into pieces, trimmed free of liver and macroscopic necrotic regions, and collected in ice-cooled beakers. Pools of cut tumor were rinsed in the cold sodium phosphate–NaCl buffer, blotted on filter paper, and thereafter processed at 2–4° (4, 15). The tissue was minced and homogenized with a Potter-Elvehjem homogenizer in 1.5 ml of sodium phosphate–NaCl buffer for each gram of tumor weight. Homogenates were centrifuged as above, yielding clear extracts containing 4.6 and 6.2 g of protein per 100 ml and 50% of the nitrogen of the whole homogenates.

The soluble proteins of liver and tumor were concentrated in some cases by dialysis

[1] The abbreviations used are: FAA, *N*-2-fluorenylacetamide (2-acetylaminofluorene); *N*-hydroxy-FAA, *N*-hydroxy-*N*-2-fluorenylacetamide.

TABLE 1

Experimental data on the binding of fluorenyl-^{14}C carcinogen to proteins of liver and primary liver tumor

Expt.	Diet	Rats		^{14}C Administration					Tissue	Weight	Electrophoresis: Protein		Electrophoresis: ^{14}C	
		No.	Weights	Compound	mc/mmoles	mg/ml[a]	μc/100 gm	μc			Applied[b]	Recovered[c]	Applied	Recovered
			gm							gm	mg	%	cpm	%
A	control	4	249-264	FAA-9-^{14}C	7.85	2.8/4.6	9.0	98	liver	41	757	81	276,000	87
B	control	3	323-330	FAA-9-^{14}C	7.36	3.1/4.9	9.8	102	liver	40	725	92	289,000	88
C	FAA[d]	10	134-186	FAA-9-^{14}C	0.55	163.7/8.2	23.7	406	liver	68	748	86	8,700[e]	77[e]
D	FAA	4	155-194	FAA-9-^{14}C	5.93	2.4/3.8	8.7	64	liver	21	535	83	104,000	101
E	FAA	5	157-171	FAA-9-^{14}C	7.21	2.5/4.1	9.8	81	liver	37	780	113	154,000	87
F_a	FAA	8	152-193	FAA-9-^{14}C	6.33	3.0/7.0	6.1	85	liver	60	685	79	89,200	93
F_b											656	90	82,900	h only
G_a	FAA	8	158-204	FAA-9-^{14}C	5.58	3.0/7.5	5.2	76	liver	47	733	84	93,600	92
G_b											527	85	55,100	h only
H_a	FAA	7	149-199	FAA-9-^{14}C	5.29	2.8/5.9	5.4	66	liver	48	489	90	75,700	92
H_b											444	85	69,800	86
H_c											435	75	69,000	88
I	FAA	9	166-212	FAA-9-^{14}C	0.65	168.1/8.4	28.6	491	liver	70	762	55[f]	79,300[e]	61[e,f]
J	FAA	5	153-198	N-OH-FAA-9-^{14}C[d]	4.82	2.7/4.6	6.1	54	liver	35	773	101	134,000	92
K	FAA	4	145-186	N-OH-FAA-9-^{14}C	6.20	2.4/5.7	6.2	62	liver	24	559	90	121,000	89
L	FAA+control	3	267-332	FAA-9-^{14}C	6.62	5.4/3.0	17.9	160	tumor	20	342	94	7,420[e]	71[e]
M	FAA+control	2	312-426	N-OH-FAA-9-^{14}C	5.98	1.4/1.9	4.8	35	tumor	40	615	88	10,400[e]	76[e]

[a] Total weight of carcinogen-^{14}C in total volume of medium listed was administered in doses of 0.50 ml per 100 gm of body weight (experiments A-K); 0.33 ml/100 gm (L); 0.25 ml/100 gm (M), as described in Methods.

[b] Based on biuret assay

[c] Based on absorption at 284 mμ

[d] FAA, N-2-fluorenylacetamide (2-acetylaminofluorene); N-OH-FAA, N-hydroxy-N-2-fluorenylacetamide

[e] Levels of radioactivity of protein residues extracted according to earlier assay procedures, as described in Methods

[f] Lower recovery resulted from the sacrifice of faster moving proteins during greater resolution of slower proteins.

for 15–19 hr against 2 volumes of 0.20 M NaCl containing 0.01 M sodium phosphate buffer, pH 7.4, and 18–22% purified clinical dextran (4, 15). In other experiments, the supernatant fluids were concentrated by freeze-drying a portion as described previously (4). The concentrated protein solutions were dialyzed for 20 hr in sodium barbital buffer, pH 8.6, ionic strength 0.02, containing 0.03 M NaCl (4, 15, 16). The resultant solutions ("column extracts") contained 8.2–10.5% (average, 9.7%) protein. Aliquots were frozen for later processing and counting of ^{14}C.

On the second day after the animals had been killed, 4.2–7.5-ml samples of fresh column extract, containing the amounts of protein and ^{14}C stated in Table 1, were subjected to zonal electrophoresis at 2.2° on a column (225 × 3.1 cm, inner diameter) of purified ethanolized cellulose (4, 15). The column was eluted for 48–60 hr into a fraction collector maintained at 6°. Concentrations and recoveries of proteins in profiles were determined from the absorbance at 284 mμ at 18° (4, 15). Fractions were then stored at −15° until processed for counting of ^{14}C.

