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# A methodology for the analysis of the preneoplastic antigen

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**A highly sensitive assay for the epoxide hydrolase activity associated with the preneoplastic antigen (PNA) has been developed based on the synthesis of *cis*-stilbene oxide labeled with tritium at ~15 Ci/mmol. This assay allows the detection of elevated epoxide hydrolase activity in the serum of humans and rodents as well as in the culture medium bathing isolated hepatocytes. The integrity of the enzymatic assay was confirmed in rodents by precipitating the serum PNA activity using an antibody raised against the rat microsomal epoxide hydrolase. Methodology for the detection of PNA in serum will facilitate evaluation of this antigen as a marker for hepatic neoplasia in man and in experimental animals.**

## Introduction

In all forms of cancer and particularly liver cancer where late detection often precludes successful therapy, early detection appears to be the key to successful therapy (1). Also in the carcinogenesis testing process for new drugs and agricultural chemicals, the long term feeding study often is the most expensive single experiment. For clinical diagnosis as well as carcinogenesis testing a series of serum tests which could indicate at an early stage which compounds ultimately will result in cancer would be very cost effective.

Unfortunately none of the serum markers proposed to date has proven to be as useful in either application as initial observations prophesied (2). Since most hepatocarcinomas express some embryonic traits, α-fetoprotein (AFP)*, a fetal glycoprotein, is commonly present in both the neoplastic tissue and serum. In spite of its value, AFP has its limitations as a marker. For instance, following treatment with a variety of chemical carcinogens AFP was detected in a minority of the preneoplastic and neoplastic lesions. In fact, some hepatocarcinomas fail to produce elevated serum levels of AFP, and others fail to do so until quite late in their evolution. AFP is normally high during pregnancy and in infants, it may be produced by a variety of tumors, and it is commonly produced in regenerating hepatic tissues (2–4). Thus, AFP is a useful research and diagnostic tool, but it clearly needs to be supplemented.

While studying the evolution of chemically induced liver cancer, Okita and Farber (5) raised antisera in rabbits against postmitochondrial supernatants from rat hepatic hyperplastic nodules. Following exhaustive absorption of this antiserum with soluble proteins from normal rat liver, there remained antibodies to a single, soluble protein termed the preneoplastic antigen (PNA). PNA was reported to correlate well with a variety of neoplastic and preneoplastic states in the rat but not with liver necrosis (5–8).

Griffin and Kizer (9) noticed that PNA was present largely in the microsomal fraction from hyperplastic nodules, but that it readily dissociated from the microsomes and could be detected as a soluble protein. They also observed that PNA could be removed from normal hepatic microsomes by detergent treatment (9,10). It was subsequently demonstrated that the PNA was in fact antigenically similar to the microsomal epoxide hydrolase (EC3.3.2.3, an enzyme which hydrates epoxides to 1,2-diols) (11). The PNA (apparently a solubilized form of the microsomal epoxide hydrolase) should not be confused with the cytosolic epoxide hydrolase, an enzyme with vastly different properties largely occurring in the cytosolic and mitochondrial fractions of liver cells (12,13).

Previous workers failed to detect the PNA in serum of animals with hyperplastic nodules or hepatocarcinoma. Since several proteins released from normal, necrotic, and/or hyperplastic liver cells can be detected in the serum, a specific and sensitive assay for the PNA based upon its ability to catalyze the hydration of epoxides was developed. Recently Griffin and Gengozian (14) have reported that PNA can be detected in the serum of rats with hyperplastic nodules using immunochemical techniques.

## Materials and methods

### *Radiosynthesis of stilbene oxides*

Within a sealed glove box equipped with a tritium monitor and catalytic scrubber (courtesy of Ray Anue, Lawrence Laboratories, Berkeley, CA) a glass ampoule containing 500 mCi of sodium borohydride (54 Ci/mmol [$^3$H], New England Nuclear, Boston, MA) was cooled with dry ice, opened resulting in an immediate but transient release of radioactive gas, and the lower part of the ampoule placed in an ice bath. The interior surface of the ampoule was washed with ethanol (95%, 200 μl) which collected in the bottom part of the ampoule yielding a colorless solution and a magnetic stir bead was added. Then 300 μl of a colorless ethanol solution containing desyl chloride (100 μmol 23 mg, Aldrich Chemical) was added to the ampoule resulting in a bright yellow solution. The solution was stirred for 20 min in the ice bath and then allowed to rise to room temperature over a period of 1 h.

The ampoule was again chilled in an ice bath and 500 μl of a 5 N sodium hydroxide solution was added in 100 μl aliquots. Shortly after each addition the reaction turned purple and then slowly cleared. After the last addition it remained purple. The reaction was allowed to come to room temperature with stirring for an additional hour. To the ampoule 178 mg of finely powdered sodium chloride was added in small aliquots, the solution extracted three times with small volumes of 20% ether in pentane, and each hyperphase passed through a plug of sodium sulfate in a Pasteur pipette and collected in a 10 x 75 mm glass tube. The volume of the solvent was reduced, and then it was spotted as a fine line on a methanol washed t.l.c. plate, (250 μl silica, $F_{254}$ Brinkman). In a separate scored channel authentic *trans*- and *cis*-stilbene ox-

