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Identification of epoxide hydrase as the preneoplastic antigen in rat liver hyperplastic nodules

(hepatocarcinogens/2-acetylaminofluorene)

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ABSTRACT A liver microsomal protein, previously referred to as preneoplastic antigen, from hyperplastic nodules of rats fed a diet containing 2-acetylaminofluorene has been identified as the enzyme epoxide hydrase [glycol hydro-lyase (epoxide-forming), EC 4.2.1.63]. Purified preneoplastic antigen from hyperplastic nodules and purified rat liver microsomal epoxide hydrase are immunochemically identical on the basis of Ouchterlony double-diffusion analysis. In addition, the purified proteins have identical minimum molecular weights in sodium dodecyl sulfate/polyacrylamide gels, and both proteins catalyze the hydration of arene oxides to dihydrodiols. Chronic feeding of 2-acetylaminofluorene to rats results in a 5- to 7-fold increase in epoxide hydrase activity in rat liver. The induced level of the enzyme is maintained in developing hyperplastic nodules and hepatomas but not in the nontumor tissue after removal of the carcinogen from the diet.

Since the discovery of an experimental model of liver carcinoma induced by *o*-aminoazotoluene (1), hyperplastic nodules have received major attention as possible precursors of liver cancer. Studies with several hepatocarcinogens, including 2-acetylaminofluorene (AAF), ethionine, nitrosamines, aflatoxin B_1, and safrole, have implicated hyperplastic nodules as precursor lesions of hepatomas (cf. 2). A more direct relationship between hyperplastic nodules and the cancers that ultimately develop came from studies by Farber and his associates (3, 4) in which procedures were described for producing well-defined hyperplastic nodules in rats treated with AAF or ethionine. A similar type of hyperplastic lesion has been proposed to be involved in the development of liver cancer in human cirrhosis (5, 6). Studies on the role of these preneoplastic lesions in the development of cancer have been hindered by the lack of biochemical markers to distinguish them from the normal cell population. A biochemical marker unique to hyperplastic nodules could prove useful as a tool to study early changes in the liver related to the onset of cancer.

Farber and coworkers (4, 7, 8) have recently described a preneoplastic antigen (PN antigen) in rat liver hyperplastic nodules induced by AAF or ethionine. This antigen was originally detected by using rabbit antibodies produced in response to a post-mitochondrial supernatant fraction from rat liver hyperplastic nodules. The polyspecific antiserum obtained was absorbed with a 9,000 × *g* liver supernatant fraction from untreated animals. After the antibody–antigen complexes were removed by sedimentation, the remaining antiserum was monospecific for a protein (PN antigen) that was not detectable in normal liver by Ouchterlony double-diffusion analysis.

The PN antigen, located primarily in the smooth endoplasmic reticulum (8), was detected immunologically in hyperplastic nodules and hepatomas induced by the hepatocarcinogens AAF, ethionine, safrole, aflatoxin B_1, nitrosamines, and aminoazo dyes but not in fetal cells, liver cells damaged by hepatotoxins, regenerating liver, or normal liver cells surrounding hyperplastic nodules (4, 7). Recently, however, Griffin and Kizer (9) showed by immunodiffusion and immunoelectrophoresis in the presence of deoxycholate that PN antigen was present in normal rat liver at approximately one-fourth the concentration found in hyperplastic nodules. The PN antigen has been purified (9) and the present report identifies the protein as the microsomal enzyme epoxide hydrase [glycol hydro-lyase (epoxide-forming), EC 4.2.1.63].

MATERIALS AND METHODS

Animals. Male Holtzman rats (150–170 g) were fed a basal diet (10) with or without 0.05% AAF for a period of 13–16 weeks according to the following protocol as originally described by Epstein *et al.* (3): 3 weeks on basal diet containing AAF, 1 week on basal diet, 2 weeks on diet containing AAF, 2 weeks on basal diet, and 3 weeks on diet containing AAF followed by removal of the carcinogen from the diet until termination of the experiments. Intermittent feeding of AAF produces a high incidence of large hyperplastic nodules in rat liver (3). Control rats received only the basal diet.

Enzymes. Animals were killed by cervical dislocation and the livers were perfused with ice-cold isotonic saline. Hyperplastic nodules were dissected out of the liver, leaving behind a rim of nodular tissue to ensure a clean separation of nodular tissue from the surrounding liver. Microsomes were prepared from hyperplastic nodules and control liver as described (8). Purification of PN antigen from hyperplastic nodule microsomes was performed by the method of Griffin and Kizer (9). Liver microsomal epoxide hydrase was purified from immature male Long–Evans rats as described by Lu *et al.* (11). The method for the production of antibodies against PN antigen has been described (4, 9) and that for purified liver microsomal epoxide hydrase will be described elsewhere.