In most experiments the liver supernatant proteins were resolved into their whole electrophoretic profiles (96 hr at 90 mamp or 114 hr at 80 mamp). The locations, relative amounts, and nomenclature of the at least 11 charge classes thus revealed were similar to those previously exhibited on boundary and zonal electrophoresis (4, 15, 16). In the 14 whole profiles from liver referred to in Table 1 (experiments A–H, J, and K), recovery of protein, as determined from absorbance at 284 mμ, ranged between 75 and 113%, and averaged 88 ± 7%. In 140 such separations in past investigations, 86 ± 5% recovery was realized (15). By comparison, 94% and 88% of the soluble proteins of the FAA-induced liver tumors were recovered (experiments L and M). In previous studies equivalent values were 76–78% in the case of extracts of primary liver tumors induced by 3′-methyl-4-dimethylaminoazobenzene (4) and 81–91% with transplanted, highly differentiated hepatomas (9).

The soluble proteins were also subjected to 1.9-fold greater electrophoretic expansion (192 hr at 90 mamp). This achieved additional partial separation of the slow h_2, middle h_2, and fast h_2 subcomponents, which had differences in mobility in free solution of 0.11×10^{-5} and 0.13×10^{-5} $cm^2\ sec^{-1}\ V^{-1}$, respectively (16). Because the faster components migrated off the column, recovery of eluted protein was reduced to 55% (experiment I) and 71% (unlisted). The combination of this and the lower resolution permitted more detailed analysis of the fluorenyl-^{14}C-proteins among the h subcomponents relative to the whole soluble protein system.

Assays of fluorenyl-^{14}C-proteins. Inasmuch as the rat does not degrade 2-fluorenamine to CO_2, and open ring metabolites have not been found, radioactivity in position C-9 was used as a marker for metabolites containing the fluorene ring of FAA (2, 3, 14).

Three methods were used to process protein fractions prior to counting. In the first (10), proteins were extracted extensively to ensure that primarily covalently bound conjugates were assayed. Proteins were precipitated with cold trichloracetic acid, washed with 1.0 M sodium acetate buffer (pH 5) and 95% ethanol at 60°, dried, combusted, and counted as $BaCO_3$ with a Geiger counter (10); experiment C was conducted in this manner. In the second method (9), proteins were precipitated and washed with cold trichloracetic acid, extracted with hot trichloracetic acid and organic solvents, dried, and counted in gel as described below; this procedure was followed in experiments I (with hot acid omitted), L, and M. It was then found that considerable loss of protein-^{14}C in these procedures could be avoided without qualitatively affecting the electrophoretic distribution of the bound ^{14}C. Accordingly, in all other experiments in Table 1, aliquots of protein fractions were simply freeze-dried, suspended in Thixcin gel, and assayed by beta scintillation spectrometry. The counting was carried out at an efficiency of 62% with a 1–2% standard deviation. When necessary, counts were

corrected for quenching by addition of internal standard.

Enzyme assays. Lactate dehydrogenase activity was assayed spectrophotometrically during the reduction of pyruvate (17). Phosphoglucomutase activity was measured by the method of Najjar (18).

RESULTS

Liver fluorenyl-^{14}C-proteins resulting from FAA-9-^{14}C administration. If metabolites of FAA interact selectively with certain target proteins in liver, a preponderance of from one to a few species of fluorenyl proteins should result. Relatively little selectivity (specificity) was found previously when rats maintained on a commercial stock diet were each administered 15 mg of FAA-9-^{14}C intraperitoneally and killed 48 hr later (10). There resulted a small degree of localization of labeled conjugates in three classes of soluble liver proteins (g, fast h_1, and fast h_2), of which two (fast h_2 and g) had somewhat higher specific activities than did the third.

In the present study, rats were fed the control diet for 5 weeks, given approximately 1 mg of FAA-9-^{14}C intragastrically, and killed 48 hr later (Table 1, experiments A and B). No concentration of conjugate was observed in any class of proteins (Fig. 1). The interaction of proteins of control livers with the fluorenyl carcinogen at low dosages was apparently nonspecific with respect to the target protein.

In contrast, there was considerable target specificity in the preneoplastic livers of animals fed FAA. In experiments C–I (Table 1), rats were fed unlabeled FAA in the grain diet for 5 weeks, and then received FAA-9-^{14}C. Forty-eight hours later, marked localization of labeled conjugates at the fast h_2 component was observed (Fig. 2). The radioactivity was confined largely to a single, tall, narrow, symmetrical peak. However, the specificity of the carcinogen-protein interactions apparently was relative, since all other charge classes contained small amounts of radioactivity.

The marked increase in specificity of carcinogen binding to liver proteins brought about by continued FAA ingestion is shown clearly in Fig. 3, which compares the specific activities profiled in Figs. 1 and 2. In contrast to the many minor peaks of specific activity in the control liver profile, particularly in the basic half, the livers from FAA-treated rats exhibited only a fast

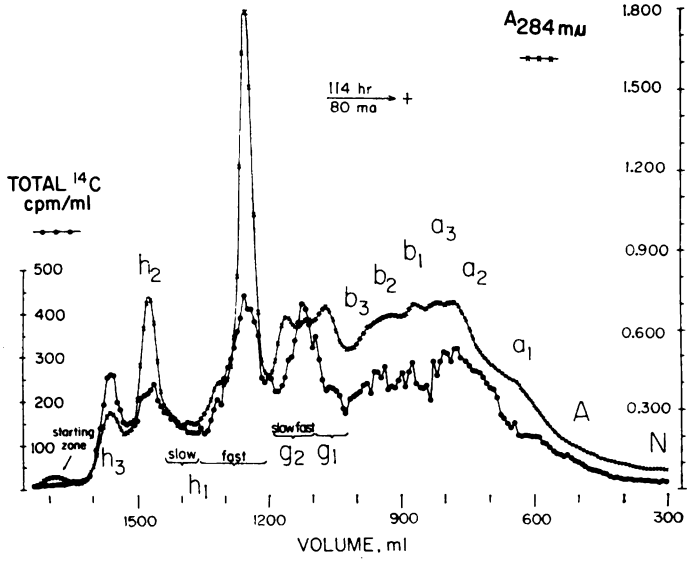

FIG. 1. *The diffuse electrophoretic distribution of the soluble liver fluorenyl-^{14}C-proteins resulting from FAA-9-^{14}C administration to rats fed control diet for 5 weeks (experiment B)*