*Abbreviations: PNA, preneoplastic antigen; AFP, alpha fetoprotein; TSO, *trans*-stilbene oxide; CSO, *cis*-stilbene oxide; $AFB_1$, aflatoxin $B_1$; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonylfluoride; LDH, lactate dehydrogenase; SGPT, glutamic pyruvic transaminase; SGOT, glutamic oxalacetic transaminase; DES, diethylsuccinate esterase; $CCl_4$, carbon tertrachloride; CPK, creatine phosphokinase.

ides (TSO and CSO) and desyl chloride standards had been spotted. The plate was developed twice in hexane:ether (10:1) to 2 cm, then to 10 cm followed by 16 cm resolving three major compounds, including an unknown compound chromatographing between CSO and desyl chloride of about equal intensity to CSO, and numerous minor bands below desyl chloride. As expected, based upon their u.v. spectra and upon isolated yield, the TSO band was much darker than the CSO band. First, the non-radioactive standards were removed totally from the plate and then the silica gel containing the TSO and CSO bands, respectively, was scraped carefully on to weighing papers and placed in separate glass tubes each containing ethanol (200 μl). To these tubes 1 ml of ether was added, the silica gel shaken, centrifuged and the supernatant passed through a small plug of sodium sulfate to remove the remaining silica gel. The process was repeated three times with 500 μl of ether, the solvent was reduced in volume, and the TSO and CSO fractions were then respotted as thin 10 cm lines on separate t.l.c. plates and developed and extracted as before. Each compound gave a single radioactive and a single u.v. dense band. Radiochemical yield was monitored at each step in the synthesis and purification. Ninety percent of the tritium reported to be in the ampoule was incorporated into the stilbene oxides of which 71% was *trans*. The cumulative radiochemical yield was 60% after the first t.l.c. and 40% after the second t.l.c. In retrospect, the second purification step was not needed for high purity material. The isolated ratio of TSO to CSO remained 7:3. The theoretical specific activity was 13.5 Ci/mmol while the experimentally determined specific activity was 15 Ci/mmol. One radioactive spot was detected on 2 dimensional t.l.c. developed with hexane:ether (10:1) followed by toluene: propanol (20:1). In each case the single u.v. dense spot co-chromatographed with the single spot detected by X-ray film and contained 97% of the radioactivity on the t.l.c. plate.

*Routine PNA assay in serum*

Unless otherwise indicated the assay for epoxide hydrolase activity was performed on unhemolized serum as described below. Serum was diluted 1:1 with 100 mM Tris-HCl buffer (pH 9.0) containing 0.5 mM phenylmethylsulfonylfluoride (PMSF) and 50 μl were placed in a 6 x 50 mm glass tube. To this was added 1 μl of an ethanol solution of CSO containing 7.5 pmol to give a final substrate concentration of $1.5 \times 10^{-7}$ M (~250 000 d.p.m. assuming a specific activity of 15 Ci/mmol). The tube was covered with parafilm or aluminium foil to minimize evaporation, shaken and incubated at 37°C for 3 h unless otherwise indicated. The reaction was terminated by adding 25 μl of methanol containing 10 mg/ml CSO and the corresponding *meso*-diol. The contents of the tube were mixed, the tubes centrifuged and 50 μl of the supernatant was spotted on Whatman LK5DF silica gel t.l.c. plates in two aliquots using a slight modification of the method of Jerina *et al.* (15). After air drying for 20 min the plate was developed to the cellulose-silica interface with methanol, dried again and developed 20 cm with toluene:*n*-propanol (20:1). When the toluene evaporated, the bands were visualized by their quenching of fluorescence when viewed with 254 nm u.v. light. The plate was sprayed lightly with water and the diol spots scraped into a scintillation vial. ACS Scintillation Cocktail (Amersham, Arlington Heights, IL) was used for analysis. The water spray reduces chemiluminescence and the hazards of radioactive dust, but the vials were held a minimum of 12 h before counting. Blank samples containing only silica gel were always used to detect chemiluminescence, while nonenzymatic hydration was monitored using buffer or buffer containing bovine serum albumin, control serum, or heat denatured positive serum. Enzymatic rates were expressed as pmols of diol produced per unit time and serum unless otherwise indicated. Thus, PNA in the experiments discussed here refers to the ability of a sample to hydrolyze CSO rather than to mass of antigen.

The radioactive metabolites which were produced by the hydration of CSO co-chromatographed with the authentic *meso*-diol in several t.l.c. systems, including one which separates the *meso*- and *erythro*-isomers. The radioactive metabolites also co-chromatographed with the *meso*-diol standard after reaction with n-butylboronic acid to form the corresponding cyclic diester.

*Immunoprecipitation assay*

Liver microsomes were prepared from an adult male Sprague-Dawley rat as previously described (16). They were resuspended to 10 ml per gram of original liver weight (10% w/v) in 76 mM sodium phosphate (pH 7.4), containing 1% w/v deoxycholate, by using a Polytron for 10 s at low speed. The mixture was then centrifuged at 105 000 *g* for 60 min and the supernatant ('solubilized microsomes') was used for the immunoprecipitation experiment.

