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Immunological Unresponsiveness to Specific Antigenic Determinants in  
Human  $\gamma$ G Globulin.\* (32085)

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Immunological tolerance to serum protein antigens can be readily induced in many animal species(1). Usually such induction is effected during the prenatal and neonatal periods and involves repeated and massive immunizations with antigen(2).

Recently, Dvorak *et al*(3,4) have described a means of rendering adult guinea pigs, with an intact immunological apparatus, unresponsive to a wide variety of antigens, by means of a single intravenous injection of a relatively small (5 mg) quantity of aqueous antigen. Such treatment arrests the development of the immune response, so that when the animals are simultaneously sensitized with the same antigen incorporated in complete Freund's adjuvant, no circulating antibody or delayed sensitivity to the antigen is detectable.

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Immunological suppression of this type is highly specific in that the immune response to other, unrelated, antigens remains unimpaired (3). It was the purpose of the present study to see whether animals could be sensitized to some antigenic determinants on an antigen, whilst being rendered "tolerant" to other determinants on the same molecule. The  $\gamma$ G globulin molecule is an ideal antigen for this purpose, being easily degraded by proteolytic digestion into fragments that have well characterized antigenic determinants. Consequently, adult guinea pigs were immunized with whole human  $\gamma$ G globulin (HGG) in complete Freund's adjuvant, and were simultaneously administered digestion fragments of  $\gamma$ G globulin by the intravenous route.

The specificity and degree of tolerance induced were studied by delayed hypersensitivity measurement, by hemagglutination, by passive cutaneous anaphylaxis (PCA) and by radioimmuno-electrophoresis using I<sup>131</sup>-labeled antigens.

*Materials.* Human  $\gamma$ G globulin was prepared from pooled normal serum by diethylaminoethyl (DEAE) cellulose (Mann Research Laboratories, New York) chromatography. The fraction eluted with 0.005 M phosphate buffer, pH 8.0, was collected, concentrated by ultrafiltration, and shown to be homogeneous by gel-diffusion precipitin analysis and by immunoelectrophoresis, employing a goat antiserum against whole human serum.

*Fragments of human  $\gamma$ G globulin.* The above preparation of HGG was digested with papain by the method of Porter(5), and the digestion mixture subfractionated with  $(\text{NH}_4)_2\text{SO}_4$  by the method of Rowe(6). The protein remaining in the supernatant at 55% saturation, was precipitated at 75% saturation, and used as the source of  $F_{ab}$ . Gel-diffusion precipitin analysis employing specific antisera showed the preparation to be free of contamination with  $F_c$  fragment.

The precipitate formed from papain digested HGG by 40% saturation with  $(\text{NH}_4)_2\text{SO}_4$  was dissolved in saline, and subjected to block electrophoresis in Pevicon C-870 (Fosfatbolaget, Sweden). Each fraction was tested against rabbit antisera specific for either  $F_c$  or  $F_{ab}$  and the fractions containing only  $F_c$  were pooled.

HGG was hydrolysed by pepsin to obtain the 5 S fragment,  $(F_{ab'})_2$ , by the method of Nisonoff *et al*(7). A 1%  $\gamma$ G globulin solution in 0.2 M acetate buffer, pH 4.5, was incubated with pepsin at an enzyme: substrate ratio of 1:100, for 16 hr at 37°C. The digestion mixture, following dialysis against 0.1 M phosphate buffer, pH 7.5, containing 0.15 M NaCl, was subjected to Sephadex G-200 (Pharmacia, Uppsala, Sweden) gel-filtration, and the fractions lacking the antigenic determinants of the  $F_c$  fragment of the molecule were pooled and concentrated.

*$\gamma$ A myeloma protein.*  $\gamma$ A myeloma protein was prepared from a patient's serum by Pevicon block electrophoresis, followed by DEAE cellulose column chromatography(8). On immunoelectrophoresis, the preparation gave a single precipitin band against anti-human serum.

*Anti-guinea pig  $\gamma$ -globulins.* Guinea pigs were immunized with ovalbumin in complete

Freund's adjuvant (Difco Laboratories, Detroit, Mich.), and the  $\gamma_1$  globulin fraction of the antisera obtained by block electrophoresis (9). Rabbits were immunized with the  $\gamma_1$  globulin fraction included in complete Freund's adjuvant. The resulting rabbit antiserum gave both  $\gamma_1$  and  $\gamma_2$  precipitin bands with the guinea pig antiserum on immunoelectrophoresis.

