Soluble Microsomal Antigens of Normal and Preneoplastic Livers

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SUMMARY

At least 8 macromolecules in the supernatant fraction of rat liver immunologically cross-react with antigens extracted from liver microsomes. These supernatant analogs of microsomal antigens have been resolved by extensive column electrophoresis and differentially characterized. One species (α) is highly acidic, 3 (β, γ1, and γ2) are of intermediate acidity, and 4 (ε1, ε2, and β) are near-neutral. The β is identical or closely related to the tissue-specific microsomal antigen, which others have found to be diminished in preneoplastic liver and absent from liver tumors induced by azoarecanogen and sometimes by N-2-fluorenylacetamide. Feeding these carcinogens, or in certain cases the control diets, affects the levels of individual supernatant analogs.

The principal soluble protein conjugates of these carcinogens present in preneoplastic livers do not cross-react with these microsomal antigens. Also, the conjugates and the coexistent supernatant analogs of the microsomal antigens differ electrophoretically. All appear to be unrelated. Thus, azo and fluorenyl are linked with these microsomal antigens or their supernatant counterparts. The findings do not support postulation of an immunologic sequence of cancer causation that is based on conjugation of carcinogen with the principal tissue-specific microsomal antigen in pre-neoplastic liver.

INTRODUCTION

The endoplasmic reticulum of rat liver is markedly affected during the hepatocarcinogenesis induced by the aminoazo dyes and FAA2 [reviewed (25)]. The membrane fraction of liver microsomes derived from the endoplasmic reticulum contains a liver-specific antigen (34, 35). Weiler (36, 37), and subsequently Nairn et al. (23), demonstrated that feeding of azocarcinogen results in the marked loss of this antigen in islands of liver cells and subsequent loss throughout the liver tumor. Hiramoto et al. later observed a similar deletion in some but not all hepatomas induced by FAA (11, 12). In addition, azo dye and fluorenyl metabolites of these carcinogens bind in vivo initially to membrane proteins (1, 14, 15) of liver microsomes (6, 13). During this feeding, the quantity of granular endoplasmic reticulum in liver diminishes as the agranular form increases (10, 26). Protein-carcinogen conjugates can later be isolated principally in the liver supernatant fraction (18, 27, 38) belonging primarily to a small electrophoretic class, h2 (29-31, 33). In contrast, subsequent multiple and also minimal deviation liver tumors lack the ability to form the h2 protein-carcinogen conjugates (17, 32, 33).

Bridging these 2 areas of study is the hypothesis by Green of an immunologic sequence of cancer causation. Postulated are the conjugation of carcinogen metabolites with microsomal tissue-specific antigen(s) in preneoplastic liver and a resultant transformation to tumor lacking this antigen (8, 9).

The present study was therefore undertaken to gain further information concerning the multiplicity of the antigens of the microsomes. Accordingly, soluble forms of a number of microsomal antigens have been found in the liver supernatant fraction. These analogs have been separated, differentially identified, and their levels during liver carcinogenesis determined. Evidence has also been obtained concerning whether or not any of these analogs or antigens, including the tissue-specific antigen, is a principal protein target of carcinogen binding in liver.

MATERIALS AND METHODS

Preparation and Fractionation of Soluble Microsomal Antigens. Rats of both sexes of the Sprague-Dawley strain (purchased from Zentralinstitut für Versuchstierzucht, Hannover, Germany) were fed a commercial stock diet (distributed as “Standardkost für Ratten” by A. Latz, Kraftfutterwerk, Euskirchen/Rhld., Germany). No difference in microsomal antigens was found between sexes and various strains. In each preparation, the liver microsomes were isolated by differential centrifugation in 0.25 M sucrose containing 0.01 M sodium phosphate, pH 7.2, using the Hogeboom-Schneider technic as modified by Vogt (35). The microsomes were suspended in 0.1 M Tris chloride and 0.005 M EDTA, pH 9.5. They were sonicated at 20,000 cycles per second for 5 min at maximal intensity in a MSE Distintegrator (distributed by MSE, London, England) at 500 watts. The suspension was centrifuged for 75 min in a Spinco L in rotor 30 at 105,000 × g, pi = 65 ×

