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STATEMENT ON VINYL CHLORIDE RESEARCH

It is well known that toxic effects are caused by absorption into the body of excessive amounts of any substance. When this occurs the capacity of the body's normal defense mechanisms are exceeded and this may result in more or less serious consequences - as with an excessive intake of alcohol. However, when the amount absorbed does not exceed this capacity the body is capable of dealing with it without toxic effects.

Research on vinyl chloride (VC) in rats has demonstrated that high doses of VC are handled in the body differently than low doses. The results indicate that low doses of VC are readily detoxified, but high doses of VC exceed the body's normal defense mechanisms and detoxification is slowed and perhaps changed. Therefore, exposure to high levels of VC in rats results in an increased susceptibility to toxic effects including cancer. From data available, it appears that induction of cancer is correlated with high doses of VC where detoxification is slowed, altered or incomplete, and that cancer is not induced at low doses where the body's normal defense mechanisms remain fully operative.

by P. G. Watanabe et al.
April 28, 1976

BOR 010670

Summary of the Studies on the Pharmacokinetics of
Vinyl Chloride in Rats and its Possible Relationship
to Oncogenicity

P. G. Watanabe, G. R. McGowan, J. A. Zempel
and P. J. Gehring

ABSTRACT

Studies on the fate of ^{14}C -labeled vinyl chloride (VC) following oral administration and inhalation exposure in rats demonstrated that the disposition of VC in the body is a function of the dose. As the dose or exposure level of VC is increased the metabolism or detoxification pathways become saturated. VC is detoxified primarily by conjugation with nonprotein sulfhydryl groups in the liver (primarily glutathione, GSH). Studies to characterize the depression of liver GSH as a function of magnitude and duration of exposure to VC have shown that a single 7 hour exposure of rats to 100 ppm or greater results in an unequivocal depression of liver GSH. Exposure to 10 ppm VC for 7 hours did not result in any depression of liver GSH. These results suggest that increasing doses of VC deplete liver GSH and consequently lower the bodys defense mechanism for detoxification of the reactive metabolites of VC. This increased susceptibility may

result in induction of carcinogenesis, and from data available, it appears that a correlation exists between doses of VC which cause tumors and those that saturate metabolic or detoxifying pathways.

Conceptually, the results of these investigations indicate that statistical projections utilizing data collected from rats exposed to high doses of VC are invalid for predicting the hazard of low level exposure because such projections assume that the dynamics governing the fate of VC in the body are unaltered; and in fact, the data presented demonstrate that the fate of VC in rats is highly dependent on the dose administered.

April 28, 1976

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Inhalation Studies in Rats, Mice and Hamsters
With Vinyl Chloride

At Industrial BIO-TEST Laboratories, Inc., a study was conducted to determine the effects from inhalation of vinyl chloride monomer to rats, mice and hamsters. The study was supported by the voluntary contributions of thirty-one VCM/PVC producing companies and is administered by the Manufacturing Chemists Association with the advice and guidance of a technical panel of scientists.

In this study, 200 rats, hamsters and mice (100 of each sex or 600 animals total per exposure group) were exposed to 50, 200 or 2,500 ppm vinyl chloride vapor 7 hours per day, 5 days per week. Mice were exposed for 9 months while rats and hamsters were exposed for 12 months. All animals were then kept for the remainder of their natural lives (up to 26 months of mice and 30 months for rats and hamsters).

There was an increased incidence in neoplasms in the liver of all 3 species at all 3 doses and the incidences are dose-related. In general, the only significant increases in rats or hamsters were in the liver. For mice there appears to be an increased incidence of all neoplasms, but particularly in the lung as well as the liver.

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Vinyl Chloride Inhalation Study

Incidence (percent) of Neoplasms - Males

Species and Organ	Control	Experimental Groups		
		T-I (50 ppm)	T-II (200 ppm)	T-III (2500 ppm)
RATS				
Liver	6.4	30.9	73.7	63.3
Kidney	0	7.4	0	0
Skin	19.0	11.2	5.0	2.0
Misc.	6.4	18.5	10.5	4.4
Pituitary				
Brain				
Mesentery				
Adrenals				
Thymus				
Lymph Node				
Genital				
MICE				
Liver	1.8	27.0	90.2	90.8
Lung	1.8	30.2	67.1	87.7
Skin	0	0	1.0	4.0
Misc.	1.8	1.1	9.3	10.8
Kidney				
Mesentery				
Lymph Node				
Genital				
HAMSTERS				
Liver	0	23.9	17.5	62.5
Skin	0	5.0	4.0	1.1
Misc.	0	15.2	7.5	8.9
Kidney				
Mesentery				
Lymph Node				
Thymus				
Genital				