Serum was obtained from Fischer-344 rats exposed to 50 p.p.b. aflatoxin $B_1$ ($AFB_1$) in the diet for 16–17 months. The immunoprecipitation assay was run with serum pooled from two animals showing high PNA activity with CSO in the standard assay. Forty μl of solubilized microsomes (diluted to 0.5% original w/v with phosphate-buffered saline – PBS) or rat serum were incubated with 10–100 μl of antiserum (1:1500 dilution in PBS) from a rabbit immunized with rat liver microsomal epoxide hydrolase (17,18). Normal rabbit serum (1:1500 dilution) was added to bring the total volume to 140 μl. The incubation time was 3 h at room temperature and 16 h overnight at 4°C. Goat anti-rabbit Ig coupled to Immunobeads (2 mg in 0.5 ml) (Bio-Rad Labs, Richmond, CA) was then added to each tube. The tubes were incubated for 2 h at room temperature and centrifuged at 1000 *g* for 5 min. The supernatant was assayed for non-immunoprecipitated enzyme activity by the standard assay except that after 40 min at 37°C, the reaction was stopped with 200 μl of dodecane and vigorous vortexing. Fifty μl of the aqueous phase was removed for liquid scintillation counting.

*Enzyme and protein assays*

The epoxide hydrolase activity on $5 \times 10^{-5}$ M CSO at pH 9.0 in tissue samples was detected by the method of Gill *et al.* (19). Alternatively, a direct enzyme-linked immunosorbent assay was used for the determination of the microsomal epoxide hydrolase (18). Lactate dehydrogenase (LDH) and glutamic pyruvic transaminase (SGPT) were assayed according to Wroblewski and LaDue (20,21), glutamic oxalacetic transaminase (SGOT) was measured by the method of Karmen (22) (Table II) or Reitman and Frankel (23) (Table III), and diethylsuccinate esterase (DES) activity was monitored by the method of Talcott *et al.* (24). AFP levels were provided by courtesy of Stewart Sell (Department of Pathology, University of Texas Medical Center, Houston, Texas). Protein was determined using a Bradford (25) assay.

*Rodent care and treatment*

Male Sprague-Dawley rats (180–210 g, Simonsen Laboratories, Gilroy, CA) were maintained in temperature and humidity controlled cages with Purina Laboratory Chow and water provided *ad libitum*. Five experimental animals were given carbon tetrachloride ($CCl_4$) as a 20% (v/v) solution in corn oil at a dose of 1 ml $CCl_4$/kg by i.p. injection. Three control animals received only corn oil. Twenty-four hours later the rats were anesthetized with carbon dioxide, blood removed by heart puncture, then the rats were dissected and the livers removed and perfused with cold buffer. Subcelluar fractions were prepared as described earlier (16).

*Hepatocyte isolation and analysis*

Hepatocytes were isolated by the biopsy perfusion method (26). They were incubated in modified Waymouth's MB752/1 (27,28) in 60 mm collagen coated culture dishes at a density of $2.5 \times 10^6$ cells/4 ml medium/dish. Fresh medium was added at 3 h after plating and experimental compounds were added in fresh medium 24 h after plating. $AFB_1$ (29) and acetaminophen (Sigma Chemical Company) were dissolved in culture medium and sterilized by filtration through a 0.45 μm membrane filter (Type TCM, Gelman). The final concentrations were $6 \times 10^{-5}$ mM or $1.6 \times 10^{-4}$ mM for $AFB_1$ and 3 or 7 mM for acetaminophen. At 24, 32 and 48 h after dosing with acetaminophen or $AFB_1$, LDH and PNA activities were monitored in the culture medium. Each assay was run in triplicate, triplicate cultures were run at each dose, and the entire experiment was repeated twice.

## Results

As with the low specific activity synthesis of the isomeric β-ethylstyrene oxides and stilbene oxides (19,30), the [$^3$H]-sodium borohydride procedure provided a high chemical and radiochemical yield. In this case it resulted in a product with a specific activity near the theoretical maximum for incorporation at a single tritium. This procedure could be used to label a wide variety of substrates for the PNA, some of which may yield much more sensitive assays.

In order to optimize the enzymatic assay, a variety of substrate concentrations were evaluated in the assay ($5 \times 10^{-5}$ to $1 \times 10^{-9}$ M) using serial dilutions of either rat microsomes (5–0.001% original liver wet weight equivalent/v) or human serum (100–1% v/v) to obtain the maximum percentage metabolism with the minimum concentration of epoxide hydrolase. In this case use of substrate concentrations much below $10^{-7}$ M failed to increase the percentage metabolism significantly. Thus, to improve assay sensitivity with this substrate one should increase the radioactivity and enzyme in the assay, while holding the substrate concentration in the high nanomolar region. Treatment of 6 x 50 soft glass tubes with either Sigmacote® or carbowax (31) did not increase the proportion of CSO in aqueous solutions at various concentrations down to $1 \times 10^{-8}$ M. Evaporative loss of CSO also was minimal.