Assay Procedures. Ten substrates for epoxide hydrase activity were used in the present study. The synthesis and specific activity of these ^{3}H-labeled substrates and the assay for epoxide hydrase activity have been described (12). The reaction mixture contained 25 μl of 0.5 M Tris buffer (pH 8.7 at 37°), 5 μl of ^{3}H-labeled substrate dissolved in acetonitrile/NH_4OH [1000:1 (vol/vol)], enzyme, and water to a final volume of 80 μl. Incubations were performed for 1–2 min at 37° and the reactions were terminated by the addition of 25 μl of tetrahydrofuran. One-third of the total mixture (35 μl) was chromatographed

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Abbreviations: AAF, 2-acetylaminofluorene; $NaDodSO_4$, sodium dodecyl sulfate; PN antigen, preneoplastic antigen.

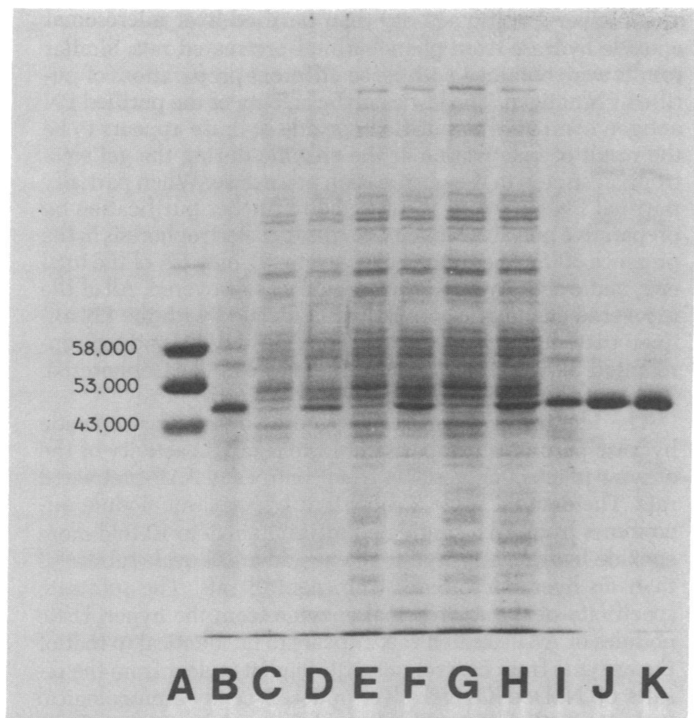


FIG. 1. Polyacrylamide gel electrophoresis of microsomes, purified PN antigen, and purified epoxide hydrase in the presence of $NaDodSO_4$. Electrophoresis was performed by the method of Laemmli (14). Samples: A, a mixture of protein standards (ovalbumin, 43,000; glutamate dehydrogenase, 53,000; catalase, 58,000); B, 0.75 μg of purified PN antigen; C, E, and G, 2, 4, and 6 μg of liver microsomal protein, respectively, from control rat liver; D, F, and H, 2, 4, and 6 μg of liver microsomal protein, respectively, from hyperplastic nodules; I, 0.75 μg of purified PN antigen; J, 0.5 μg of purified epoxide hydrase; K, mixture of PN antigen (0.75 μg) and purified epoxide hydrase (0.5 μg).

on silica gel thin-layer plates and the radioactive dihydrodiol or glycol products were separated and quantified as described (12). All assays were performed in duplicate.

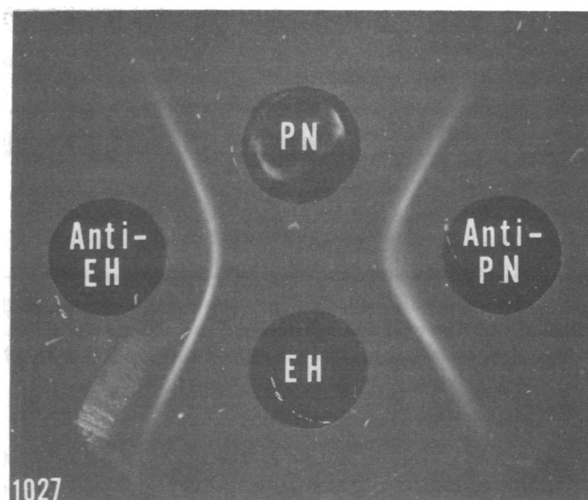
The immunodiffusion medium for Ouchterlony double-diffusion analysis consisted of 0.9% agarose, 1.0 M glycine-NaOH (pH 7.4), 0.2% Emulgen 911 (Kao-Atlas Co., Japan), 0.08 M NaCl, and 0.015 M sodium azide. The gel thickness was approximately 1 mm with wells 4 mm in diameter. The immunodiffusion plates were incubated at 23° in a humid atmosphere for 2–3 days.