*Methods. Immunodiffusion.* Double diffusion in agar was performed by the method of Ouchterlony(10). After incubation for 48 hours at room temperature, the plates were washed and precipitin bands stained with amido-Schwartz 10B. Radioimmuno-electrophoresis was carried out by the method of Yagi *et al*(11). Antigens were labeled with  $\text{I}^{131}$  by the chloramine-T method of McConehey and Dixon(12). Serum samples were electrophoresed according to the method of Scheidigger(13) and precipitin lines developed using a rabbit antiserum specific for guinea pig  $\gamma_1$  and  $\gamma_2$  globulins. Following washing,  $\text{I}^{131}$ -labeled antigens were added to the antibody troughs. The plates were then washed for 2 to 3 days and wrapped with cellophane (Saran-wrap). Radioautographs were obtained by placing X-ray film (Kodak Industrial X-ray film, Type KK) in contact with the plates.

*Hemmagglutination.* Hemagglutination was carried out by the method of Daniel *et al*(14) employing tannic acid coated sheep erythrocytes. Cells coated with HGG,  $F_c$  and  $F_{ab}$  were used.

*Delayed hypersensitivity.* Delayed hypersensitivity measurements were evaluated 24 hrs following intradermal challenge with 10  $\mu$ g protein. The level of induration and erythema were arbitrarily assessed on a plus score system.

*Passive cutaneous anaphylaxis.* PCA titrations were determined by the method of Ovary(15). Serial 3-fold dilutions of guinea pig antiserum were introduced intradermally into the shaven backs of guinea pigs, in 0.1 ml aliquots. Three hours after intradermal injection, 1 mg aqueous antigen in 0.5 ml 1% Evans blue dye was administered intravenously. The highest dilution of antiserum which gave a definite 'blueing' reaction in the

TABLE I. The Effect of Intravenous Administration of Aqueous Human  $\gamma$ G Globulin Digestion Fragments on the Immune Response to a Concomitant Sensitization with the Whole  $\gamma$ G Globulin Molecule.\*

Intravenous antigen	Immune Response								
	Delayed Hypersensitivity			PCA titers			Precipitating antibodies first detectable		
	HGG	F <sub>ab</sub>	F <sub>c</sub>	3 wks after immunization			HGG	(F <sub>ab</sub> ) <sub>2</sub>	F <sub>c</sub>
None	++	++	+++	810	270-810	270	4 wk	5 wk	4 wk
F <sub>c</sub>	++	+++	—	810-2400	270-810	90	4 wk	5 wk	—†
F <sub>ab</sub>	++	—	+++	810	90	810	4 wk	—	4 wk
(F <sub>ab</sub> ) <sub>2</sub>	+	—	++	810	30	270	4 wk	—	5 wk
$\gamma$ A	+	—	+	2400	90	810	4 wk	—	4 wk
HGG	—	—	—	<30	<30	<30	—	—	—
F <sub>ab</sub> + F <sub>c</sub> (Digestion mixture)	++	—	—	810-2400	<30	<30	4 wk	—	—

\* Each animal received 60  $\mu$ g HGG in complete Freund's adjuvant *via* the hind footpads.

† —not detected in a period up to 9 weeks.

skin was taken as the titer of the serum.

*Experimental results.* Adult guinea pigs weighing approximately 400 g were immunized in the rear footpads with 60  $\mu$ g of human  $\gamma$ G globulin (HGG) in complete Freund's adjuvant. Immediately following this stimulatory dose of antigen, the animals (in groups of 6) were given an intravenous dose of 5 mg aqueous antigen comprising some digestion fragment of HGG. The immune response was followed at weekly intervals by gel-diffusion precipitin analysis, by delayed hypersensitivity (DHS) measurement and radioimmuno-electrophoresis using HGG, F<sub>c</sub> and F<sub>ab</sub> as test antigens. On occasions, hemagglutination and PCA titrations were also carried out.