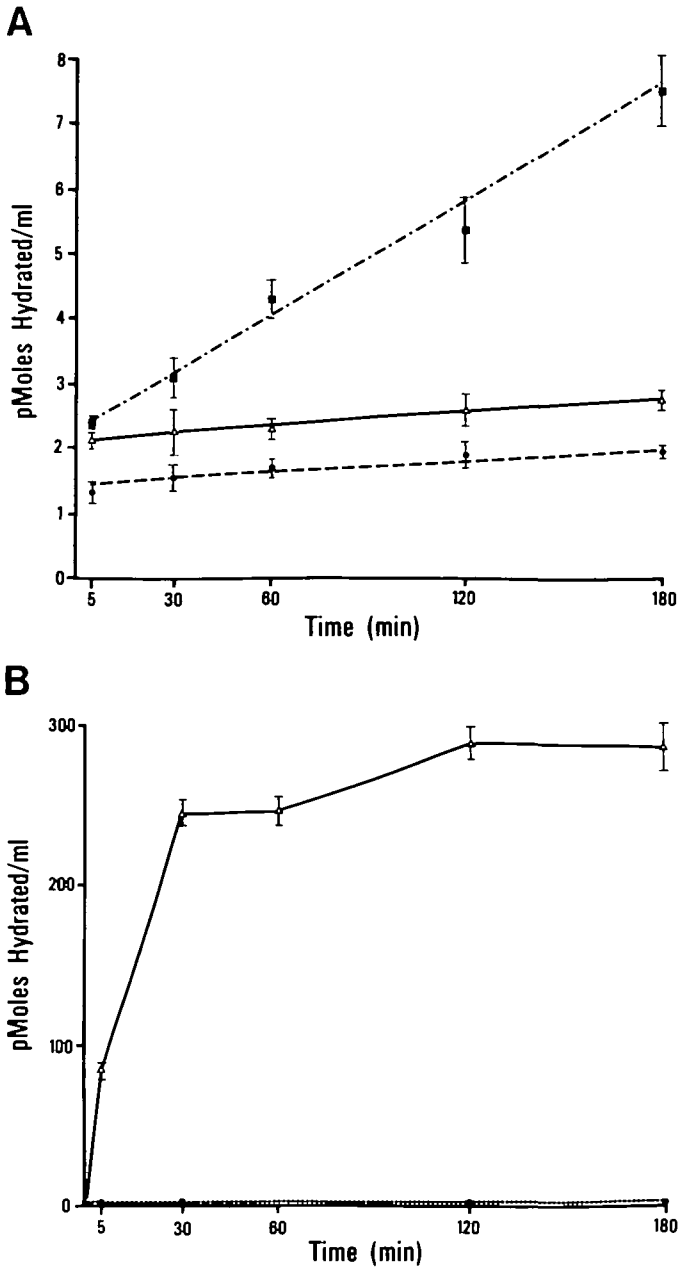


Fig. 1. A. Time dependence of hydration of CSO in buffer (●—●), serum from an apparently healthy patient (△—△), and serum from a patient with hepatocarcinoma (■—·—·—■). The rate is expressed in pmol of CSO hydrated to the corresponding *threo*-diol per ml serum. Bars represent standard deviations of triplicate determinations. B. Time dependence of hydration of CSO in serum from an apparently healthy patient (●—●) and serum from a patient following overdose with acetaminophen (△—△). Bars represent standard deviations of triplicate determinations.

The assay was linear with protein concentration (data not shown), yet the use of >50% serum in the assay resulted in problems with emulsion or build up of biomass at the origin of the t.l.c. plate. To circumvent these problems one can extract the incubation mixture with ether which allows a larger sample size to be used. Alternatively, a 1:1 dilution of the serum in buffer was used and serum proteins were precipitated with methanol following incubation. The latter method was used because it allowed termination of hydration and addition of unlabeled standards simultaneously, rapid evaporation from the cellulose prelayers, effective extraction of radioactivity from precipitated protein, and resulted in clean tracks on the t.l.c. plate. As shown in Figure 1A the assay appeared reasonably linear with time with the serum from cancer patients. Three hour incubation times were selected to maximize the amounts of radioactivity in the diol zone produced enzymatically relative to other sources of background. However, as shown by the results from the serum of a patient suffering from an overdose of acetaminophen (Figure 1B), rates do decrease as the substrate is depleted.

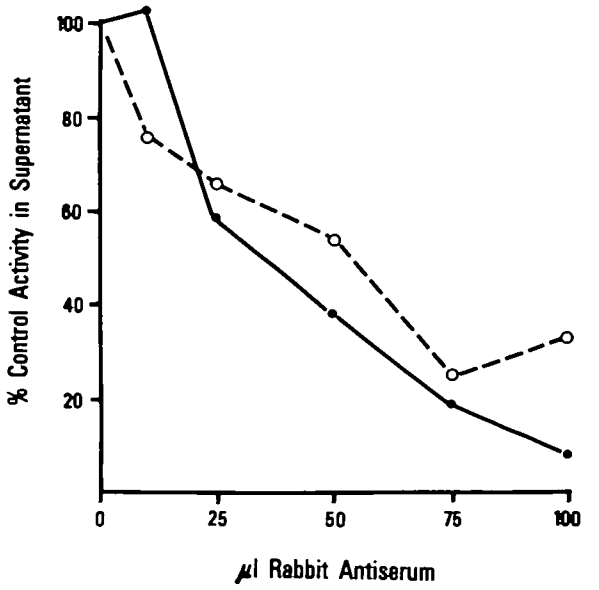


Fig. 2. Immunoprecipitation of epoxide hydrolase activity. Solubilized microsomes (● – ●) and rat serum (○—○) were incubated with rabbit anti-rat microsomal epoxide hydrolase antiserum (1:1500) and the amount of non-immunoprecipitated enzyme activity in the supernatant was determined with [$^3$H]*cis*-stilbene oxide as substrate – 100% activity refers to the amount of activity present when the immunoprecipitation was carried out with non-immune antiserum. The rates of hydrolysis in control samples were 154 pmol/h/ml for microsomes and 23 pmol/h/ml for serum. The serum was obtained from rats exposed to 50 p.p.b. $AFB_1$. Respective protein concentrations were 0.1 mg/ml and 75 mg/ml. Each point represents the mean of at least duplicate determinations. Deviations from the mean were always <10% when three values were used. Lines derived from a modified partition assay (19) and the standard t.l.c. based PNA assay were indistinguishable.