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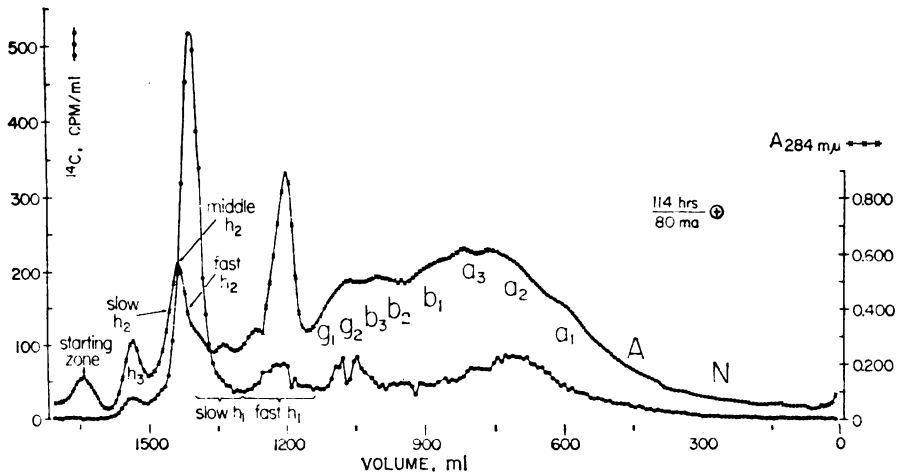

FIG. 2. *Specific fast h_2 fluorenyl-^{14}C-proteins resulting from administration of FAA-9-^{14}C in livers of rats fed FAA diet for 5 weeks (experiment F_a)*

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h_2 peak in an otherwise low, flat distribution. Although in the FAA experiment less than one-third as much ^{14}C was applied to the column at the start of electrophoresis, the specific activity of the fast h_2 proteins there exceeded that of any peak in the control profile (Table 1, experiments B and F_a).

In order to examine the electrophoretic homogeneity of the fast h_2 fluorenyl-^{14}C-proteins further, the slow components were additionally resolved nearly 2-fold in an otherwise similar experiment (experiment I, Table 1). The principal conjugate again migrated as part of the fast h_2 component in a monodisperse, symmetrical, prominent fashion (Fig. 4).

In other experiments, however, with nearly equal frequency and for unknown reasons, the specific fluorenyl-protein was slightly more anionic and belonged almost totally to the slow h_1 class. This type of

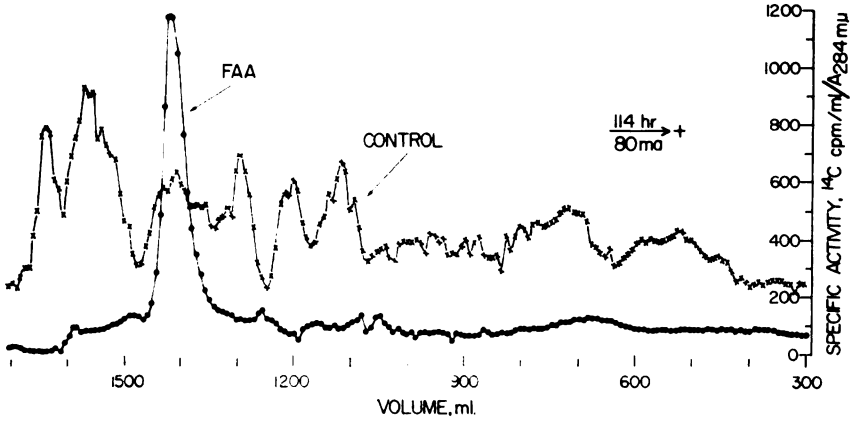

FIG. 3. *Comparison of specific activities throughout the electrophoretic profiles of the soluble fluorenyl-^{14}C-proteins of control livers and FAA-induced preneoplastic livers*

The distributions derive directly from the profiles in Figs. 1 and 2. The electrophoretic column in the FAA experiment contained only 31% of the ^{14}C present in the control experiment (Table 1, experiments B and F_a). For matching electrophoretic components, the FAA liver distribution should be displaced 35 ml toward greater elution volumes.

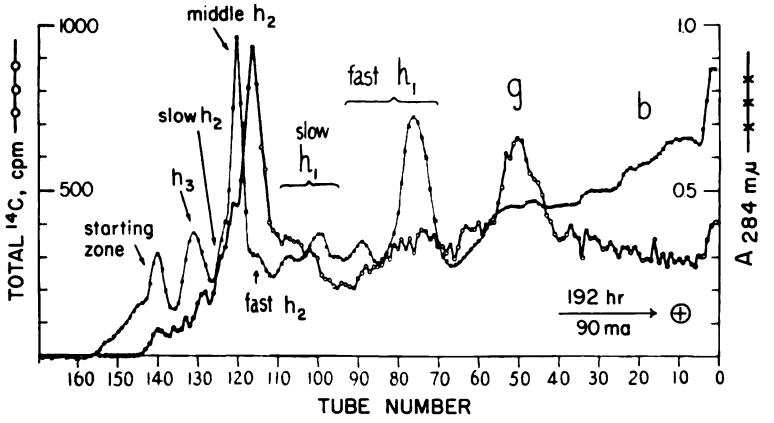

FIG. 4. *Electrophoretically expanded h region, showing specific fast h_2 fluorenyl-^{14}C-proteins resulting from FAA-9-^{14}C administration to rats fed FAA diet for 5 weeks (experiment I)*