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2 Abbreviations: FAA, N-2-fluorenylacetamide (2-acetylamino-fluorene); Tris, trihydroxyaminomethane; EDTA, ethylenediaminetetraacetate; 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene.
10⁶/min² [see (7) for use of pi, the performance index]. A fraction of the microsomal extract was precipitated with ammonium sulfate between 20 and 50% saturation. Each 6 gm of sediment was dissolved in 300 ml of 0.025 M NH₄OH, dialyzed against 4.5 liters of 0.025 M NH₄OH with at least 4 changes, and freeze-dried. The powder was dissolved in 0.14 M NaCl containing 0.01 M sodium phosphate, pH 7.2, yielding a solution of 1% protein for use in gel immunodiffusion.

**Preparation of Antisera to Soluble Microsomal Antigens.** Twenty mg of the above powder in 1 ml of the saline-phosphate buffer were mixed with an equal volume of Freund's complete adjuvant (supplied by Difco Laboratory, Detroit, Michigan). Ten mg of powder in this suspension were injected i.m. into each thigh of albino rabbits weighing 2 to 2.5 kg. After 3 weeks, the rabbits were bled for the 1st time (50-60 ml/rabbit). A high titer of antiserum was usually obtained after 2 months. Antisera were stored at —24°C. After 10 bleedings the antisera were pooled, divided into 1-ml samples, and refrozen. A different aliquot of these nonspecific antisera was used in each series of experiments.

Absorbed tissue-specific antisera were prepared by reacting 100 ml of pooled antisera with 10 mg of kidney microsomes (isolated as above) for 24 hr at 4°C. After centrifugation, supernatant antisera were similarly absorbed 4 additional times. Antiserum against rat serum globulins was prepared as above. Globulins were precipitated from serum at 50% saturated ammonium sulfate. After dialysis in 0.14 M NaCl containing 0.01 M sodium phosphate, pH 7.2, they were similarly mixed with Freund's adjuvant and injected into rabbits.

**Immunoelectrophoresis of Fractionated Microsomal Extract.** Immunoelectrophoresis of the fractionated microsomal extract was carried out at room temperature on glass slides (2.5 × 7.5 cm) using an LKB (LKB-Produkter AB, Stockholm, Sweden) apparatus. Samples were electrophoresed for 90 min (250 volts; 3 ma each slide) in 1% agar (supplied by Difco Laboratory, Detroit, Michigan) in pH 8.6 buffer containing 0.0125 M sodium Veronal, 0.0025 M Veronal, 0.0125 M sodium acetate, and 1:10,000 merthiolate. Immediately after electrophoresis, separated antigens were reacted with antiserum at room temperature for 12 to 30 hr. Resultant bands were then photographed (Fig. 1).

**Diets.** Each of 4 experiments with normal rats used 4–5 males which were raised on a stock diet of Wayne Lab Blox. In each of 7 experiments, 5 adult male rats were fed for 18–21 days the semisynthetic Diet 3 of Miller and Miller (19). The diet included 18% casein, 1.0 mg riboflavin per kg, and

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**Chart 1.** Representative electrophoretic profile of the soluble proteins of rat liver. Protein components are labeled N through h₃. Pools of fractions (— • —) selected for immunochemical assay are numbered 1 to 31 at regions named in Table 1.
Soluble Microsomal Antigens of Liver

Table 1

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<th>Pools*</th>
<th>Average protein concentrations (%)</th>
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Electrophoretic localizations of the soluble liver proteins cross-reacting with antigens of liver microsomal extract.

a Numbered pools correspond to the electrophoretic regions of the profile shown in Chart 1.
b Brackets span widths of electrophoretic regions in which cross-reactions occur; bars indicate locations of maximum intensities of reactions.
c Pools containing the minor species of azoproteins and fluorenylproteins after feeding azocarcinogen (33) and N'-2-fluorenylacetamide (31, 32), respectively.
d Pool containing the principal species of fluorenylproteins after feeding AT-2-fluorenylacetamide (31, 32). (See also Footnote 3.)
e Pool containing the principal species of azoproteins after feeding azocarcinogen (33). (See also Footnote 4.)

0.058% of the liver azocarcinogen 3'-Me-DAB. Azo dye was absent in the 3'-Me-DAB-control diet.