A primary development of the t.l.c. plate in methanol followed by toluene:propanol resulted in very narrow, u.v. dense bands. For routine studies only the diol bands were scraped since the epoxide but not the diol evaporated slowly from the silica gel. Of greater significance, large amounts of CSO evaporated from the prelayer with two apparent rates.

As indicated in Figure 2, the majority of the epoxide hydrolase activity in rat serum and from solubilized rat microsomes was precipitated by rabbit anti-rat microsomal antibodies (17,18,32). The extent of precipitation of both samples was very similar with several different dilutions of rabbit serum added indicating that the majority of the hydrolytic activity is due to enzymes with common antigenic determinants. Using these samples the partition assay (19,30) was compared with the t.l.c. assay for each of the data points generated with the microsomal enzyme. No difference was found in the rate of CSO hydration indicating that, with caution, the partition and t.l.c. assays can be used interchangeably.

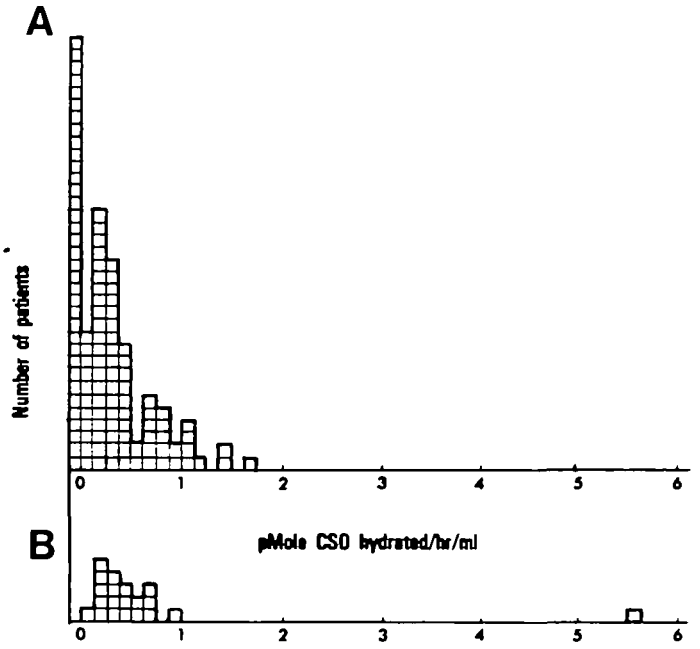


**Fig. 3. A.** Apparent PNA activity in sera from 117 apparently normal patients. PNA activity is expressed as pmol CSO hydrolyzed/h/ml serum. In each case SGOT and DES values were apparently normal as well. **B.** Apparent PNA activity in sera from 20 patients from a cancer clinic. The study was carried out in a blind fashion and only the patient with 5.6 pmol/h/ml serum had diagnosed hepatoma. The other 19 patients averaged $0.11 \pm 0.06$ pmol/h/ml serum and suffered from (number of patients, activity ± standard deviation) AIDS/Karposi Sarcoma (5, $0.17 \pm 0.04$), breast cancer (5, $0.13 \pm 0.07$), small cell lung cancer (1, 0.058), squamous cell lung cancer (1, 0.062), squamous cell oral cancer (1, 0.046), testicular cancer (1, 0.17), multiple myeloma (1, 0.037), prostate cancer (1, 0.085), stomach cancer (1, 0.032), lymphoma (1, 0.04), and cervical cancer (1, 0.10).

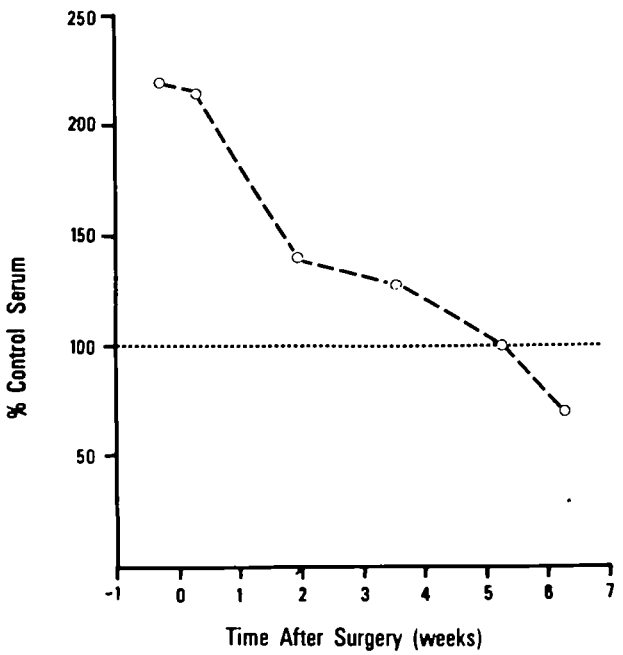


**Fig. 4.** Decrease in human serum PNA levels following surgery to remove diseased tissue. Patient was a 52 year old Oriental woman with hepatocellular carcinoma. Activity is expressed as percent of control serum from normal patients.