*Effect of intravenous administration of the whole HGG molecule.* A control series of animals was subjected simply to a single dose of 60  $\mu$ g HGG in complete Freund's adjuvant into the rear footpads. Hemagglutination and PCA measurements showed an antibody response as early as 2 weeks later. DHS measurements, effected by evaluating the skin reaction 24 hours after intradermal challenge with 10  $\mu$ g antigen, showed that the immune response was directed against antigenic determinants found in both the F<sub>ab</sub> and F<sub>c</sub> portions of the HGG molecule. This finding was also demonstrated by hemagglutination and PCA titrations. Precipitating antibodies were initially detected by gel-diffusion precipitin analysis, 4 weeks after stimulation. They were likewise directed against both the F<sub>ab</sub> and F<sub>c</sub>

portions of the HGG molecule (Table I). Radioimmuno-electrophoresis, employing I<sup>131</sup>-labeled HGG, F<sub>c</sub> and F<sub>ab</sub> showed that the major antibody response in each case was of the  $\gamma$ <sub>1</sub> type, although much  $\gamma$ <sub>2</sub> antibody was also demonstrable (Fig. 1).

When an intravenous administration of 5 mg HGG accompanied the dose of 60  $\mu$ g HGG in complete Freund's adjuvant *via* the footpad, the immune response was delayed, and of an extremely low order. No sensitization to HGG or its papain digestion fragments was detectable by DHS measurement. PCA titers, against HGG, 3 weeks after immunization, were less than 1:30 compared with control (no intravenous HGG) titers of greater than 1:2000. Precipitation and hemagglutinating antibodies failed to develop throughout the period of study (10 weeks) (Table I).

It is thus apparent that intravenous administration of aqueous antigen causes a suppression of both the  $\gamma$ <sub>1</sub> and  $\gamma$ <sub>2</sub> antibody response to below the levels of detection.

*Effect of intravenous administration of F<sub>c</sub>.* No delay in the immune response was noted following intravenous administration of 5 mg F<sub>c</sub>, along with the footpad injection of HGG, compared with the control group receiving HGG alone. Hemagglutination and PCA titrations, as well as DHS measurements, demonstrated an immune response 2 weeks after immunization. The specificity of the response however was strikingly different (Table I). DHS measurements demonstrated most clearly that the response in this case was

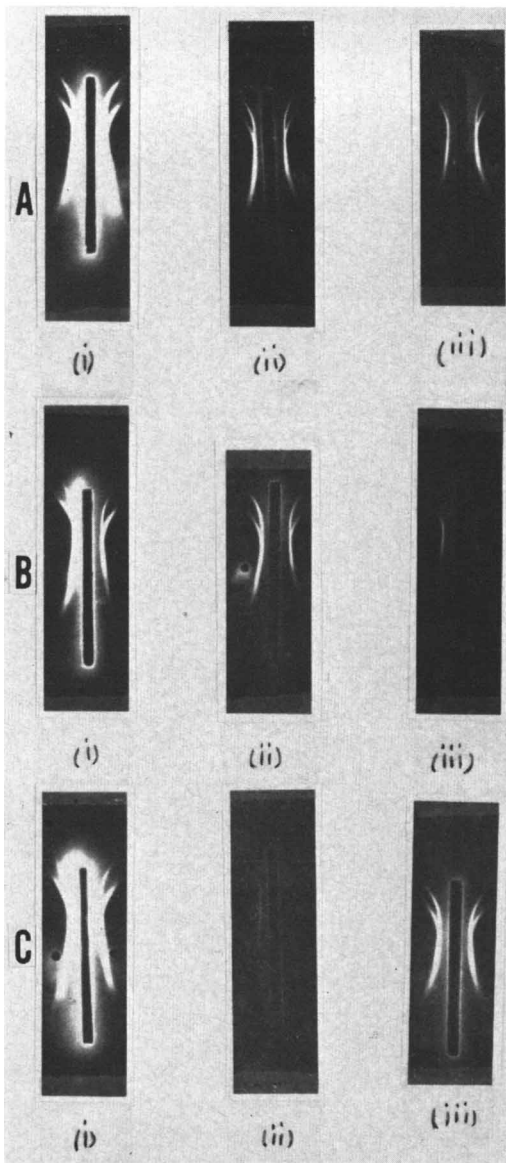


FIG. 1. Radioimmuno-electrophoresis of guinea pig sera obtained 4 weeks after immunization. All animals received 60  $\mu$ g HGG *via* the hind footpad; in addition group B received 5 mg  $F_{ab}$  and group C 5 mg  $F_c$  intravenously. The  $\gamma_1$  and  $\gamma_2$  precipitin bands of the sera were specifically developed with a rabbit antiserum. After washing, radioactively labeled (i) HGG (ii)  $F_c$  and (iii)  $F_{ab}$  were applied to the antibody troughs.