In each of 4 experiments, 4-7 adult male rats were fed a grain diet (20) containing 0.036% of the hepatocarcinogen FAA for 5 weeks. The carcinogen was absent in the FAA-control diet.

Isolation and Column Electrophoresis of the Liver Supernatant (Soluble) Proteins. The liver supernatant (soluble) proteins from these rats (all Carworth CFN) were prepared, processed, and electrophoretically resolved cold as described (33). Livers were perfused portalily with 0.08 M sodium phosphate buffer, pH 7.8, + 0.075 M NaCl, and disrupted with a Potter-Elvehjem homogenizer in this buffer (one ml = one gm of liver). Homogenates were centrifuged at 105,000 × g (average) for 1 hr, yielding 32-53 ml of clear supernatant fluid (4.2-6.0% protein) containing 40-47% of the nitrogen of the homogenates. These soluble proteins were concentrated by dialysis for 15-19 hr against 2 volumes of 0.20 M NaCl + 0.01 M sodium phosphate buffer, pH 7.4, + 22% purified clinical dextran. Additional dialysis for 20 hr in sodium Veronal buffer, pH 8.6 and ionic strength 0.02, + 0.03 M NaCl, followed. Protein solutions were adjusted at 4°C to pH 8.6200, and centrifuged for 20 min at 103,000 × g (average) to remove a slight turbidity. The supernatant proteins at 11.6% (9.1-15.2%) were diluted with Veronal-chloride buffer to 8.9% (8.3-10.6%) (column extract). For immunochemical assay, aliquots thereof were diluted with 2 volumes of 0.20 M NaCl + 0.01 M sodium phosphate, pH 7.4, and dialyzed overnight in this buffer. After biuret
analysis of protein concentration, samples were frozen for later use.

Starting on the 2nd day after animal sacrifice, 7.5 ml of column extract was electrophoresed at 80 ma for 114 hr at 2.2°C in a column (225 × 3.1 cm, i.d.) of purified ethanolized cellulose (33). Elution into a fraction collector maintained at 6°C followed for 48–60 hr. Concentration and recoveries of protein in eluates were determined at 284 ma (33). Up to 31 pools of fractions throughout each electrophoretic profile (Chart 1) were concentrated at 1–4°C by alternate dialyses in 0.07 m NaCl + 0.01 m sodium phosphate, pH 7.4, and pervaporations. After final dialysis in 0.20 m NaCl + 0.01 m sodium phosphate, pH 7.4, the concentrates contained 1.5–2.0 ml of 0.1–0.7% protein (biuret). On the 13th–15th day, the pooled fractions were stored at —15°C. In most experiments, frozen samples were flown to the European laboratory for immunochernical analysis. Otherwise, samples were assayed in the originating laboratory.

Gel Immunodiffusion. Gel diffusion was performed on glass slides (7.5 × 2.5 cm) containing 2.5 ml of 1.5% agar in 0.85% NaCl and 0.5% phenol. Pure agar for gel immunodiffusion was obtained from Behring Werke, Marburg, Germany. Samples in gels were allowed to react for 24 hr at room temperature.

Immunodiffusion experiments were performed in 4 ways. Fig. 2 depicts the 1st arrangement, serial gel diffusion. Parallel rows of holes, 4 mm in diameter, were spaced 4 mm apart. Consecutive electrophoretic fractions 1–31, designated in Chart 1 and Table 1, were placed in the wells of the middle row. Holes in the upper row contained unabsorbed antiserum against the fractionated microsomal extract, while absorbed (tissue-specific) antiserum was in the lower row of holes. The 2nd arrangement is illustrated in Fig. 3a. In this hexagonal geometry, holes were 4 mm in diameter, with 5 mm of agar between those central (unabsorbed antisera) and those outer (supernatant proteins). Unabsorbed antisera to the fractionated microsomal extract were thus reacted with the electrophoretically separated soluble proteins of liver. Identity with respect to immunodiffusion is indicated by continuity of precipitation bands in the gel. In the 3rd configuration (Fig. 3b), the same was done, except that the middle hole contained absorbed antiserum. In the 4th setup, an individual fraction of soluble liver proteins was placed in the middle well. The upper 3 holes contained absorbed antiserum, while the lower 3 holes had unabsorbed antiserum. The arrangement is similar to serial gel diffusion, but weak reactions appear more distinctly in this configuration. Using these 4 arrangements, over 1200 reactions were carried out on different electrophoretic fractions of liver soluble proteins.