**Table I.** PNA levels in patients with diagnosed liver cancer

| Patient number | Sex | Age | Hepatitis B surface antigen | Liver cancer | AFP[a] (ng/ml) | PNA activity in serum (pmol/h/ml) |
|---|---|---|---|---|---|---|
| 1 | F | 55 | (+) | + | >25 000 | 1.22 |
| 2 | M | 53 | (−) | + | <10 | 0.34 |
| 3 | M | 30 | (+) | + | | 2.24 |
| 4 | M | 38 | (+) | + | >48 000 | 8.22 |
| 5 | M | 29 | (+) | + | >48 000 | 7.72 |
| 6 | M | 42 | (+) | + | 32 | 1.24 |
| 7 | F | 52 | (−) | + | | 0.66 |

[a]Normal AFP levels are <20 ng/ml.

Use of the radioenzymatic assay for PNA in the serum of 117 apparently normal individuals indicated that the PNA activity averaged $0.30 \pm 0.36$ pmol CSO hydrolyzed/h/ml serum (median 0.21) (Figure 3A). Similar low levels were found in laboratory personnel. The data base was too small to discern significant trends with age, race or sex. When serum from a cancer clinic was analyzed in a blind fashion, 19 patients with a variety of cancers (but no known hepatic involvement) had serum PNA levels averaging $0.11 \pm 0.06$ pmol/h/ml serum. The single patient with hepatoma had a level of 5.6 pmol/h/ml serum.

The analysis of serum from seven patients awaiting surgery for liver cancer yielded variable results (Table I). With the exception of patients I-4 and I-5 who had very high PNA activity and I-2 with very low PNA activity, PNA levels were increased only slightly above control levels. Although these data represent a very small sample size, it should be noted that the two highest values were associated with the highest levels of AFP and the two lowest values were hepatitis negative. When PNA levels were monitored in the serum of a patient following surgery, the rate of CSO hydration was found to decrease with time (Figure 4).

The data in Table II indicate that serum PNA levels can be elevated following an overdose with acetaminophen. In patients II-1 – II-3 it can be seen that PNA levels may remain high even as serum levels of acetaminophen drop. Patient 7 had the highest levels of PNA so far detected in human serum. Only value II-7e is comparable with other values in this study since the PNA activity exceeded the linear region of the routine assay. Patient II-8, suspected of acetaminophen overdose, had low PNA values which correlated well with SGOT, SGPT, and DES esterase levels. Of 22 CPK positive patients high serum PNA levels were found in only two samples (II-9, II-10). These high PNA levels correlated with high SGOT, SGPT and DES esterase levels and with diagnosed liver involvement.

As shown in Table III, SGOT values increase dramatically in the serum of rats exposed to necrotic doses of carbon tetrachloride. Similarly, PNA activity increases in the serum whether it is monitored by the radioenzymatic assay or an ELISA based on rabbit anti-rat microsomal epoxide hydrolase assay. At this short time after dosing a slight increase of CSO hydration at pH 9.0 was seen in the cytosol of treated rats. However, there was a significant decrease in microsomal epoxide hydrolase.

As shown in Figure 5, LDH activity in the culture medium of rat hepatocytes increased with time following exposure to two doses each of $AFB_1$ and acetaminophen. The increase also appeared dose-dependent at 32 and 48 h. Similarly, PNA activity increased in response to these materials with the most

**Table II.** PNA levels in patients without diagnosed cancer

| Patient number | A. Acetaminophen overdose [Acetaminophen][a] mg/ml | Serum PNA (pmol/h/ml) | SGOT[b] units/ml | SGPT[c] units/ml | DES esterase[d] nmol/min/ml |
|---|---|---|---|---|---|
| 1.a. | 67 | 2.28 | | | |
| 1.b. | 36 | 2.28 | | | |
| 2.a. | 179 | 5.30 | | | |
| 2.b. | 146 | 0.78 | | | |
| 2.c. | 70 | 1.44 | | | |
| 3.a. | 257 | 2.61 | | | |
| 3.b. | 186 | 3.12 | | | |
| 3.c. | 122 | 1.48 | | | |
| 3.d. | 53 | 1.52 | | | |
| 3.e. | 29 | 3.33 | | | |
| 3.f. | 7 | 3.98 | | | |
| 3.g. | <2 | 2.51 | | | |
| 4. | 14 | 3.86 | | | |
| 5. | 97 | 2.80 | | | |
| 6. | 29 | 3.49 | | | |
| 7.a. | (0 h)[e] | >500[f] | 6510 | 924 | 964 |
| 7.b. | (3 h) | >500 | 7440 | 877 | 1314 |
| 7.c. | (4 h) | >500 | 5506 | 648 | 1493 |
| 7.d. | (5 h) | >500 | 6080 | 656 | 1200 |
| 7.e. | (7 h) | 500 | 6138 | 869 | 1429 |
| 8. | – | 0.116 | 8.75 | 3.34 | 4.76 |

| Patient number | B. CPK positive Comments | Serum PNA (pmol/h/ml) | SGOT[a] units/ml | SGPT[b] units/ml | DES esterase[c] nmol/min/ml |
|---|---|---|---|---|---|
| 9. | Myocardial infarction with secondary liver necrosis | 80.4 | 5304 | 846 | 771 |
| 10. | Alcoholic with hepatic cirrhosis and varices, cholelithiasis and nephrolithiasis, gastric ulcer | 50.5 | 2467 | 230 | 537 |
| 11–30 | No apparent liver involvement | 0.18 ±0.42 | 105 ±103 | 25.4 ±22.9 | 3.14 ±19.1 |

[a]Serum levels of acetaminophen concentration following admission to the hospital.
[b]Expressed as Karmen units (22).
[c]Expressed as Wroblewski-LaDue units (21).
[d]Rate of metabolism of diethylsuccinate (24).
[e]Time after first admission to hospital.
[f]Metabolism of CSO in the standard 3 h assay was out of the linear region. Values are estimated based on a time course of the 7 h sample.

substantial difference occurring at ~32 h following dosing.

