

The Principal Liver Carcinogen-Protein Conjugate after a Single Dose of Hepatic Azocarcinogen¹

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SUMMARY

Two days after a single intragastric dose of the hepatic carcinogen, 3'-methyl-4-dimethylaminoazobenzene, the principal azoprotein in rat liver cytosol was the relatively basic slow *h₂-5S* azoprotein. The principal species of azoprotein at 2 days was the same as that previously found after azocarcinogen was fed for 2.5 through 11 weeks. Apparently, the same one liver protein serves as the principal protein target of azocarcinogen throughout most of the duration of the liver carcinogenesis.

INTRODUCTION

Weeks of feeding of hepatocarcinogenic azo dyes leads to the presence of a principal species of protein-carcinogen conjugate in rat liver cytosol. This conjugate, termed slow *h₂-5S* azoprotein, is relatively basic and has recently been purified and partly characterized (12). It derives from an unknown target protein, which apparently has been detected immunologically in the cytosol of normal rat liver (11).

On the other hand, Ketterer *et al.* (6) purified a liver azoprotein, described as the principal azoprotein, from rats given a single dose of azocarcinogen. However, that azoprotein, which derives from ligandin (7, 8), is not the slow *h₂-5S* azoprotein (12, 15). In addition, Sugimoto and Terayama (19-21) have reported that, after a single dose of azocarcinogen, the principal azoprotein in heat-treated liver cytosol is nonbasic and is not the slow *h₂-5S* azoprotein. The question therefore arose of whether or not there is principal protein target of the azocarcinogens that is specific to the beginning events in liver carcinogenesis, one that would give rise to a principal conjugate other than slow *h₂-5S* azoprotein. The present study undertook to answer this question.

MATERIALS AND METHODS

Administration of Carcinogen. In each experiment, 9 to 13 CFN rats (Carworth Farms, New City, N. Y.) were fed a control semisynthetic diet (Diet 3 of Miller *et al.* (10) without carcinogen for 7 days. Each rat (♂♂, 108 to 138 g; ♀♀, 142 to 218 g) was then given 3'-Me-DAB² (Eastman

Kodak Co., Rochester, N. Y.; m.p., 120.0-120.6°) as a single intragastric dose at a level of 25 mg dye per 1 ml corn oil per 100 g weight of rat. The male rats in each experiment thus received an average of 30 to 41 mg of the carcinogen; the females were given an average of 43 to 51 mg. After 48 hr with the control diet available, the animals were sacrificed.

Resolution of Cytosol Proteins. All steps were carried out at 1-4°. The rat livers were perfused and their cytosol extracts were prepared using 0.075 M NaCl containing 0.08 M sodium phosphate, pH 7.8 (13, 17).

In 2 experiments in which the cytosol macromolecules were resolved according to molecular size, fresh liver cytosol extracts at 45 and 48 mg/ml protein concentrations from 10 male and 9 female rats, respectively, were applied to columns (*ca.* 215 x 3.4 cm, inside diameter) of Sephadex G-200 gel (Pharmacia Fine Chemicals, Piscataway, N. J.) in 0.20 M NaCl containing 0.01 M NaPO₄, pH 7.4, and eluted (13, 14).

In 7 electrophoretic experiments, the liver cytosol proteins from 9 female and 9 to 13 male rats were concentrated by dialysis against a 20% solution of previously dialyzed dextran (clinical grade; M.W. 80,000) (13). After dialysis against 0.02 ionic strength sodium Veronal buffer, pH 8.6, containing 0.03 M NaCl, the proteins (102 to 106 mg/ml) were resolved into charge profiles by zonal electrophoresis in columns of purified ethanolyzed cellulose (225 x 3.4 cm, inside diameter) in the Veronal-chloride buffer for 96 hr at 90 ma (13, 17).