RESULTS

Microsomal Antigens among Supernatant Proteins of Liver. The question arose whether or not the microsomal antigens are also present in the supernatant fraction of livers of rats fed stock and hepatocarcinogenic diets. The question particularly applied to the tissue-specific antigen present in the microsomes of normal rat liver (35). In his studies (36, 37), Weiler (Eberhardt Weiler, personal communication) did not detect the tissue-specific antigen in the soluble liver fraction. However, the concentration of the whole liver extract was possibly low. Therefore, the liver soluble proteins were reexamined at higher concentrations, revealing the presence of analogs of microsomal antigens therein (see column extract in Fig. 2). This finding suggested the possibility of differentiating and characterizing the antigens of the microsomal extract in terms of their immunologic cross-reactions with electrophoretically resolved supernatant liver proteins. In addition, the identification of microsomal-like antigens throughout the column electrophoretic profile of the soluble liver proteins permitted comparison with the gel immunophoretogram of the fractionated microsomal extract itself. Results with immunodiffusion and electrophoreses of both microsomal extract and supernatant liver proteins suggest that the extracted microsomal antigens are closely related to, if not identical to, their cross-reacting liver supernatant proteins.

Gel Immunoelectrophoresis of the Fractionated Microsomal Extract. Fig. 1 shows the partial resolution of the fractionated microsomal extract achieved by gel immunoelectrophoresis. Two types of experiments are compared by juxtaposing the pictures of only the halves of the gel containing resolved microsomal extract. The upper portions of the picture and diagram show the reactions of resolved microsomal extract with anti-microsomal serum previously absorbed with kidney microsomes. Apparent are 1 intensely reacting antigen of intermediate negative charge and 2 antigens of greater negative charge. The former is the tissue-specific β antigen; the latter 2 are the α antigens. The lower halves in Fig. 1 show the immunophoretogram of fractionated microsomal extract as developed by reactions with unabsorbed antiserum. Visible in addition to the above are 3 nonspecific antigens, γ, δ, and ε.

Column Electrophoresis of Supernatant (Soluble) Liver Proteins. Chart 1 presents the zonal electrophoretic profile of the soluble liver proteins. In 15 experiments, recovery of protein in electrophoretic eluates averaged 87 ± 5%. No significant difference in protein profile could be attributed to the diets of the rats. The 31 pools designated in Chart 1 and Table 1, representing regions throughout the profile, contained concentrations of protein listed in Table 1. Column extract, at 3.3% (average), and profile pools were tested for cross-reaction with absorbed and unabsorbed antimicrosomal sera using mainly serial gel diffusion (Fig. 2). The combined use of the extensive column electrophoresis and serial gel diffusion achieved, in effect, an immunophoresis of considerable electrophoretic resolution without molecular sieving.

Differentiation of the Microsomal Antigens among the Supernatant Proteins. At least 8 analogs of the microsomal antigens were consistently identified in the electrophoretic profile of the liver supernatant proteins by their cross-reactions with the fractionated microsomal extract.

Chart 1, Tables 1 and 2, and Fig. 2 describe the profile locations thus involved. Table 2 summarizes further the distinctions between the supernatant analogs. Additional proteins cross-reacted to small extents, but their presence was not reproducible.

The 8 cross-reacting supernatant proteins could be grouped into 3 general charge types. The microsomal α antigens cross-react with the highly acidic and highly negatively charged...
Soluble Microsomal Antigens of Liver

The analogs of the microsomal antigens among the supernatant proteins, A through α-A (pools 2 and 3). The tissue-specific β and the 2 γ antigens cross-react with the weakly acidic supernatant proteins of intermediate charge (β at β3 through γ1-β3 in pools 13 and 14; γ1 at β2 through β2-β2 in pools 11 and 12; γ2 at γ1 in pool 13). Finally, the microsomal δ, ε, γ, and η antigens cross-react with various supernatant near-neutral k proteins of little negative charge (δ at faster h1-slow g2 through fast h1-faster h1 in pools 18, 19, and 20; ε1 at lower h1 in pool 24; ε2 at slow h2 in pool 27; γ at h2 in pool 31).

