1. **FINAL PERFORMANCE REPORT**

Department: Human Biological Chemistry and Genetics

Institution: The University of Texas Medical Branch
Galveston, Texas 77555-0647
Project Title: Plasma Proteins: Markers of Chemical Exposure
Project Period: 09.01.89 to 08.31.93
Principal Investigator: G.A.S. Ansari, Ph.D.
Co-Principal Investigator: Jose C. Gan, Ph.D.
Grant Number: OH-02149

R01-OH-02149
End-Date-08-31-1993
Grants-Other

List of Publications

Gan JC, Ansari GAS: Inactivation of Plasma α_1-proteinase Inhibitor by Acrolein: Adduct Formation with Lysine and Histidine Residues. Mol Toxicol 2:137-145, 1989.

Sanduja R, Ansari GAS, Boor PJ: 3-Hydroxypropyl-mercapturic Acid: A Biological Marker for Exposure to Allylic and Related Compounds. J Appl Toxicol 9:235-238, 1989.

Gan JC, Ansari GAS: Inactivation of Plasma α_1-proteinase Inhibitor by Aldehydes Found in Cigarette Smoke. SAAS Bull Biochem Biotech 3:32-36, 1990.

Gan JC, Oandasan A, Ansari GAS: *In Vitro* Covalent Modification of Serum Albumin by Acrolein. Chemosphere 23:939-947, 1991.

Thakore K, Gan JC, Oandasan A, Ansari GAS: Quantitation of Blood Protein Adducts of Acrolein by Tritiated Bromohydride Reduction. Toxicol Methods 2:295-305, 1992.

Kaphalia BS, Ansari GAS: Covalent Binding of Ethylene Dibromide and Its Metabolites to Albumin. Toxicol Letters 62:221-230, 1992.

Kaphalia BS, Bhat HK, Khan MF, Ansari GAS: Tissue Distribution of Monochloracetic Acid and Its Binding to Albumin in Rats. Toxicol Ind Hlth 8:53-61, 1992.

Trieff NM, Ficklen D, Gan J: *In Vitro* Inactivation of Glucose-6-Phosphate Dehydrogenase from Human Red Cells by Acrolein: A Possible Biomarker of Exposure. Toxicol Letters 69:121-127, 1993.

Thakore K, Gan JC, Ansari GAS: Rapid Plasma Clearance of Albumin-Acrolein Adduct in Rats. Toxicol Letters (In Press).

Significant Findings

Covalent binding of acrolein to albumin (Chemosphere 23:939-947, 1991

We have studied the covalent binding of acrolein to human serum albumin. Amino acid analysis showed four new ninhydrin positive peaks. These were identified as lysine and histidine adducts utilizing methods we developed earlier.

The adducts formed as a function of increasing concentration of acrolein was dose dependent between 1 to 10 mM range. The lowest concentration of acrolein used to quantitate the adducts was 1μ M at an albumin concentration of 1 mg/ml. The effects of acrolein on the ultraviolet absorption and the biological function of the protein to bind fatty acid or bromocresol green were also studied. At acrolein concentrations of 2.5 to 10 mM, we observed an increased absorption at 280 nm by about 80%. This data suggest that more tyrosine and/or tryptophan

residues were exposed due to the unfolding of the protein as a result of covalent modification of the lysyl and histidyl residues. In spite of significant numbers of modified lysyl and histidyl residues in albumin, the serum protein did not lose the ability to bind either palmitic acid or bromocresol green. Apparently, the alteration in the secondary or higher ordered structure (unfolding) of albumin did not affect its capacity to bind these ligands. These studies indicate that even substantial covalent modification may not result in the loss of biological activity of albumin.

Acrolein adducts with α_1-proteinase inhibitors were also studied and a lysine adduct formation was confirmed by mass spectrometry (Mol. Toxicol. 2:137-145, 1989; SAAS Bull. Biochem. Biotech. 3:32-36, 1990). *In Vivo* covalent interaction of ethylene dibromide and chloroacetic acid also resulted in the formation of albumin adducts (Toxicol. Letters 62:221-230, 1992; Toxicol. Ind. Hlth. 8:53-61, 1992). These studies indicate that covalent binding to plasma proteins can be identified and quantitated but the purification and characterization methods are cumbersome and therefore can not be used for routine analysis. However, such thorough characterization is essential for these protein adducts to be used for the development of biological assays.

Rapid Quantitation of Acrolein and Crotonaldehyde Modified Albumin (Toxicol. Methods 2:295-305, 1992):

We developed a rapid and sensitive method for the estimation of acrolein adduct of albumin which can subsequently be used to measure acrolein exposure.