Expansion is 1.9-fold greater than that in Fig. 2. Fractions were extracted with solvent prior to counting.

conjugate is shown in Fig. 5A (experiment H_c). It matched that of the fast h_2 class with respect to monodispersity, prominence, and symmetry (see below). In other instances, both species of conjugate were present together in varying relative amounts. Figure 6 shows such a profile derived from the livers of four rats (experiment D). The last two columns in Table 2 list the relative amounts of fluorenyl-^{14}C in column extracts which were present in the fast h_2 and slow h_1 components in all the experiments reported.

In the profiles containing predominantly one specific conjugate of either type, the carcinogen-protein adduct behaved as a single species of macromolecule. The degree of electrophoretic monodispersity of the fast h_2 or slow h_1 conjugate was comparable to that of a single protein. The shape of the distribution shown in Fig. 2 simulated those of two single enzyme species, phosphoglucomutase and the principal isozyme (V) of rat liver lactate dehydrogenase, each superimposed from different electrophoretic locations in matching experiments (Fig. 7). The shapes coincide, except at the base, where minor amounts of nonspecific fluorenyl-^{14}C-proteins are localized at adjacent components.

The two specific conjugates are weakly basic proteins, the fast h_2 being slightly more basic than the slow h_1. The protein classes to which they belong make up only a small fraction of the soluble liver proteins. The fast h_2 component constitutes $1.68 \pm 0.08\%$, and the slow h_1 $3.9 \pm 0.2\%$, or almost half these values if referred to the total amount of the proteins in preneoplastic livers from FAA-treated rats (16). Their free boundary electrophoretic mobilities are 0.699×10^{-5} and 0.913×10^{-5} cm^2 sec^{-1} V^{-1}, respectively. The degree of localization of fluorenyl conjugates in these classes is indicated in Table 2 [(component ^{14}C $\times$ 100)/column extract ^{14}C]. When one principal conjugate was present, it contained up to 33% (fast h_2) or 37% (slow h_1) of the ^{14}C of the extract applied to the column. Both together comprised between 32% and 43% of the total (experiments D–H_c). However, such division of bound carcinogen-^{14}C into its components does not adequately describe relative specificity, as previously noted (4). Broad components are excessively weighted, and individual species residing between modes are not completely included in any one component. A more accurate index of relative specificity is the comparison of peak levels of ^{14}C content relative to that at the fast h_2 or slow h_2, whichever is greater, valued as 100. (Component nomenclature is used only to locate maxima.) Such a comparison is made in Table 3. Each of the two specific conjugates stands out by a factor of at least 3. [This conclusion is based on experiments (D–H_c) which were processed

TABLE 2

Distribution of bound fluorenyl-^{14}C among soluble proteins of liver and primary liver tumor

Expt.	Homogenate ^{14}C x 100 / dose ^{14}C	Component ^{14}C x 100 / dose ^{14}C		Component ^{14}C x 100 / homogenate ^{14}C		Component ^{14}C x 100 / supernate ^{14}C		Component ^{14}C x 100 / column extract ^{14}C	
		fast h_2	slow h_1	fast h_2	slow h_1	fast h_2	slow h_1	fast h_2	slow h_1
	%	%	%	%	%	%	%	%	%
A	1.60	0.03	0.01	1.6	0.6	2.2	0.9	7	3
B	1.65	0.02	0.01	1.5	0.6	2.0	0.8	7	3
C						4.7[a]	2.7[a]	34[a]	19[a]
D	0.56	0.03	0.04	5.4	7.3	7.7	10.5	18	25
E	0.74	0.01	0.05	2.0	7.3	2.2	8.4	7	25
F_a	0.71	0.03	0.01	4.2	0.8			33	6
F_b		0.03	0.01	3.9	0.8			33	7
G_a	0.61	0.03	0.01	4.2	1.6	6.2	2.3	24	9
G_b		0.01	0.01	2.3	1.5	3.4	2.1	22	14
H_a		0.01	0.03					6	37
H_b		<0.01	0.03					2	35
H_c		<0.01	0.03					4	36
I				2.0[a]	1.2[a]	2.9[a]	1.7[a]	11[a]	7[a]
J	1.66	0.03	0.10	1.9	5.8	2.3	7.3	7	22
K		0.06	0.05					22	17
L	0.02	insignificant	insignificant	insignificant	insignificant	insignificant	insignificant	insignificant	insignificant
M	0.01	insignificant	insignificant	insignificant	insignificant	insignificant	insignificant	insignificant	insignificant

[a] Levels of radioactivity of protein residues extracted according to earlier assay procedures, as described in Methods