Protein was determined by the method of Lowry et al. (13) and sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis was performed as described by Laemmli (14).

RESULTS AND DISCUSSION

Griffin and Kizer (9) have purified the PN antigen from AAF-induced hyperplastic nodules and have established that this protein is the PN antigen originally described by Farber and associates (4, 7, 8). Three approaches were used in the present study to identify the purified PN antigen as epoxide hydrase. The PN antigen was compared to purified rat liver epoxide hydrase in regard to (i) minimum molecular weight on $NaDodSO_4$ gels, (ii) immunological properties, and (iii) catalytic activity.

The $NaDodSO_4$ gel electrophoretic pattern of the purified antigen revealed that this protein has the same mobility as purified liver microsomal epoxide hydrase prepared from



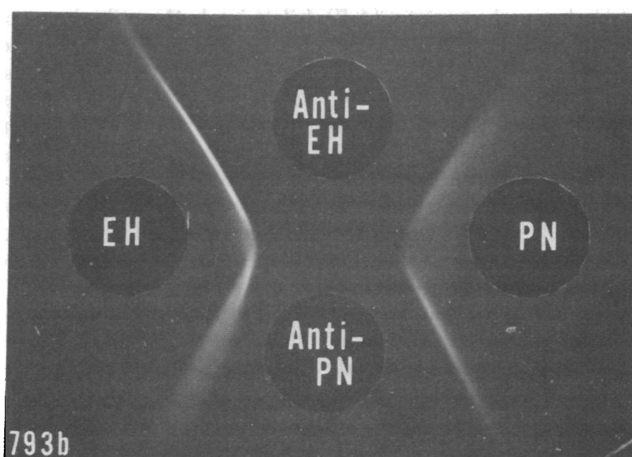


FIG. 2. Ouchterlony immunodiffusion plates with purified epoxide hydrase (EH) (0.13 mg/ml) and purified PN antigen (0.24 mg/ml). Anti-epoxide hydrase was prepared in sheep against purified rat liver epoxide hydrase. The immunoglobulin fraction was purified from immune serum and was used at a concentration of 5 mg of protein per ml. Antibody against PN antigen was produced in rabbits by the method of Farber and associates (4, 7) and the immune serum was used without further purification.

phenobarbital-pretreated rats (Fig. 1).[§] Both proteins had a minimum molecular weight of 49,000 in the $NaDodSO_4$ gel system of Laemmli (14). When the two proteins were mixed and applied to the gel, only one major protein-staining band was observed. Compared to control liver microsomes, liver microsomes from hyperplastic nodules induced by AAF had a markedly increased concentration of a specific protein with the same minimum molecular weight as PN antigen and epoxide hydrase. These results are consistent with the results of Griffin and Kizer (9) that PN antigen is present in hyperplastic nodule microsomes to at least a 4-fold greater extent than in control microsomes when determined by electroimmunoassay.

Because the $NaDodSO_4$ gel electrophoretic profile indicated that PN antigen might be the microsomal enzyme epoxide hydrase, we compared the immunological properties of these proteins. Fig. 2 shows that PN antigen and purified epoxide hydrase from phenobarbital-treated rats are immunochemically identical in Ouchterlony double-diffusion analyses. The im-

[§] The purified PN antigen shown in Fig. 1 (samples B and I) has a small amount of contaminating protein between 53,000 and 58,000.

Table 1. Epoxide hydrase activity of purified PN antigen

Purified enzyme	Substrate	Dihydrodiol formed, nmol/mg protein/min
PN antigen	Benzo[*a*]pyrene 4,5-oxide	36
Epoxide hydrase		535
PN antigen	Benz[*a*]anthracene 5,6-oxide	62
Epoxide hydrase		714

PN antigen was purified from hyperplastic nodules by the method of Griffin and Kizer (9) and epoxide hydrase was purified from the livers of phenobarbital-pretreated rats by the method of Lu *et al.* (11).

munoprecipitin bands produced by the reaction of each protein form a line of identity against anti-rat liver epoxide hydrase or antibody monospecific for PN antigen prepared by the method of Farber and associates (4, 7). Likewise, both antibody preparations recognized identical antigenic sites in PN antigen and epoxide hydrase. The absence of spurs at the intersection of the precipitin bands demonstrates that the PN antibody recognizes only a single protein (epoxide hydrase) in the PN antigen preparation. Neither the antibody prepared against epoxide hydrase nor the antibody prepared against PN antigen inhibited the catalytic activity of microsomal or purified epoxide hydrase—i.e., the immunoprecipitate retained full catalytic activity toward various alkene and arene oxides as has been found for the rat enzyme (data not shown).