In Vitro Studies: Human plasma albumin samples were incubated with increasing concentration of acrolein (0.025 to 10 mM) at 37°C in 0.1 M phosphate buffer, pH 7.2, for 2 hr. After exhaustive dialysis, the modified protein was treated with [^{3}H]-$NaBH_4$ to reduce the aldehyde adduct to corresponding alcohol. The radioactivity was measured after exhaustive dialysis and nmoles of carbonyl function/mg protein calculated. The response was found to be linear. This method is about four fold more sensitive compared to the amino acid analysis method in detecting covalent binding of acrolein to albumin. Although not as reactive, crotonaldehyde (a metabolite of butadiene) had a similar capacity to bind covalently with albumin as acrolein.

In Vivo Studies: Rats were treated with acrolein by gavage (13 mg kg^{-1}) and killed at 1,2,4, and 6 hr. Blood samples were collected and analyzed for adducts of plasma proteins and hemoglobin as described above. The adducts of both plasma proteins and hemoglobin were found to be significantly higher than in the controls.

The efficacy of this method needs to be established in humans before it can be used for molecular dosimetry. This method will be nonspecific in terms of the identity of the aldehyde but could serve as a primary screening procedure before a detailed analysis is attempted.

Susceptibility to Degradation of Covalently Modified Albumin (Toxicol. Letters In Press)

In Vitro Studies: Albumin which was covalently modified by acrolein or crotonaldehyde as described above was incubated with elastase. Proteolysis in both cases occurred at a faster rate than with the native protein. Albumin treated with elastase degraded between 20 - 25% while covalently modified albumin with 5 mM acrolein or crotonaldehyde degraded 98 and 91%, respectively. A dose response was observed between 0.025 - 5 mM range in both cases.

In Vivo Studies: Biosynthetically [^{3}H]-lysine labeled rat albumin was incubated with 5 mM acrolein in 0.1 M phosphate buffer pH 7.2 in order to prepare radioactively labeled protein adducts. The acrolein-adducted albumin and native albumin were injected intravenously into a group of rats. The disappearance of radioactivity was followed in plasma. In contrast to that of native albumin (t 1/2 of 12 hr), the corresponding estimated half-life of the adducted-protein was about 6 hr. Moreover, at the end of 33 hr after injection only 6.26% (5.5%, TCA-insoluble; and 0.9%, TCA-soluble) remained in the plasma vs. 20.4%, (19.4% TCA-insoluble; 1.1%, TCA-soluble) with native protein. Thirty-three hrs after the injection only 4.4% of the radioactive dose was associated with plasma albumin in the adducted protein, while 16.2% remained in the plasma when native albumin was used. The radioactivity data from the liver indicates that more degradation is occurring with the adducted protein (4.5%, in TCA-soluble function) than in the native protein (1.5%, TCA-soluble).

These studies show that chemically modified proteins may be degraded at faster rates than native proteins. Further studies are needed to substantiate this observation using several other protein adducts which will provide the time frame in which such protein adducts can be measured.

Development of Enzyme-linked immunosorbent assay (ELISA) for styrene (Unpublished Results):

Styrene oxide, a metabolite of styrene was used to develop this assay.

Binding of Styrene Oxide to Human Albumin:

Human albumin (Sigma Chem. Co., St. Louis), 1 mg/ml in 0.1 M phosphate buffer (pH 7.4), was incubated with 0.1, 0.5, 1.0, 2.5, 5.0 and 10 mM of ^{14}C-labeled Styrene Oxide (a metabolite of styrene responsbile for covalent binding, specific activity 0.052 uCi/umole) for 4 hrs at 37°C and extensively dialyzed against cold water until background level of radioactivity is achieved in the dialysate. Protein was determined in the dialyzed albumin. Radioactivity was determined and covalent binding of styrene oxide expressed as nmole/mg protein was calculated and plotted against the styrene oxide concentration (fig. 1). The binding of stryene oxide increased from 0.1 to 10.0 mM concentration range.