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Vinyl Chloride Inhalation Study

Incidence (percent) of Neoplasms - Females

Species and Organ	Experimental Groups				
	Control	T-I (50 ppm)	T-II (200 ppm)	T-III (2500 ppm)	T-IV (2500 ppm)
RATS					
Liver	4.8	39.5	76.3	78.5	76.7
Kidney	0	1.3	1.3	2.6	0
Skin	64.0	56.0	23.0	13.1	25.0
Misc.	17.9	13.2	9.2	8.9	6.8
Pituitary					
Brain					
Mesentery					
Adrenals					
Thymus					
Lymph Node					
MICE					
Liver	0	36.1	82.6	85.9	
Lung	4.3	43.1	60.5	81.2	
Skin	2.0	37.0	17.0	20.0	
Misc.	2.1	38.9	5.8	8.2	
Kidney					
Mesentery					
Lymph Node					
Genital					
HAMSTERS					
Liver	11.4	18.2	26.6	60.6	
Skin	0	2.0	15.3	1.0	
Misc.	11.4	9.1	8.9	6.1	
Kidney					
Mesentery					
Lymph Node					
Thymus					
Genital					

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* Food Available During Exposure.

Interim Report on Mortality
and Gross Observations
in Rats, Mice and Hamsters
With Vinyl Chloride

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Interim Report on Mortality
and Gross Observations
in Rats, Mice and Hamsters
With Vinyl Chloride

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D. E. Gordon and J. C. Calandra

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At Industrial BIO-TEST Laboratories, Inc., a study was conducted to determine the effects from inhalation of vinyl chloride monomer to rats, mice and hamsters. The study was supported by the voluntary contributions of thirty VCM/PVC producing companies. It is administered by the Manufacturing Chemists Association (MCA), with the advice and guidance of a technical panel of scientists from the sponsoring firms.

The protocol for the study was designed by the technical panel. The study was designed as a life-span study in these species which usually means 1-1/2 to 2 years for the "in-life" portion, with tissue examination to follow this period of time. All animals were allowed to live the remainder of their natural lives. Therefore, the total time of the study was longer than 2 years.

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In this study, 200 rats, hamsters and mice (100 of each sex or 600 animals total per exposure group) were exposed to 50, 200 or 2,500 ppm vinyl chloride vapor 7 hours per day, 5 days per week. Mice were exposed for 9 months while rats and hamsters were exposed for 12 months. All animals were then kept for the remainder of their natural lives.

The rats were COBS Charles River; mice were CDI Swiss Charles River; and the hamsters were Golden Syrian from the same source. Food was removed during exposure, water was present and no bedding was used at any time.

The usual procedure in conducting inhalation studies is not to have food present in the chamber during exposure. In at least some of Dr. Maltoni's studies, food was present in the chamber. The question was raised as to whether ^{contaminated food} would influence the onset or incidence of tumors. Therefore, a separate group of 100 female rats was exposed to 2,500 ppm VCM while food was in the chamber.

All 600 animals per level were exposed in a single chamber. The chambers were operated under dynamic conditions. The chambers were sealed and were operated under slight negative pressure. This was done so that, in case of an accidental leak, room air would flow into the chamber instead of contaminated air flowing out of the chamber.

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The concentrations in the chamber were analyzed using gas-liquid chromatography. An automatic sampling device allowed samples to be drawn from 5 locations in each chamber. The concentrations at all locations in each chamber were monitored every day during the total period of operation of the chamber.

Moribund and dead animals received gross autopsies and many gross lesions were photographed. The major organs of all animals were fixed for histological examination.

There were no particularly adverse effects on body weights or, elements or enzymes measured in the blood.

The data for mortality or survival are presented in Tables I, II and III. It can be seen that the last survivors of mice were sacrificed or died in month 26, while the last survivors of rats or hamsters were sacrificed or died in month 30. While these tables indicate "mortality", it should be noted that the numbers also include those animals sacrificed in extremis.