directed almost exclusively against antigenic determinants located in the  $F_{ab}$  region of the  $\gamma$ G globulin molecule; 10  $\mu$ g  $F_c$  administered intradermally caused neither induration nor erythema. PCA and hemagglutination data

likewise illustrated this effect, as did radioimmuno-electrophoretic analysis employing  $I^{131}$ -labeled HGG,  $F_{ab}$  and  $F_c$ . Thus, PCA titers were of the order of 1000 when HGG or  $F_{ab}$  were used as antigens, but were approximately 10 times less when  $F_c$  was used as challenging antigen (Table I). Hemagglutination titers were in excess of 1000 when HGG or  $F_{ab}$  were used as 'coating' antigens on the red cell surface, but were negligible when  $F_c$  was used. Precipitating antibodies directed exclusively against the  $F_{ab}$  portion of the HGG molecule were initially detectable 4 weeks after immunization. The specificity of the antibody produced in this group of animals, compared with those animals receiving HGG alone, is well illustrated by the radioimmuno-electrophoretograms using  $I^{131}$ -labeled HGG,  $F_{ab}$  and  $F_c$  shown in Fig. 1.

Thus, following suppression of HGG immunization by intravenous administration of  $F_c$ , both the DHS response and the circulating antibody were directed almost exclusively against antigenic determinants in the  $F_{ab}$  portion of the HGG molecule.

*Effect of intravenous administration of  $F_{ab}$ ,  $(F_{ab}')_2$  and  $\gamma A$  globulin.* Again, no absolute delay in immune response against HGG was noted when intravenous administration of either the  $F_{ab}$  monomer or dimer accompanied the sensitizing dose of HGG. Once again, however, the specificity of the response was markedly different from that of the control group of animals receiving HGG alone. DHS measurements showed that when either  $F_{ab}$  or  $(F_{ab}')_2$  were given intravenously as suppressive antigens, these animals were sensitized solely against the  $F_c$  portion of the  $\gamma$ G globulin molecule (Table I). PCA titrations, 3 weeks after immunization, likewise illustrated this effect showing much higher titers when  $F_c$  or HGG were used as challenging antigens (titers  $\sim 800$ ) than when  $F_{ab}$  was used (titers 30-90) (Table I). Radioimmuno-electrophoretic analysis using  $I^{131}$ -labeled  $F_{ab}$  as antigen, revealed only very low amounts of antibody directed against this portion of the molecule. This was in direct contrast to the amount of antibody observed using radioactively-labeled  $F_c$  or HGG as antigens (Fig. 1).

The rate of production of precipitating antibodies against the  $F_c$  portion of the molecule seemed little affected by the intravenous regime. Precipitating antibodies against both the whole HGG molecule and its  $F_c$  fragment were detectable 4 weeks after immunization, the same time as was observed in animals receiving HGG alone. Unlike the control group, however, no precipitating antibodies against either the  $F_{ab}$  monomer or dimer were detectable in these animals.

When 5 mg human myeloma  $\gamma A$  globulin was given intravenously, an overall effect similar to that caused by administration of the  $F_{ab}$  portion of the  $\gamma G$  globulin molecule was noted; the major antibody response being directed against the  $F_c$  portion of the  $\gamma G$  globulin molecule (Table I).

*Effect of intravenous administration of papain-digested HGG.* When HGG was subjected to papain digestion, and the resulting digestion mixture containing  $F_{ab}$  and  $F_c$  fragments, (separated from undigested HGG by passage through Sephadex G-100), was used to induce tolerance by intravenous dosage, far different results were obtained than when the intact molecule was used as suppressing antigen.

Three weeks after antigenic stimulation, PCA titers against HGG were of the order of 1000, while when  $F_{ab}$  or  $F_c$  were used as test antigens, either separately or as a mixture, no PCA activity was demonstrable (Table I). DHS measurements likewise showed that the animals were sensitized against the intact HGG molecule, but to neither the  $F_{ab}$  nor  $F_c$  fragments (Table I).