Analyses of Proteins and Azoproteins. Protein concentration in molecular size profiles was measured spectrophotometrically at 280 nm, in electrophoretic profiles at 284 nm (17), and in pooled protein solutions by biuret reaction. Total bound azo dyes were assayed in 88% formic acid at 525 nm, while interfering substances (hemochromogens) were measured at 400 nm (3, 17). Firmly bound azo dyes were analyzed according to the following modification of the method of Miller and Miller (9): The proteins (<20 mg) were precipitated in 10% trichloroacetic acid, washed with 10 ml of 1.0 M sodium acetate buffer, pH 5; extracted 4 times in 7 ml of 95% ethanol at 60° for 12 to 15 hr; dissolved in 0.5 ml of 0.2 M KOH at 60° for 30 min; neutralized with 0.1 ml of 1 M HCl; freeze-dried; dissolved in 1 ml of 88% formic acid; clarified by centrifugation if hazy; and analyzed in a spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio) at 525 and 400 nm (3, 17).

Recoveries of proteins and azo dyes in profiles were 84% or more, as previously reported (13-15, 17).

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²The abbreviation used is: 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene.

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RESULTS

Molecular Size Profiles of Liver Cytosol Azoproteins.

The molecular size profiles of the liver cytosol macromolecules resembled those previously reported for the liver cytosols of normal and azocarcinogen-fed rats (13, 14).

The principal molecular size species of azoprotein was a 5S macromolecule in the liver cytosols of rats of either sex (Chart 1). A smaller amount of azoprotein was also present at the 4S component. This distribution of total bound azo dyes after a single dose of azocarcinogen is similar to that reported for liver cytosol after 2.5 to 3 weeks of ingestion of azo dye (13, 15).

Electrophoretic Profiles of Liver Cytosol Azoproteins.

The 7 electrophoretic patterns of liver cytosol proteins in the present study resembled those previously reported for normal and azocarcinogen-fed rats (17).

The slow h_2 azoprotein was the sole principal charge species of azoprotein in liver cytosol 48 hr after the single intubation of azocarcinogen in rats of either sex. This was the case in 3 of the 4 electrophoretic profiles in which the azoproteins were assayed for total bound azo dyes. Chart 2 demonstrates such an azoprotein distribution. The high absorption at 525 nm and low absorption at 400 nm at the position of slow h_2 component attest to the high concentration of bound azo dyes there. In the 4th profile (not shown), there were 3 principal species of very closely migrating h azoproteins, which were present in nearly equal amounts. These azoproteins migrated as middle h_2 , slow h_2 , and ch_3 proteins. These protein components were previously described (13, 17, 18). The resolution between each adjacent pair of the 3 species corresponded to a mobility difference

equal to only approximately 0.1×10^{-5} sq cm/V sec in free-boundary electrophoresis (18).

The charge species of azoproteins were also examined in 4 experiments in which the cytosol proteins after electrophoresis were analyzed for firmly bound dyes, *i.e.*, those resistant to extraction. In all 4 profiles, the slow h_2 azoprotein was the principal species of azoprotein. Chart 2 also demonstrates this result, in the case of a profile the azoproteins of which were analyzed by both methods of dye analysis. There were small amounts of the middle h_2 and ch_3 azoproteins in 2 profiles.

DISCUSSION

The slow h_2 -5S azoprotein is the principal azoprotein in liver cytosol during the very early stage of liver carcinogenesis by azocarcinogen. This azoprotein is the dominant species of carcinogen-protein conjugate in male and female rats as early as 2 days after a single intragastric dose and as late as 11 weeks of feeding azocarcinogen (15, 17, 18). The inference is therefore that the same one liver protein serves as the principal protein target of azocarcinogen throughout most of the duration of the liver carcinogenesis. This constancy of target protein of azocarcinogen is in marked contrast with the inconstancy of the target proteins of aminofluorenyl carcinogens. With the latter, the carcinogen-protein conjugates are of great multiplicity during the 1st few days of carcinogen administration (1, 16) but are highly specific at 5 weeks of feeding (16).