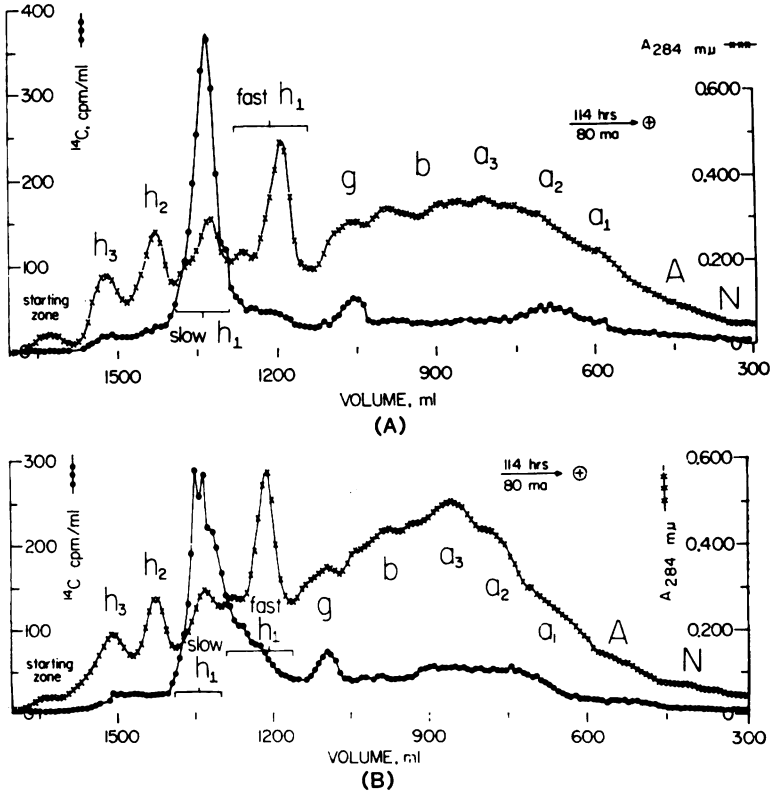

FIG. 5. *Specific slow h_1 fluorenyl-^{14}C-proteins of rats fed FAA for 5 weeks and then given FAA-9-^{14}C*

A (upper). Liver extract was concentrated by freeze-drying (experiment H_a).

B (lower). Instability of the slow h_1 conjugate. Liver extract was dialyzed against cold sodium phosphate–NaCl at pH 7.8 for 2 days, and then freeze-dried (experiment H_b).

for minimum loss of ^{14}C, and disregards values obviously enlarged as a result of overlap by adjacent principal conjugate (footnote *b*, Table 3).]

In experiments conducted to determine the factors that control which of the two types of specific conjugate predominates in any given profile, the slow h_1 conjugate

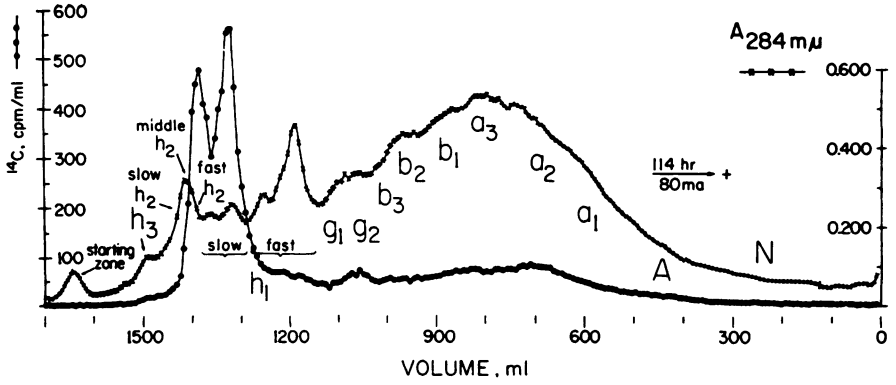

FIG. 6. *Bimodal distribution of specific h fluorenyl-^{14}C-proteins resulting from FAA-9-^{14}C administration to rats fed FAA diet for 5 weeks (experiment D)*

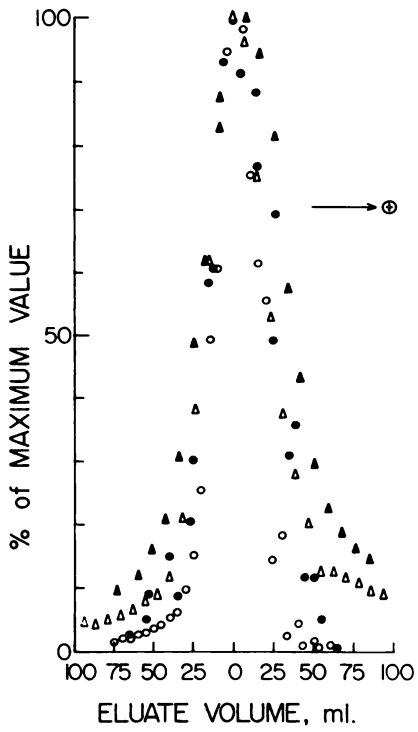

FIG. 7. *Comparison of electrophoretic distributions of fast h_2 fluorenyl-^{14}C-protein (experiment F_a) (△), slow h_1 fluorenyl-^{14}C-protein (experiment H_a) (▲), rat liver phosphoglucomutase (●), and rat liver lactate dehydrogenase V (○) from different profile locations*

The profiles are normalized as percentages of peak level vs. volume of column eluent (4, 15). Shown are two distributions of the liver *h* fluorenyl-^{14}C-proteins, two of phosphoglucomutase, and two of lactate dehydrogenase V.

was found to be labile under mild conditions. In a three-part experiment, an aliquot of a fresh liver extract was concentrated by freeze-drying (experiment H_c). It yielded a relatively homogeneous slow h_1 conjugate (Fig. 5A). The second sample of the extract was concentrated at 1–4° by dialysis for 1 day against the sodium phosphate–NaCl buffer (pH 7.8) used for liver homogenization, containing 18% dextran (4, 15) (experiment H_a). An identical homogeneous slow h_1 conjugate was present. However, when the third portion (experiment H_b) was dialyzed for 2 days against the same buffer (without dextran) and then freeze-dried, the conjugate became noticeably heterogeneous, apparently during the second day of dialysis. Conversion to subcomponents more anionic than slow h_1, up to and including fast h_1, was observed (Fig. 5B). This finding implicated some instability as responsible for the dual nature of the specific *h* conjugates, and suggested the possibility that the less anionic fast h_2 component might be the more native of the two species.