## Discussion

The failure of several previous workers to detect PNA in the serum of animals with hepatocellular carcinoma was probably the result of using either older immunoprecipitation assays or tritiated styrene oxide as a substrate for the radioenzymatic assay. Radioimmunoassays and enzyme linked immunoassays should be capable of detecting the antigen with much greater sensitivity than the previous methods, but then a separate antibody would be needed for most species (14,18). Therefore a radiochemical-enzymatic assay was developed. CSO offers numerous advantages over styrene oxide as a substrate for both the microsomal epoxide hydrolase and PNA (12,13,33). However, a variety of other compounds including arene oxides (34) could also be used in this assay.

It seems likely that the epoxide hydration detected in human serum is homologous to the PNA described in the rat by Farber and co-workers (5–8). CSO can be used to detect microsomal epoxide hydrolase in its bound and solubilized form from both normal and neoplastic tissue of a variety of species (19,35). The immunoprecipitation data shown in Figure 2 indicate that the majority of the PNA activity in the serum of rats fed $AFB_1$ can be precipitated by an antibody against the rat microsomal epoxide hydrolase. These data do bring up a potential limitation of the radioenzymatic PNA assay in that it only monitors catalytically active epoxide hydrolase. Gill *et al.* (18) reported that an enzyme linked immunosorbent assay for the microsomal epoxide hydrolase detected both catalytically active and inactive enzyme. Thus, an immunoassay specific for the PNA might offer some advantages over the radioenzymatic assay if a substantial portion of the PNA in serum is inactive or is inactivated before analysis (14).

The number of patients was too low to carry out an extensive epidemiological study. However, several trends are evident. Analysis of the sera of 117 apparently healthy patients as well as repeated analysis of the sera from investigators in this laboratory revealed very low levels of PNA (Figure 1A). Similarly, when a blind study was performed on the sera from twenty patients suffering from a variety of diagnosed cancers, high levels of PNA were found only in the serum of the single patient with hepatocellular carcinoma (Figure 1B).

The analysis of the serum from seven patients with diagnosed liver cancer at U.C. Davis is less convincing (Table I). Some patients had very high levels of PNA while others had levels which were only slightly elevated. There is only a tentative correlation between PNA levels in patients with cancer and hepatitis versus those without hepatitis. Certainly a further study of patients suffering from hepatitis and cirrhosis is warranted. The majority of the liver cancer patients

**Table III.** Effect of carbon tetrachloride treatment on PNA and related markers in the rat

| Treatment[a] | SGOT[b] (units/ml) | mEH (PNA) in serum | | mEH (PNA) in liver cytosol (nmol/min/g liver) | mEH in liver microsomes (nmol/min/g liver) |
|---|---|---|---|---|---|
| | | Enzyme assay (pmol/h/ml) | ELISA[c] (% inhibition) | | |
| Control | 82 ± 2 | 0.0 | 11 ± 12 | 2.05 ± 0.33 | 121 ± 20 |
| $CCl_4$ treated | 2660 ± 1330 | 45 ± 19 | 46 ± 24 | 2.57 ± 0.52 | 62 ± 24 |

[a]Reported values ± standard deviation are indicated for three control and five experimental animals treated with 1 ml/kg $CCl_4$ and analyzed 24 h later.
[b]Expressed in Reitman and Frankel units (23).
[c]Assay performed as described by Gill *et al.* (18). Values are expressed as percent inhibition in the ELISA of 10 μl of serum. This value corresponds to ~2.5 μg of microsomal epoxide hydrolase per ml serum.