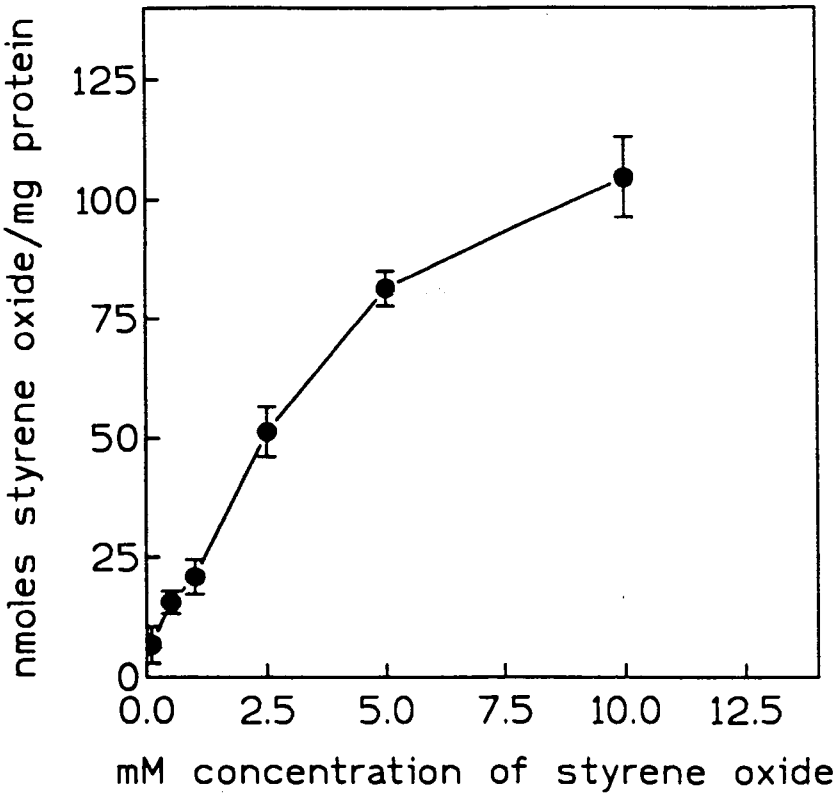

Fig. 1. Binding of styrene oxide to human albumin *in vitro.*

Preparation and detection styrene oxide - specific antibodies:

(i) Preparation of styrene oxide (SO) - albumin (A) conjugate:

Five mg of A was incubated with 10 mM of styrene oxide as described above. After incubation, the sample was dialyzed extensively and lyophilized. The protein concentration was adjusted to 1 mg/ml.

(ii) Generation of polyclonal antibodies in rabbit:

New Zealand white rabbit was used to raise the antibodies against SO-A. A small volume of blood was withdrawn from the rabbit to obtain pre-immune serum. For immunization, 0.5 ml of SO-A (~ 500 μg protein) was mixed thoroughly with 0.5 ml of Freund's complete adjuvant and injected subcutaneously at 6-7 sites on the back of the rabbit. Three weeks later a booster injection of 250 μg of the conjugate in Freund's incomplete adjuvant (total volume 0.5 ml) was given at 4-5 sites. Ten days later, the rabbit was bled and the serum was separated by standard procedure.

(iii) ELISA procedure for the detection of antibodies:

Briefly, 96-well microtiter plates were coated with 50 μl of antigen (A or SO-A 1 μg/well), suspended in bicarbonate buffer, overnight at 4°C. Plates were washed thoroughly with PBS-Tween 20 (0.05%) and non-specific binding sites were blocked with 100 μl of 1.5% BSA (in PBS). After washing 50 μl of the diluted primary antibody (serum) was added and incubated at 37°C for 2 hrs. The plates were washed again and 50 μl of 1:1000 diluted enzyme conjugated secondary antibody (goat anti-rabbit IgG-HRP) was added and incubated at 37°C for 1 hr. Appropriate controls were included during the assay. Finally, the plates were washed with PBS-Tween 20 and then 100 μl of HRP substrate (Bio Rad) was added to each well. The optical density (O.D.) was read immediately at 450 nm.

The results in Table 1 indicate that antibody has specificity for both A as well as SO-A and thus, indicating the formation of SO-A specific antibody.

Table 1. Binding pattern of A and SO-A with the primary antibody*

Antigen	**0.D at 450 nm**
A	0.29± 0.03
SO-A	0.41 ± 0.03**

* Antibody dilution was 1:1000
** ~ 40% more binding than A.
O.D. values are mean of 16 wells.

Table 2. Comparative antibody titers for A and SO-A:

Antibody dilution	(O.D. at 450) A	(O.D. at 450) SO-A	% increase over A
1:1000	0.25 ± 0.02	0.38 ± 0.02	+52
1:2000	0.29 ± 0.01	0.40 ± 0.04	+38
1:4000	0.20 ± 0.01	0.30 ± 0.01	+50
1:8000	0.22 ± 0.01	0.29 ± 0.01	+32
1:16,000	0.12 ± 0.02	0.17 ± 0.01	+42

Results indicate that both at the highest as well as lowest concentrations, the antibody has more

affinity for the SO-A and thus, further indicates its specificity for SO-A.

Since antibody showed binding with both A and SO-A, absorption of the antibody with A should reduce the binding with A. In order to test this, 1:1000 diluted antibody (serum) was incubated with 100 μg/ml A at 37°C for 30 min. After centrifugation at 4000xg for 10 min., the supernatant was used as antibody source. ELISA analysis indicated that even after absorption with excess antigen (A), the antibody had 34% more binding with SO-A and the O.D. values with A were reduced to the lower cut off levels.

Further purification and specificity of these antibodies is being evaluated using other epoxides as well as unrelated chemicals.

Other Studies:

Efficacy of glucose-6-phosphate dehydrogenase as a marker of chemical exposure has also been studied (Toxicol. Letters 69:121-127, 1993). We have also shown that 3-hydroxypropyl mercapturic acid in urine can serve as a marker of chemical exposure to allylic compounds (J. Appl. Toxicol. 9:235-238, 1989).