Liver fluorenyl-^{14}C-proteins derived from N-hydroxy-FAA-9-^{14}C. The question whether the specificity of protein binding observed with labeled FAA would apply to its more active metabolite, *N*-hydroxy-FAA, then arose. FAA was fed to rats, and *N*-hydroxy-FAA-9-^{14}C was administered (experiments J and K). Again, conjugates were present mainly at the fast h_2 and slow h_1 components. Figure 8 contains a profile in which both were prominent (experiment K). Here 22% of the radioactivity applied to the column was associated with the fast h_2 component, while 17% was associated with the slow h_1 (Table 2). In experiment J, the specific conjugate was located mainly at the slow h_1 component (22% slow h_1, 7% fast h_2). In these profiles, and in others which involved the second method of processing before counting (not reported), no other prominent conjugate was present. This is confirmed by the findings reported in Table 3, where the relative specificities of the various components are shown. In addition, the slower components were further resolved as in experiment I, Fig. 4. The only specific conjugate was associated with the fast h_2. It therefore appears that the proximate carcinogen *N*-hydroxy-FAA, like its parent FAA, gives rise to specific fast h_2 and/or slow h_1 fluorenyl-proteins in preneoplastic livers of FAA-fed rats.

Distribution of liver fluorenyl-^{14}C-proteins. The increase in specificity of the protein conjugates could have resulted either from an elevation in the content of the specific conjugates or, inversely, from a corresponding decrease in the proportion of nonspecific conjugates. To adjust for the unequal amounts of carcinogen-^{14}C administered in the various experiments, the content of bound carcinogen-^{14}C in the fast

TABLE 3

Relative specificities of the soluble fluorenyl-^{14}C-proteins of FAA-induced preneoplastic livers of rats given FAA-9-^{14}C or N-hydroxy-FAA-9-^{14}C

Values are the relative peak contents of ^{14}C compared to the maximum level, taken as 100, in each profile (see the text).

Expt.	Carcinogen-^{14}C	h_3	Slow h_2	Middle h_2	Fast h_2	Slow h_1	Fast h_1	g	b	a_2	a_1	A	N
C[a]	FAA-9-^{14}C	21	32	56[b]	100	41	42	47	41	60	46	49	25
D	FAA-9-^{14}C	3	8	21[b]	85	100	10	13	12	15	9	4	1
E	FAA-9-^{14}C	5	7	15	28	100	17	21	12	20	11	5	3
F[a]	FAA-9-^{14}C	6	12	38[b]	100	11	15	16	10	17	9	4	1
G[a]	FAA-9-^{14}C	10	21	52[b]	100	52[b]	28	34	17	26	15	6	3
H[a]	FAA-9-^{14}C	4	9	9	9	100	9	22	13	18	10	4	2
H[b]	FAA-9-^{14}C	6	8	6	10	100	28	26	16	19	15	5	2
H[c]	FAA-9-^{14}C	5	6	8	8	100	1	2	9	14	8	3	1
I[a]	FAA-9-^{14}C	20	32	48[b]	100	37	41	70	33				
J	*N*-Hydroxy-FAA-9-^{14}C	8	12	24[b]	41[b]	100	19	19	13	23	8	6	2
K	*N*-Hydroxy-FAA-9-^{14}C	14	22[b]	56[b]	100	85	27	24	21	32	12	3	2

[a] In experiments C and I, protein residues were processed according to earlier procedures prior to counting of ^{14}C (see METHODS).

[b] Values are enlarged because of insufficient resolution from adjacent principal conjugates in the electrophoretic profiles.

h_2 and slow h_1 conjugates was expressed as a percentage of the ^{14}C administered [(component ^{14}C × 100) / administered ^{14}C]. By this index, more ^{14}C was bound in the specific conjugates after FAA feeding than in the corresponding control components (Table 2). (Values of the subdivisions of experiments F, G, and H should be summed when comparing these experiments. Experiments C and I are not considered, since they involved the earlier methods of processing protein precipitates.)

Thus, the fraction of the FAA-9-^{14}C dose associated with both the fast h_2 and slow h_1 components increased from 0.03–0.04% in the control experiments (A and B) to 0.06–0.10% after FAA feeding (D–H). When *N*-hydroxy-FAA-9-^{14}C was given, both components contained still more radioactivity, namely, 0.11–0.13% (experiments J and K). This increase occurred in the FAA-fed rats despite the fact that their livers actually contained less than one-half as much fluorenyl-^{14}C metabolites relative