The analogs of the microsomal antigens among the supernatant proteins were further differentiated according to their reactions with the absorbed (tissue-specific) antiserum against the microsomal antigens and antiserum against rat serum globulins (Table 2). Of the 8 analogs encountered, only those of the α and β antigens react with the absorbed antimicrosome serum (Table 2, Figs. 1–3). These 2 analogs can be differentiated further using antiserum against serum globulins. The α analogs cross-react with serum globulins; that of the β antigen does not. As shown in Table 2, reaction with antiserum against serum globulins can also be employed to distinguish between the analogs of the nonspecific microsomal antigens. Thus, analogs of the γ1 and δ react; the others do not.

Another means of differentiating between the microsomal antigens is by band position in gel immunodiffusion reactions (Fig. 3, Table 2). Differences in the concentrations of the various antigens in the fractionated microsomal extract and their analogs in liver extract (column extract) cause differences in the relative positions of the precipitation bands in the gel. If antiserum (central hole) is reacted with serially diluted samples of an antigen (outside holes), then the precipitation band shifts toward the antigen wells (toward position 1). In the hexagonal arrangement (Fig. 3a) in the reaction of microsomal extract with nonspecific (unabsorbed) antiserum, the position 1 band is the precipitation line of the δ antigen. Next is the α antigen, which sometimes overlaps with the δ antigen. Position 3 is that of the β antigen. Then, in succession, are γ1, ε2, ε1, γ2, and η (some not shown). This order in the case of microsomal extract is different from that of reactions between the liver supernatant proteins (column extract) and nonspecific antiserum against microsomal extract (Fig. 3a, Table 2). In the latter reactions, for example, the β is 1st, and the ε2 is last.

In this way, it is possible to compare the concentrations of the same antigen (analog) in the 2 mixtures. A lower position number signifies a lower concentration. (Different antigens cannot be compared.) From the positions of the β band at 3 and 1 with the microsomal and liver extracts, respectively, the relative concentration of the β analog appears to be considerably less among the liver supernatant proteins than does the β antigen among the microsomal proteins. As a result, there is a characteristic crossover by the β band in the reactions of these 2 mixtures with the microsomal antiserum (Fig. 3a, Table 2). The β band bends in the direction of the well containing liver supernatant proteins and crosses the δ band. Closer to the middle hole is a 2nd crossover. The ε2, at position 8 (nearest to the middle hole containing antiserum) in the reaction of the liver supernatant proteins, crosses over to position 5 (microsomal extract). The γ2 band crosses in reverse, coming from position 7 (microsomal extract) to position 5 (soluble liver proteins).

Effects of Carcinogens and Diets. Feeding of the present carcinogens and diets caused changes in the amounts of the analogs of the microsomal antigens in the liver supernatant fraction. Levels are indicated as intensities of cross reaction under comparable conditions, as follows: 0, absent; 1, weak; 2, medium; 3, strong. Weak reactions include precipitations so faint that the gel bands are only slightly deflected toward the wells containing supernatant proteins. Table 3 presents average values (to the nearest 0.5) of the levels of the 8 supernatant analogs in rats fed the 5 diets.

Of the 8 antigen analogs encountered, only those of the α, β, ε2, and δ displayed changes in amount attributable to carcinogen and diet. Less of the α analog was present after carcinogen feeding. In contrast, the supernatant form of tissue-specific β antigen was usually elevated in carcinogen-fed rats. The ε2 analog was detectable only with rats fed FAA and its control diet, apparently the result of the control grain diet. The most striking change attributable to carcinogen was the sharp elevation in the level of the δ analog. It is yet to be determined if this effect is specific for carcinogens.

Table 2

<table>
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<th>Antigens</th>
<th>Gel band positionsa</th>
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Differential characterization of the antigens of normal rat liver microsomes.

a Reactions with nonspecific microsomal antiserum.
b Assayed as column extract (see text).
c Liver microsomal antiserum absorbed with kidney microsomes.