Table 1 shows that the purified PN antigen metabolizes arene oxides to dihydrodiols which, with the results of $NaDodSO_4$ gel electrophoresis and antibody studies, establishes this protein as epoxide hydrase. However, the purified PN antigen had a much lower specific activity than purified liver microsomal epoxide hydrase from phenobarbital-pretreated rats. Similar results were obtained with three different preparations of purified PN antigen. The low catalytic activity of the purified PN antigen compared to purified epoxide hydrase appears to be the result of inactivation of the enzyme during the gel electrophoresis step in the purification procedure. When partially purified PN antigen was subjected to further purification by preparative polyacrylamide (7%) disc gel electrophoresis in the presence of 0.2% sodium deoxycholate (9), only 5% of the total enzymatic activity applied to the gel was recovered. All of the recovered enzymatic activity was associated with the PN antigen that eluted from the gel. The purified PN antigen represented 39% of the protein that had been electrophoresed. Total protein recovery from the gels was 89%.

The identification of PN antigen as microsomal epoxide hydrase permits a comparison of the catalytic activity of the enzyme in intact microsomes from control and AAF-pretreated rats. The data in Table 2 show that hyperplastic nodule microsomes from AAF-pretreated rats contain 6- to 10-fold more epoxide hydrase activity for alkene and arene oxide substrates than do liver microsomes from control rats. The substrate specificity of the microsomal enzyme from the hyperplastic nodules of AAF-treated rats appears to be identical to that of the enzyme from control rats. Although it is clear from the results of $NaDodSO_4$ gel electrophoresis and immunological techniques that hyperplastic nodules and hepatomas from AAF-pretreated rats contain a higher concentration of epoxide hydrase, the markedly enhanced catalytic activity of the enzyme may be due to a combination of increased protein content and enzyme activation. Interestingly, the nonhyperplastic liver tissue (after removal of the hyperplastic nodules) had less epoxide hydrase activity than the hyperplastic nodules. The liver surrounding the hyperplastic nodules had an increased epoxide

Table 2. Epoxide hydrase activity in hyperplastic nodules, hepatoma, and liver microsomes from control and AAF-pretreated rats

Substrate	Microsomal epoxide hydrase activity, nmol product/mg protein/min			
	Control	HPN	Nonhyperplastic	Hepatoma
	Experiment 1			
Octene 1,2-oxide	8.4	54.9	23.2	
Styrene 7,8-oxide	3.2	22.7	—	
Dibenz[*a,h*]anthracene 5,6-oxide	0.1	0.7	0.3	
3-Methylcholanthrene 11,12-oxide	0.4	2.1	0.9	
Phenanthrene 9,10-oxide	22.6	165.2	60.6	
Benz[*a*]anthracene 5,6-oxide	4.6	37.3	—	
Benzo[*a*]pyrene 4,5-oxide	3.4	23.4	—	
Benzo[*a*]pyrene 7,8-oxide	3.0	13.4	—	
Benzo[*a*]pyrene 9,10-oxide	1.6	9.1	—	
Benzo[*a*]pyrene 11,12-oxide	0.2	1.3	0.7	
	Experiment 2			
Octene 1,2-oxide	13.4	93.2		87.8
Styrene 7,8-oxide	5.1	32.9		31.3
Dibenz[*a,h*]anthracene 5,6-oxide	0.1	1.1		1.2
3-Methylcholanthrene 11,12-oxide	0.4	3.2		3.3
Phenanthrene 9,10-oxide	23.2	215.7		227.7
Benz[*a*]anthracene 5,6-oxide	6.6	56.4		59.1
Benzo[*a*]pyrene 4,5-oxide	5.0	36.6		36.7
Benzo[*a*]pyrene 7,8-oxide	2.4	16.5		17.7
Benzo[*a*]pyrene 9,10-oxide	1.3	12.7		13.9
Benzo[*a*]pyrene 11,12-oxide	0.2	1.5		1.6

Liver microsomes were prepared from control and AAF-treated rats. Hyperplastic nodules (HPN) were prepared from the pooled livers of 5–10 rats at 14 weeks after start of treatment with AAF. At 11 weeks of the treatment protocol, AAF was removed from the diet and the animals were killed 3 weeks later. Nonhyperplastic liver microsomes were prepared from liver after removal of the hyperplastic nodules. These microsomes contain some hyperplastic tissue because a rim of nodular tissue was left behind to ensure clean separation of hyperplastic nodules from the surrounding nonnodular liver. A single hepatoma was used to prepare microsomes 6 mo after treatment with AAF had ceased. Microsomes from control rats were prepared from the pooled livers of five rats after 14 weeks on the basal diet.