That there is a target protein that interacts with azocarcinogen to form a single, principal macromolecular entity,

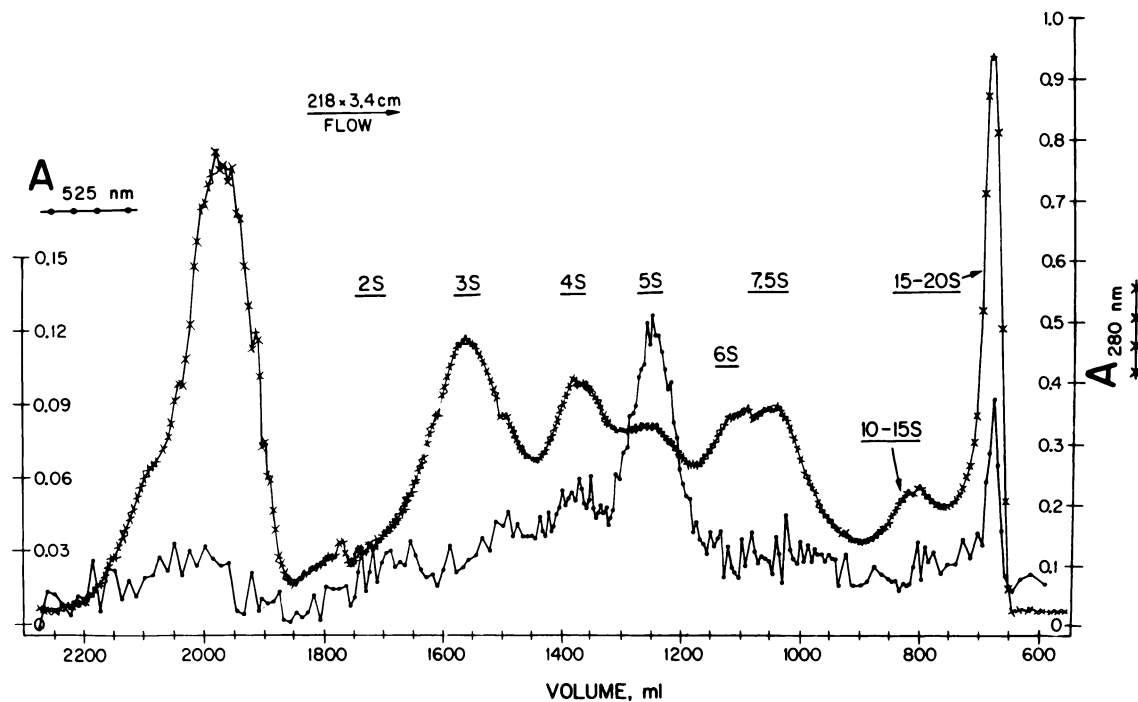


Chart 1. Molecular size profiles of the liver cytosol proteins and azoproteins of 10 male rats 48 hr after single doses of azocarcinogen. The principal nature of the 5S azoprotein is shown. Protein concentration was measured spectrophotometrically at 280 nm and total bound azo dyes at 525 nm. The methods are described in the text.