Table 3

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Intensities of the reactions of the supernatant analogs of the liver microsomal antigens with unabsorbed antimicrosomal serum. Intensities indicate relative amounts of analogs in liver supernatants. FAA, N-2-fluorenylaceticamide (2-acetylaminofluorene); 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene.
None of the 8 microsomal antigens appears to be the principal target of the binding by the azo dye and fluorenyl metabolites of the present carcinogens. This conclusion is based on the assumption that liver supernatant analog conjugated with carcinogen metabolite would cross-react with unconjugated microsomal antigen. The electrophoretic profile localizations of the soluble protein carcinogen conjugates of livers of rats fed aminoazo dyes and FAA have been reported (Table 1). Thus, both pools 4 (a1) and 17 (slow g2) consistently contain the 2 minor species of the liver soluble azoproteins and fluorenylproteins (31–33). Likewise, pools 25 (fast h2) and 27 (slow h2) possess the principal fluorenylproteins and azoproteins, respectively (31–33). When the antigen analogs and the soluble carcinogen-protein conjugates have different electrophoretic properties. For example, the supernatant analog of the tissue-specific ß migrates between the electrophoretic classes h2 and g1-b2, both of which have among the least amount of the carcinogen-protein conjugates (31, 33). Further, all 8 antigen analogs appear to be immunologically unrelated to the conjugates. The case nearest to correspondence relates to the e2 analog and the slow h2 azoproteins. However, these 2 are different, as judged by their diverse response to carcinogen feeding. The e2 analog is absent, whereas the slow h2 azoproteins are present, in the soluble liver fraction of azocarcinogen-fed rats. In FAA-fed rats, the e2 analog at the slow h2 and the fluorenylproteins at the fast h2 have different profile locations.

DISCUSSION

The microsomal and supernatant fractions of rat liver appear to share at least 8 antigens in common. Their detection, as described, is quite reproducible if the antibody titer is significantly high, with the possible exception of the e2. A mixture as heterogeneous as the microsomal fraction would ordinarily be unsuited to be used as antigen. Ribosomes, smooth and rough membranes, soluble proteins, lysosomes, and mitochondria may be present. Complications are reduced by the avoidance of mitochondria by the method of Vogt (34, 35). Further, the situation is greatly simplified by the fact that, unlike the supernate, the microsomal fraction, fortunately, contains only a few strongly reacting antigens, permitting their unambiguous identification.

The most interesting microsomal antigen is the tissue-specific ß. It, alone, reacts intensely with kidney-adsorbed antiserum against liver microsomes, but not with antiserum against serum globulins. Weiler found that the antigen does not react with antiserum against the particulate fractions of lung, heart, spleen, testicle, and kidney. Absorption of the antiserum against liver microsomes, made tissue-specific by absorption with kidney microsomes, strongly stains normal liver. After additional absorption with the solubilized fraction of liver microsomes, reaction with normal liver almost completely disappears. The same consideration applies to complement fixation. Thirdly, that the extracted ß microsomal antigen cross-reacts with only 1 supernatant macromolecule, favors there being only 1 extracted microsomal tissue-specific antigen. Fourthly, highly purified ß antigen appears to be associated with 1 enzymatic activity (below). Hence, apparently no tissue-specific antigen in liver microsomes other than the extracted ß antigen was detected in previous studies.

The ß antigen extracted from liver microsomes has recently been purified to the point of giving 1 band in polyacrylamide gel electrophoresis. The antigen is a protein with a sedimentation constant of 10 (5). The precipitation bands of this and the other antigens do not react with Sudan Black, indicating the absence of lipoprotein. Esterase (EC.3.1.1.1) activity is present. The present study shows that at least the supernatant analogous of the antigen, if not the antigen itself, is immediately negatively charged at pH 8.6.

The ß antigen is not tissue-specific in the strict qualitative sense (5). A more apt term for the antigen is probably “tissue-distinctive.” While the liver ß antigen does not react with antiserum against kidney microsomes, Frank (unpublished studies; coworker of H. Friedrich-Freksa) recently found that extraction of kidney microsomes yields antigen which absorbs antibodies against the liver ß antigen. In agreement, a constituent in kidney microsomal extract gives a very weak band continuous with that of the liver ß antigen in gel immunodiffusion. The quantity of the kidney antigen in relation to protein is only about a fiftieth of that in liver. Frank also found that the content of ß antigen in the liver of newborn rats is about as low as in adult rat kidney. At 3 weeks of age, the amount of ß antigen in liver rises markedly. Thus, the difference in

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3 An instability sometimes occurs in which the principal fluorenyl proteins, rather than being only at the fast h2, are also or only at the slower h1 (pool 24) (S. Sorof and M. Young, unpublished study). That the supernatant analog of the e1 antigen peaks only and consistently at the slower h1 position appears to rule out the e1 antigen as a possible target of fluorenyl carcinogen.