Table 3. Induction of rat liver epoxide hydrase by AAF

Weeks on test diet	Epoxide hydrase activity, nmol product/mg protein/min	
	Octene 1,2-oxide	Benzo[*a*]pyrene 4,5-oxide
0	14.9	5.8
1	69.1	31.6
3	74.8	35.0
8	37.7	15.9

Rats (150–170 g) were fed a diet containing 0.05% AAF for 3 weeks, a control diet for 1 week, a diet containing 0.05% AAF for 2 weeks, and a control diet for 2 weeks (8 weeks on test diet). Epoxide hydrase activity was determined on the liver microsomes from two rats at each time period.

hydrase level compared to liver from control rats but at least part of this increased activity was due to residual hyperplastic nodule tissue. These results and the very high epoxide hydrase activity in an AAF-induced hepatoma 6 mo after removal of AAF from the diet indicate that the increased epoxide hydrase levels in these liver lesions are maintained without continuous exposure to the carcinogen.

Further information on the nature of the induction of epoxide hydrase by AAF is shown in Table 3. Liver microsomal epoxide hydrase was markedly induced during the first week of treatment with AAF according to the discontinuous feeding schedule of Farber and associates (2–4) before any evidence of hyperplastic nodule formation was observed. This result is consistent with the reported detection of PN antigen by immunological techniques during the first 3 weeks of treatment with AAF (4, 9). Removal of the carcinogen from the diet at 6 weeks of treatment resulted in a decrease in epoxide hydrase activity during the next 2 weeks. Based on the results in Tables 2 and 3, the pattern of induction of epoxide hydrase in hyperplastic nodules by AAF can be summarized as follows. Inclusion of 0.05% AAF in the diet for 1–3 weeks causes a maximum induction (5- to 6-fold) of liver microsomal epoxide hydrase activity. This induction requires continued exposure to the carcinogen because removal of AAF from the diet resulted in a decrease in epoxide hydrase activity to near control levels. However, at some time during the development of hyperplastic nodules, the induced epoxide hydrase level becomes "fixed" and does not require the continued presence of AAF in the diet. Hepatomas maintain the maximal induced level of epoxide hydrase activity 6 months after AAF feeding has ceased. Epstein *et al.* (15) have shown that metabolites of AAF remain bound to liver glycogen and DNA in hyperplastic nodules and liver cancer cells for weeks to months after removal of the carcinogen from the diet. This prolonged binding does not occur in nonhyperplastic or non-neoplastic liver surrounding the tumor cells. Whether or not this bound metabolite(s) of AAF plays a role in the maintenance of the elevated level of epoxide hydrase activity in these tumor cells is unknown.

The prolonged elevation of epoxide hydrase in hyperplastic nodules and hepatomas caused by the hepatocarcinogen AAF raises the intriguing question of the significance of this induction. Epoxide hydrase is known to play a pivotal role in the activation and detoxification of the carcinogenic polycyclic aromatic hydrocarbons by the cytochrome *P*450-dependent monooxygenase system (16–18) but epoxide hydrase has no known role in the metabolism of AAF or ethionine and nitrosamines which also increase the levels of PN antigen in liver. In the case of the hepatocarcinogens AAF, safrole, aflatoxin B_1, nitrosamines, and aminoazo dyes, the cytochrome *P*450-dependent monooxygenase system is known to be involved in the conversion of these compounds to ultimate carcinogens (19, 20). This enzyme system is markedly decreased in hyperplastic nodules (21), spontaneous hepatomas, and hepatomas induced by hepatocarcinogens (22–24). What then is the significance of the marked induction of epoxide hydrase in hyperplastic nodules and in hepatomas induced by AAF? The normal physiological role of epoxide hydrase is unknown, but it would be of considerable interest if epoxide hydrase were involved in the regulation of cell growth and neoplasia. At the present time, this question remains unanswered but the identification of the PN antigen as epoxide hydrase should provide further impetus for studies designed to determine the physiological importance of markedly elevated epoxide hydrase in hyperplastic nodules and in hepatomas induced by hepatocarcinogens.

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