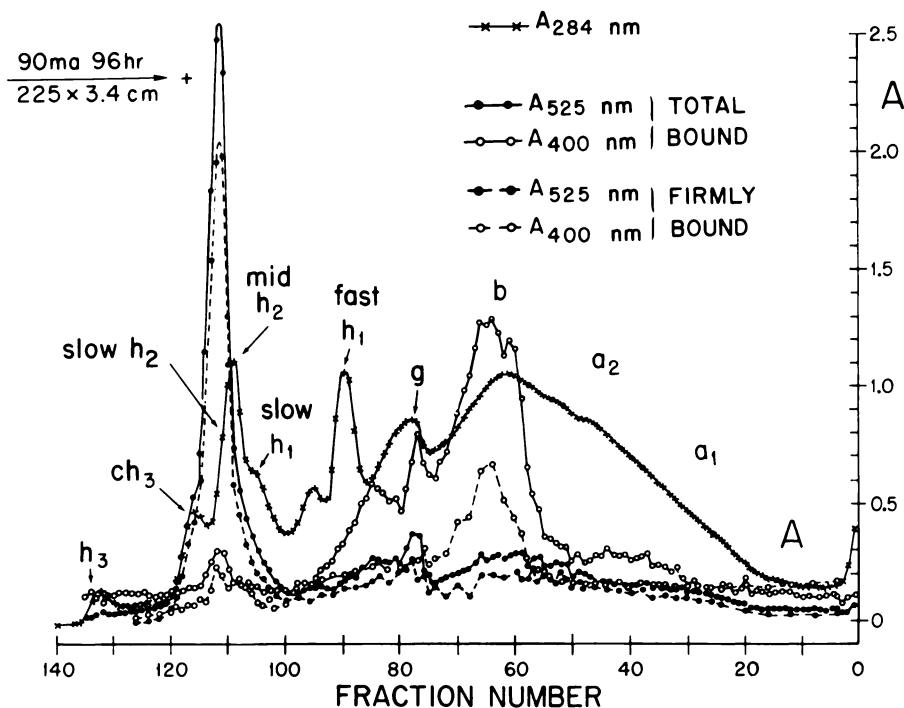


Chart 2. Zonal electrophoretic profiles of the liver cytosol proteins and azoproteins of 10 male rats 48 hr after single doses of azocarcinogen. The principal nature of the slow h_2 azoprotein is shown. Total bound azo dyes were assayed directly in formic acid; firmly bound azo dyes were analyzed in formic acid after extraction. Spectrophotometric measurement of protein concentration was at 284 nm, azo dyes at 525 nm, and interfering hemochromogens at 400 nm. The methods are described in the text.

the h_2 -5S azoprotein, is supported by the following findings. (a) Specific antiserum (11) against purified h_2 -5S azoprotein (12) reacts only with a protein in normal rat liver cytosol that is 5S in the molecular size profile and is h_2 in the electrophoretic charge profile (unpublished findings). (b) That there is a single, principal azoprotein entity, slow h_2 -5S azoprotein, is supported by the similar content of bound dyes in the principal species of azoprotein as resolved according to molecular size and charge. The 5S azoprotein has 62% of the bound dye of liver cytosol proteins after 2.5 to 3 weeks of feeding 3'-Me-DAB (15); the slow h_2 azoprotein correspondingly has 47% (17). All other azoprotein species have much less dye. (c) The slow h_2 azoprotein has been isolated, and found to be 5S azoprotein (12). In addition, the 5S azoprotein has been isolated and was found to be mostly slow h_2 azoprotein (unpublished findings).

Ketterer *et al.* (6) have purified an azoprotein, described as the principal azoprotein of liver cytosol, from rats 16 hr after a single i.p. dose of the azocarcinogen, 4-dimethylaminoazobenzene. This particular azoprotein is an azo dye derivative of ligandin (7, 8), the liver protein that noncovalently and covalently binds various organic substances (2, 8). Azo-ligandin is not the slow h_2 -5S azoprotein (12, 15). Azo-ligandin is a minor azoprotein in the liver cytosol of dye-fed rats; we have described it as the h_3 -4S azoprotein (13, 15, 17, 18). The present report demonstrates that, also at 2 days after a single intragastric dose of azocarcinogen, the slow h_2 -5S azoprotein, rather than azo-ligandin, is the principal azoprotein in liver cytosol. Ketterer (5) has recently noted the presence of an azoprotein C with a molecular weight

greater than that of azo-ligandin, and which has a large portion of the bound azo dye of liver cytosol. Its identity and relative content of azo dyes are yet to be reported. It is possible that azoprotein C may be the slow h_2 -5S azoprotein.

The evidence in the present paper is at variance with the conclusions of Sugimoto and Terayama (19-21) in regard to the type of principal azoprotein in liver cytosol existent very early during liver carcinogenesis by aminoazo dye. In their studies, 40 hr after the administration of a single intragastric dose (40 mg) of 3'-Me-DAB, the supernatant of heat-treated (55°, 3 min) liver cytosol contained a principal azoprotein which was nonbasic and isoelectric at pH 5.2 (Fraction 1). Only after the continuous feeding of the carcinogen for 2 or more weeks were the primary species of azoprotein "slightly basic and more basic" (Fractions 4, 6, and 7). These azoproteins were isoelectric at pH 8.0 to 8.6 and considered to include the slow h_2 azoprotein. The heat-treated cytosol proteins were not examined electrophoretically by them. At the present time, their conclusions are not in accord with those of 2 studies. In unheated liver cytosol, the principal azoprotein 48 hr after a single dose of azocarcinogen is the slow h_2 -5S azoprotein, just as after long-term feeding (this report). Further, according to a preliminary account by Ketterer (4), electrophoresis of the proteins of heated liver cytosol showed that most of the azoproteins are basic, just as in unheated cytosol.

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