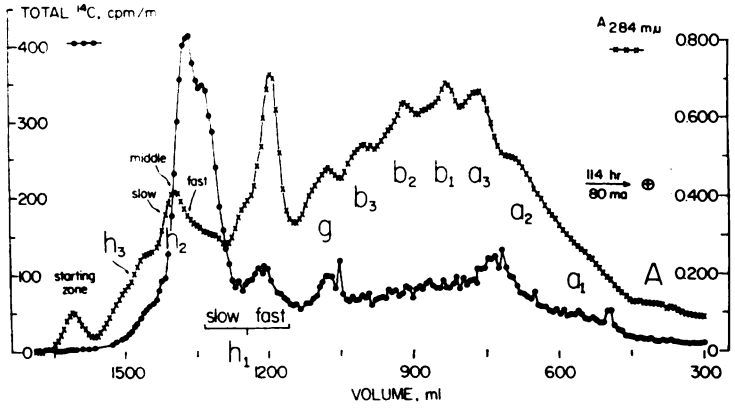

FIG. 8. *Specific fast h_2 and slow h_1 fluorenyl-^{14}C-proteins of liver resulting from N-hydroxy-FAA-9-^{14}C administration to rats fed FAA diet for 5 weeks (experiment K)*

to the dose of FAA-9-^{14}C as did the control livers. Thus, as shown in Table 2, the percentage of homogenate ^{14}C relative to the dose ^{14}C was reduced from 1.60–1.65% in control livers to 0.56–0.74% in the livers from animals fed FAA. Administration of *N*-hydroxy-FAA-9-^{14}C in experiment J raised the value to the control level.

Other indices of increased fluorenyl-^{14}C content in the specific conjugates were the increased fractions of the ^{14}C of the liver homogenates and supernatant fluids associated with the fast h_2 and slow h_1 proteins. The ^{14}C in these cytochemical fractions (homogenates and supernatant fluids) was derived from free as well as bound fluorenyl metabolites. Whereas the fast h_2 and slow h_1 adducts derived from FAA-9-^{14}C together contained 2.1–2.2% of the ^{14}C of control liver homogenates (experiments A and B), these conjugates contained 9.3–12.7% after FAA feeding (experiments D–G). Administration of *N*-hydroxy-FAA-9-^{14}C resulted in a content of 7.7% (experiment J). Furthermore, whereas the fast h_2 and slow h_1 conjugates observed after the administration of FAA-9-^{14}C contained 2.8–3.1% of the ^{14}C of the control liver supernatant fluids (experiments A and B), these adducts had 10.6–18.2% after FAA feeding (experiments D–G). Administration of *N*-hydroxy-FAA-9-^{14}C after FAA ingestion resulted in the association of 9.6% of the radioactivity of the liver supernatant fluid with these two conjugates (experiment J).

Still another indication that the greater content of fluorenyl-^{14}C associated with the fast h_2 and slow h_1 proteins accompanied greater specificity was the increased fraction of the column extract ^{14}C which was bound to these proteins. This index considered only labeled metabolites which were associated with the soluble liver macromolecules [(component ^{14}C × 100)/column extract ^{14}C]. The addition of FAA to the diet for 5 weeks elevated the percentage of the ^{14}C of the soluble liver proteins which was contained in the fast h_2 and slow h_1 components from 3- to 4-fold, i.e., from a level of 10% in the control experiments (A and B) to 32–43% in FAA-9-^{14}C experiments (D–H_c), and 29–39% after *N*-hydroxy-FAA-9-^{14}C administration (experiments J and K).

Fluorenyl-^{14}C-proteins in FAA-induced liver tumors. To examine whether FAA-induced liver tumors would form the specific *h* conjugates, rats bearing such tumors and maintained on the control diet were given FAA-9-^{14}C (experiment L) or *N*-hydroxy-FAA-9-^{14}C (experiment M). The latter compound was administered because of the possibility that the tumor cells might lack the ability to absorb or to *N*-hydroxylate the former. The fractions were processed by

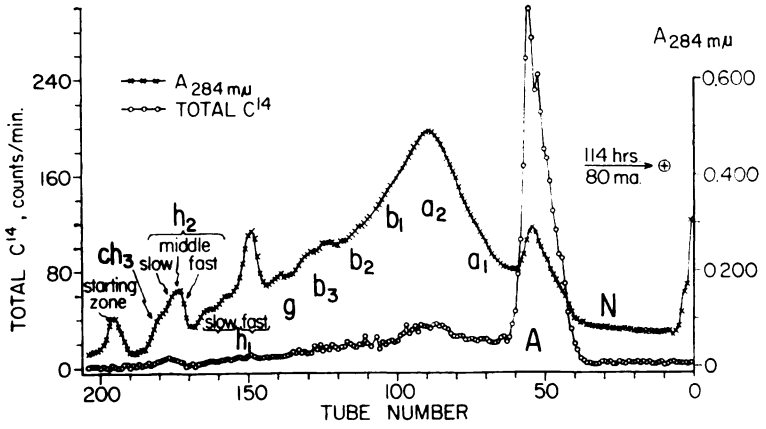

FIG. 9. *Lack of h conjugates among the soluble fluorenyl-^{14}C-proteins in primary liver tumors resulting from FAA-9-^{14}C administration*

Rats were fed the FAA diet for 3 months, followed by the control diet for 5⅓ months (experiment L).

the second procedure (METHODS), by which *h* specific proteins are demonstrable in preneoplastic liver.

Both carcinogens produced similar distributions of the labeled conjugates from liver tumors. The profile derived from FAA-9-^{14}C (experiment L) is shown in Fig. 9. The only labeled conjugates present in large amounts were those of the *A* component, which migrated at a rate close to that of serum albumin. Apparently, therefore, primary liver tumors induced by FAA do not form specific *h* fluorenyl conjugates.

DISCUSSION

Fluorenyl carcinogens interact highly selectively with certain target proteins to produce principally one or two soluble liver fluorenyl proteins *in vivo*. The specific conjugate formed is a fast h_2 or a slow h_1 protein, or both. Together they contain about one-third of all the soluble protein-bound fluorenyl metabolites of rat liver. The quantity and duration of feeding of the fluorenyl carcinogens required for these adducts to become manifest is not known. Approximately 1 mg of FAA did not promote their accumulation in control rats, 15 mg did so to only a small degree (10), and 5 weeks of FAA ingestion caused considerable accumulation. This range of dosage produced at these two *h* components a 3–4-fold increase in relative content of bound fluorenyl-^{14}C, a 2-fold rise in specific radioactivity, and an elevation in the relative amounts of protein [fast h_2, 42%; slow h_1, 15% (16)]. The development of this marked enhancement in the selectivity of protein binding requires a change in target specificity, from many proteins to primarily one or two.