This finding was confirmed by gel-diffusion precipitin analysis. Sera obtained from animals 4 weeks after injection of a mixture of  $F_{ab}$  and  $F_c$  intravenously, gave precipitin bands against HGG but not against  $F_{ab}$  (monomer or dimer) or  $F_c$ , or a mixture of  $F_{ab}$  and  $F_c$ . Moreover, the precipitin band between HGG and these antisera was not prevented by prior diffusion of the sera against a mixture of  $F_{ab}$  and  $F_c$ , before insertion of intact HGG as antigen. Using  $I^{131}$ -labeled HGG,  $F_{ab}$  and  $F_c$ , the much more sensitive technique of radioimmuno-

phoresis again revealed both  $\gamma_1$  and  $\gamma_2$  antibodies directed solely against the intact molecule. In order to verify this specificity, sera were mixed with a papain digest containing both  $F_{ab}$  and  $F_c$ , and the resultant mixture re-analyzed by radioimmuno-electrophoresis. Again, a radioactive precipitin band was detected when  $I^{131}$ -HGG was used as antigen, but no such band was obtained with either  $I^{131}$ - $F_{ab}$  or  $I^{131}$ - $F_c$ .

These findings indicate that the antigenic determinant(s) demonstrated here are not represented in either the  $F_{ab}$  or  $F_c$  portions of the HGG molecule.

*Discussion.* The data presented here clearly show that in the guinea pig at least, tolerance to some antigenic determinants can be induced, concomitant with sensitization to other determinants on the same antigen molecule. The animals' immune mechanism is apparently capable of 'subtracting' those determinants shared by both suppressive and sensitizing antigens from those found in the sensitizing antigen alone. Thus, when the  $F_c$  portion of HGG is administered as suppressing antigen, simultaneously with a sensitizing dose of whole HGG, the immune response is basically to the  $F_{ab}$  portion of the molecule. On the other hand, when either  $F_{ab}$ ,  $(F_{ab}')_2$  or human  $\gamma A$  globulin, which shares common light chains with HGG, are used as suppressants, the response is basically against those determinants found uniquely on the HGG molecule, *i.e.*, the  $F_c$  portion. As the  $(F_{ab}')_2$  dimer was more effective at inducing immunological suppression to this part of the molecule than was the monomer, it would appear that tolerance is induced more readily by compounds of greater antigenicity. The relative inefficiency of a  $\gamma A$  myeloma protein in rendering tolerance to the  $F_{ab}$  portion of HGG may reflect paucity of one of the light chain types of HGG in this preparation. Thus, tolerance to only one of the two light chain types present in HGG may have been induced. The ineffectiveness may also reflect the differences in the heavy chain determinants of the  $F_{ab}$  portions of human  $\gamma A$  and  $\gamma G$  globulin.

When papain digested HGG, containing both  $F_{ab}$  and  $F_c$  fragments but no undigested

material was used as suppressing antigen, antibodies directed against the whole intact molecule but against neither  $F_{ab}$  nor  $F_c$  were demonstrable. Whether this response, represents an antibody directed against the configurational structure of HGG, or whether it demonstrates a primary structural determinant destroyed by papain has been examined exhaustively. These findings will be published elsewhere.

The findings presented here, parallel very closely those found when rabbits, tolerant to one antigen, are challenged with a cross-reacting antigen. Thus, Curtain(16) found that when rabbits tolerant to a Bence-Jones protein were immunized with the related myeloma  $\gamma G$  globulin, the antibody response was directed only to the antigenic determinants specific for the myeloma protein. Similarly, Weigle(17) has shown that the initial antibody response of rabbits rendered tolerant to BSA and challenged with HSA, was directed specifically against HSA. Despite the close antigenic similarity between HSA and BSA, little or no antibody against their shared determinants was detectable.

Although all the methods used to assess the immune suppression demonstrated the same general pattern, estimation of the degree of tolerance induced, and its absolute specificity, was found to vary. Thus, DHS measurements demonstrated that the effect of  $F_{ab}$  or  $F_c$  administration was absolute, and specific: that when either of these portions was administered intravenously, no sensitization to this part of the molecule could be shown. On the other hand, PCA (Table I) and radioimmuno-electrophoretic data (Fig. 1) demonstrated that small amounts of antibody were formed to those determinants introduced intravenously, and that therefore the tolerance was only partial. Such discrepancies may represent a further example of non-correlation between DHS and circulating antibody, or may simply be an extension of the sensitivity or threshold limits of the systems used for testing.