4 The slow h2 proteins also contain the principal soluble liver azoproteins 48 hours after a single intragastric dose of 3′-Me-DAB (S. Sorof, M. Young, and P. L. Fetterman, unpublished data).

5 K. U. Hartmann, R. Süss, and H. Friedrich-Freksa, unpublished data.
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Harold P. Morris for a kind gift of this tumor.

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In immunophoresis and electrophoresis in polyacrylamide gel, rat serum displays 2 bands which move faster than albumin. These pre-albumins react both with the tissue-specific microsomal antiserum and antisera against serum globulins, and may be the two α antigens. On the other hand, in column electrophoresis the stable α analog of liver supernate migrates slightly slower than does serum albumin.

The occurrence of the α antigens in microsomal extract conceivably could result from a contamination of serum proteins. This seems improbable, since many other serum components are absent from the microsomal fraction according to tests with antisera globulins. Therefore, in agreement with the view of Perlmann et al. (24), the α antigens may be inherent in liver. A plausible hypothesis seems to be that the two α antigens may be serum pre-albumins produced in the liver microsomes.

Of all the nonspecific antigens in liver microsomal extract, the δ reacts most intensely with unabsorbed antiserum. The reaction is even stronger than that of the β. In immunophoresis and in polyacrylamide gel electrophoresis of the microsomal extract, the α antigen migrates as a very sharp band. However, where present in liver supernatant electrophoretic fractions, the δ analog extends over a broad region and gives a diffuse band in serial gel diffusion (Table 1; Fig. 2, upper), possibly because of chromatography on the ethanolized cellulose. A peculiarity of the δ antigen is that it also cross-reacts with extract of spleen microsomes. It is improbable that this presence in spleen microsomes results from a serum contamination, since other components which cross-react with serum globulins are absent. Microsomal δ antigen, purified to a point of giving 1 band in polyacrylamide gel electrophoresis, has an esterase (E.C.3.1.1.1) activity which is much more sensitive to inactivation by disopropylfluorophosphate than is the esterase activity of the solubilized β antigen.

Relatively little is known about the other nonspecific antigens. Differentiation between the supernatant analogs of the σ1 and σ2 was sometimes obscure. In some experiments, the σ1 analog extended into the electrophoretic region specified for that of the σ2 in Table 1. The soluble microsomal antigens observed by D’Amelio and Perlmann was to be present in the h2 and h3 proteins were apparently the analogs of the σ2 and σ1 (4).

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Fig. 1. Immunophoretic analyses of fractionated extract of normal rat liver microsomes. Two different runs were carried out under the same conditions. Upper halves of photograph and diagram show the reaction against tissue-specific antiserum (absorbed with kidney microsomes). Lower halves show the reaction against unabsorbed antiserum. See text for medium and conditions of immunophoresis.

Fig. 2. Serial gel immunodiffusion of the supernatant liver proteins cross-reacting with the soluble microsomal antigens of liver. Upper series, the wells in the middle row contain fractions of supernatant liver proteins in the order of decreasing negative electrophoretic mobility (from left (fast) to right (slow)). The 1st central hole at the left contains the column extract used for electrophoresis (Table 1). The holes in the top row contain unabsorbed antiserum against microsomal extract; those in the bottom row contain tissue-specific antiserum (absorbed with kidney microsomes). The supernatant liver proteins are those from rats fed N-2-fluorenylacetamide for 5 weeks. Lower series, same as above, except that the rats were fed the appropriate control diet for 5 weeks.
Fig. 3. Hexagonal arrangements of gel immunodiffusion analysis. (a) Left: Middle hole contains unabsorbed antiserum against extract of normal liver microsomes. The upper 3 holes contain the supernatant liver proteins (column extract) of rats fed FAA for 5 weeks. The lower 3 holes have extract of normal liver microsomes. (b) Right: The same arrangement as in a, with the exception that the middle hole contains tissue-specific (absorbed) antiserum against extract of normal liver microsomes.
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