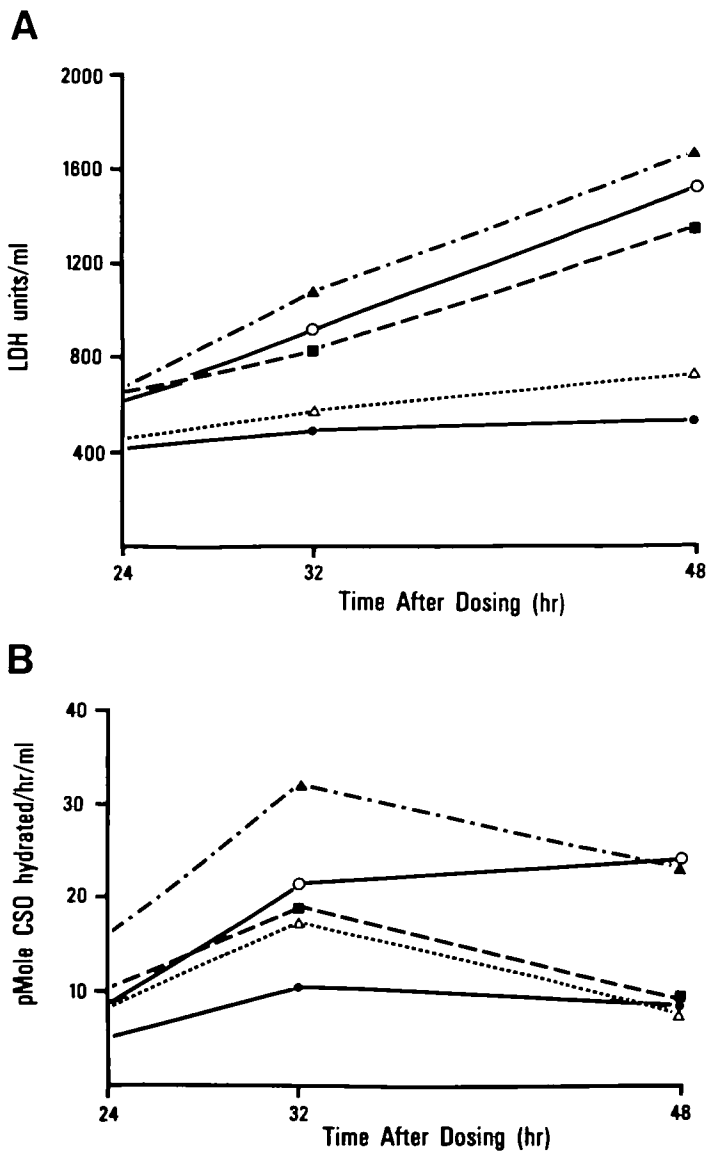


**Fig. 5. A.** Dose and time-dependent release of LDH activity from control hepatocytes and hepatocytes treated with two doses each of acetaminophen and $AFB_1$. LDH activity is expressed as units per ml at various times after doses. Control (● – ●), 3 mM acetaminophen (△—△), 7 mM acetaminophen (■—■), 6 x $10^{-5}$ mM $AFB_1$, (○ – ○), and 1.6 x $10^{-4}$ mM $AFB_1$ (▲—·—·—▲). Each point is the average of two separate experiments. **B.** Dose and time-dependent release of PNA activity from control hepatocytes and hepatocytes treated with two doses each of acetaminophen and $AFB_1$. See **A** legend for key to lines.

with unexpectedly low levels of PNA were critically ill and had relatively little hepatic tissue of normal appearance in their biopsies. It is possible that as the disease progresses the initially high levels of PNA drop. Since tissue from some liver tumors was found to be very low in either membrane bound epoxide hydrolase or PNA (35), production and release of PNA into the blood may be low as the disease becomes very advanced. One exception was patient I-2 who was diagnosed as having moderately advanced liver cancer without hepatitis yet had very low levels of both PNA and AFP.

It is critical to determine if increased serum PNA concentration is due specifically to the carcinogenic process or to more generalized hepatic necrosis. For instance, the high levels of PNA in the serum of some patients suffering from acetaminophen overdose and those CPK positive patients with liver damage may, however, bring into serious question the general utility of the PNA assay if used alone for cancer diagnosis (Table II). In this regard a time course of PNA release into the serum during carcinogensis versus cell injury may provide useful data.

Only a very limited study has been done with rodents (Cullen *et al.*, unpublished results), but serum PNA levels have some correlation with time of exposure and dose of aflatoxin $B_1$ in rats. Of additional concern are the apparently high levels of PNA in the serum of rats exposed to carbon tetrachloride (Table III). The data in Figures 1B and 5 and Tables II and III certainly indicate that the effects of a variety of necrogenic agents on serum PNA levels should be examined in both rodents and in humans. The observations on the effects of acetaminophen on serum PNA levels in man also correlate with the *in vivo* effects of carbon tetrachloride in the rat and with the rapid release of PNA from rat primary hepatocyte cultures when exposed to aflatoxin and acetaminophen (Figure 5). These data also indicate that the radioenzymatic assay for PNA is sufficiently sensitive to investigate the mechanism of its release from cultured hepatocytes.

In spite of these limitations, our results indicate that serum tests for PNA may have the potential to complement AFP and other markers in a battery of tests. For instance no PNA was detected in the serum of a patient in her 27th week of pregnancy while this same patient was found to have 69 ng of AFP per ml serum. Thus, PNA could be a potential marker for liver cancer at a time when the production of AFP by cancer cells could be simulated by fetal and placental production of this protein.

In two other cases PNA levels were monitored both before and after surgery in the blood of an adult woman and infant male both suffering from hepatocellular carcinoma. In both cases marked decreases in serum PNA levels were noted in serum collected following surgery. In the former case PNA levels began to rise again very shortly and the patient was ultimately readmitted six weeks later with high AFP levels and suspected metastases. In the second case PNA levels dropped to control values and the infant is apparently healthy two years post-surgery. In a final case a four month old female was suspected of having hepatocellular carcinoma based upon a variety of tests including initial observations at biopsy; however, PNA levels in the serum and tissue were found to be very low. Further histological work resulted in a diagnosis of hemangioendothelioma from which the infant has apparently recovered following surgery.

The above examples illustrate that serum PNA levels can be used to monitor the success of a surgical procedure at a time when AFP may be very high due to liver regeneration. It also illustrates that serum PNA can be monitored in infants even in cases where moderate levels of AFP continue to circulate.

It is obvious that many more studies are needed to correlate serum PNA levels with disease states in both human and animal models. However, even from the eclectic data presented in this manuscript cautions regarding the diagnostic value of serum PNA levels are obvious.

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