Despite earlier findings(3) that tolerance induced in the manner described here primarily affects  $\gamma_2$  antibody, while little affecting the  $\gamma_1$  response, the impression gained

from these studies was that the levels of both  $\gamma_1$  and  $\gamma_2$  antibodies were significantly lowered and that the depression of the  $\gamma_2$  antibody level (being initially of lower concentration) is more easily rendered below the level of detection. The demonstration of antibody response essentially specific for the intact antigen molecule might suggest that this is a general phenomenon representing an early manifestation of immunological response to antigenic stimulation. This is being pursued with a number of other antigens.

The technique of concomitant suppression and sensitization to different antigenic determinants on the same antigen molecule would seem to afford an excellent means of defining more precisely those antigenic determinants responsible for many autoimmune phenomena, *e.g.*, rheumatoid arthritis and thyroiditis, in which a body constituent, becomes altered and, thereby, autoantigenic.

*Summary.* Immunological unresponsiveness to HGG fragments was induced by intravenous administration of 5 mg soluble antigen. Concomitantly the animals were sensitized to the intact HGG molecule, by injection of 60  $\mu g$  protein, in complete Freund's adjuvant, into the rear footpads. The immune response was followed at weekly intervals by delayed hypersensitivity measurements, by PCA titrations, by gel diffusion precipitin analysis and by radioimmuno-electrophoresis. It was found that when either  $F_{ab}$  or  $F_c$  fragments were given intravenously, together with an injection of HGG in the footpad, the animal became tolerant to those antigenic determinants represented in the fragments, but was simultaneously sensitized to the other antigenic sites on the molecule.

When papain digested HGG (free from undigested material) was administered intravenously, however, antibodies directed against the whole intact molecule, but not against either  $F_{ab}$  or  $F_c$  fragments, were demonstrable. It would appear that the animals' immune mechanism is capable of 'subtracting' those determinants shared by both suppressive and sensitizing antigens, from those found in the sensitizing antigen alone.

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### Species Variations in the *N*- and Ring-Hydroxylation of 2-Acetylaminofluorene and Effects of 3-Methylcholanthrene Pretreatment.\* (32086)

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Rats treated with the carcinogen 2-acetylaminofluorene (AAF) excrete major fractions of the administered compound in the urine as the *N*-, 3-, 5-, and 7-hydroxy derivatives (1-3) and in the bile as *N*-hydroxy-AAF(4). The liver endoplasmic reticulum is a major site of the enzyme systems which perform these hydroxylations, all of which require TPNH and oxygen(5-7). The activity of the hepatic aromatic ring-hydroxylases increases approximately 10-fold when weanling rats are treated 24 hours prior to assay with 1 mg of 3-methylcholanthrene (MC)(5), and administration of MC with AAF inhibits the carcinogenicity of AAF in the rat (8,9). Liver preparations from a variety of other rodent species also hydroxylate AAF (5-7).

The relative amounts of ring- and *N*-hydroxylation of AAF are of importance, since ring-hydroxylation leads to non-carcinogenic metabolites(10-12), while *N*-hydroxylation is

one of the steps required for activation of the amide(11,13). This paper presents data on the relative amounts of hydroxylation at the *N*-, 3-, 5-, and 7-positions of AAF by liver microsome preparations from weanling rodents either with or without prior treatment with MC. Marked species differences were found in the relative amounts of hydroxylation of AAF at these positions before and after the administration of MC.

*Materials and methods. Animals.* Male weanling animals were obtained from the following dealers in Madison, Wis.: albino rats, Holtzman Rat Co.; Syrian golden hamsters, Con Olson Co.; Ha/ICR mice, A. R. Schmidt Co.; guinea pigs, O'Brien Co.; New Zealand rabbits, Willard Voss. They were fed *ad libitum* a grain diet(14) which, for the guinea pigs, was supplemented with 1 g of ascorbic acid/kg. In those experiments in which puromycin or actinomycin D was injected, food was withheld from all animals after these injections were started.

*Chemicals.* *N*-Hydroxy-AAF(11,15), 2-acetylaminophenanthrene(16), and *N*-hydroxy-2-acetylaminophenanthrene(17) were synthesized in this laboratory. 7-Fluoro-AAF and its *N*-hydroxy derivative were generously furnished by Dr. T. Lloyd Fletcher, University

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