The two specific conjugates apparently originate in a common target protein. Otherwise the occurrence of only one of these species in some experiments would require the presence of one type of target protein in some rats and a second kind in others. The dual nature presumably results from alteration of a common target protein or a common conjugate. The possibility of a common conjugate is supported by the lability of the slow h_1 adduct, generating more anionic species. It should also be noted that the electrophoretic mobilities of the two conjugates are close [0.21×10^{-5} cm^2 sec^{-1} V^{-1} (16)], and that only one molecular size of specific fluorenyl-protein has been found in such liver extracts (12).

It seems improbable that the specificity resulted from selection as targets those proteins with exceptionally rapid rates of turnover. Fluorenylamines, azocarcinogens (4), ethionine (19), and amino acids[2] all have a distinctive distribution of adducts among the soluble liver proteins. If selectivity resulted from the exceptionally high turnover of particular proteins, such diversity would not be expected.

Activated carcinogen, which is required for covalent protein binding (20), was apparently present in normal (10) and control livers after the administration of low levels of FAA-9-^{14}C. This was evidenced by the formation of a wide variety of fluorenyl-proteins. Nevertheless, relatively little or no specificity resulted. That such selectivity may not reside in the initial activation reaction of FAA, i.e., its *N*-hydroxylation, is suggested by the finding that the administration of *N*-hydroxy-FAA-9-^{14}C yielded specific *h* conjugates resembling those derived from FAA-9-^{14}C. However, the possibility cannot be excluded that this similarity stemmed from reduction of *N*-hydroxy-FAA-9-^{14}C to the parent labeled compound (2, 20). Nor is exceptional chemical reactivity of the receptor protein the factor probably responsible for the specificity, since fully activated fluorenyl carcinogen reacts relatively nonspecifically *in vitro* (20). It therefore seems reasonable that the unusual susceptibility of the target protein *in vivo* results from its specific involvement in a particular biological system. In this connection, DeBaun *et al.* (21) have speculated whether the specificity *in vivo* may result in part from the high reactivity and short half-life of the fully activated carcinogen, FAA-*N*-sulfate, and whether the sulfotransferase responsible for its formation may itself be the principal target. Recently, normal rat liver *h* pro-

[2] Unpublished observations.

teins were found to be devoid of this activity (22). The possibility exists, however, that the target protein may be transformed to an *h* protein as a result of interaction with carcinogen. In any event, the present study indicates that a marked qualitative and/or quantitative change occurs in response to the continued feeding of the carcinogen, with respect to the activated carcinogen or target protein, or both. The apparent dependence of the increased *N*-hydroxy-FAA excretion on regenerative liver growth (23, 24) may be relevant in this regard. It would be interesting to compare the dependence of enhancement of conjugation specificity and liver growth on the amount and duration of administered carcinogen.

The specificity of protein binding was noted 2 days after the feeding of carcinogen for 5 weeks. Since our preliminary reports, Barry and colleagues (25, 26) have described early preferential protein binding. DEAE-cellulose chromatographic fraction B, composed of weakly basic soluble liver proteins and proposed to contain h_2, retained a gradually increasing concentration of bound carcinogen following the administration of FAA over a period of 18 days. The 4.1 S sedimentation coefficient and the estimated 60,000 molecular weight of the proteins of fraction B reported by these authors, however, differ from those of the specific *h* fluorenyl-proteins [7.5 S and mol wt 150,000 (12)] in the present study. The question of the possible identity of these two sets of conjugates therefore needs to be investigated, particularly since a wide variety of soluble fluorenyl-proteins are formed after administration of a low dose of carcinogen. Even after the prolonged feeding of carcinogen in the present study, all of the charge classes of soluble liver proteins contained low levels of conjugates, as did all the molecular size classes (12). Collectively the nonspecific adducts comprise approximately two-thirds of all the soluble protein-bound carcinogen-^{14}C (Table 2, experiments D–H). A similar situation exists with the soluble liver azo-proteins formed during azo dye carcinogenesis (4). This multiplicity of species seriously complicates findings based on unfractionated conjugates.

Most of the soluble fluorenyl-^{14}C-proteins of unperfused primary liver tumors induced by FAA have electrophoretic mobilities close to that of serum albumin. Analogous conjugates have been found in the sera of these tumor-bearing rats,[2] the sera of normal rats given fluorenyl carcinogens (27, 28), and extracts of four transplanted, highly differentiated hepatomas (9). These primary and transplanted liver tumors, both originally caused by fluorenyl carcinogens, fail to form specific *h* fluorenyl-proteins. Whether this inability reflects a lack of receptor protein or activated carcinogen, or both, is unknown.

The ability of the preneoplastic tissues to form specific *h* conjugates of the fluorenylamines, azo dyes, and polycyclic aromatic hydrocarbons, and the inability of the subsequent tumors to do so (see above), may be unrelated to each other. This might be the case if, for example, conjugation during preneoplasia serves primarily to initiate temporary but necessary biochemical perturbations, as during cell proliferation, which in turn might permit or promote carcinogen-induced alterations of a genetic nature. On the other hand, the inability of the tumors to form specific *h* conjugates might be the result of neoplastic progression.

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