In rats and mice (and somewhat in hamsters) there was a dose-related increase in mortality. This is well illustrated by examining the time to 100% mortality in the tables. For example, in mice the months in which there was 100% mortality were 11 at 2,500 ppm, 14 at 200 ppm, 16 at 50 ppm and 20 (male) or 26 (female) controls. In rats the months were 16 at 2,500 ppm (both fed and fasted), 21 at 200 ppm, 29 (male) or 30 (female) at 50 ppm and 30 for controls.

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Preliminary examination of tissues from mice which had died after about 6 or 7 months of exposure indicated possible tumors in the livers, lungs, mammary glands and/or skin. At that time there was no evidence of tumors in the rats or hamsters.

A tabulation of incidences of neoplasms for males and females is presented in Tables IV and V. The males and females were tabulated separately to reveal any differences between the 2 sexes. A comparison of the data indicates that there are no real differences between males and females with regard to type or incidence of neoplasms.

The data from these tables indicate an increased incidence in neoplasms in the liver of all 3 species at all 3 doses and the incidences are dose related. In general, the only significant increases in rats or hamsters were in the liver. For mice there appears to be an increased incidence of all neoplasms, but particularly in the lung as well as the liver.

In many instances the incidences at the 2 higher levels (200 and 2500 ppm) are about the same. This might be interpreted as evidence of lack of dose-response. However, these incidences are very high and probably indicate nearing the plateau of the dose-response curve at 200 ppm.

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The incidences (liver) at 50 ppm are well above control values which indicates that this is a definite effect level.

A comparison of tumor data of fed and fasted (during exposure) female rats exposed to 2500 ppm VCM leads to the conclusion that there was no significant difference between the 2 groups. For example, the incidences of liver tumors were 77 and 78%. Although the incidence of skin tumors was 25% in the fed animals vs. 13% in the fasted animals, it should be noted that incidence in this group of fasted animals was the lowest of any group (control 64%, 50 ppm 56% and 200 ppm 23%).

It was stated previously that there were 100 animals per group (male or female). It will be noted in the tables of incidences of neoplasms that the denominator in most cases is less than 100. There are several reasons for this. As soon as an animal dies, autolysis starts. If an animal is necropsied soon after death the tissues are worth saving for histological examination. If, however, several hours elapse from death to necropsy, the tissues may not be saved if the pathologists feels there is too much postmortem autolysis.

The animals were housed in groups. If an animal (particularly mice, hamsters & rats) dies in a cage with other animals, cannibalism

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usually occurs unless the dead animal is removed immediately.

When the chambers were open (no exposure) and an animal was observed to be dead, it was removed and tissues were saved. If an animal died overnight, it frequently was lost for histology.

There is one other important aspect for inhalation studies compared to other studies such as feeding. The chamber is sealed during exposure. Even if an animal is observed to be dead in the chamber, there is no way to remove it until the end of the exposure. This results in loss of tissues for histology.

In most of the chronic inhalation studies now being conducted, all animals are housed individually to prevent many of these losses.

The denominator for the skin usually is 100 (or slightly less such as 98 or 99). If an animal is cannibalized, the skin usually is left. Even though there is autolysis or degeneration of other organs and tissues, the skin is not changed enough to present meaningful evaluation. Therefore, the denominator for the skin is higher than for other organs or tissues, and does allow accountability of the number of animals in the study.

It might also be emphasized that in the usual chronic toxicity or carcinogenic study at the present time 50 animals per group are judged adequate to start a study. With life-time studies in rodents such as rats, mice or hamsters, a number of animals

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are expected to be lost for good histological examination. The number of 50 is used to assure that an adequate number, usually 20 to 25, are available for examination at the end of the study.

The VCM study was designed with twice as many animals to assure that the usual expected number of 20 to 25 would be available at the end of the study. It will be noted that the denominator in all cases exceeds the 20 to 25, and it far exceeds the 20 to 25 in almost all cases.

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TABLE I

TEST MATERIAL: Vinyl Chloride (Ethylene Derived)

Chronic Vapor Inhalation Toxicity Study - Albino Rats,
Albino Mice and Golden Hamsters

Summary of Mortality Data - Albino Rats

Month Number	Cumulative Number of Mortalities at End of Month								
	UC		T-I		T-II		T-III		T-IV
	M	F	M	F	M	F	M	F	F
1	0	0	0	0	0	0	0	0	0
2	0	0	2	0	1	0	0	0	0
3	0	1	2	0	1	0	0	4	0
4	0	2	4	1	3	0	1	4	0
5	2	3	5	1	6	1	3	4	0
6	3	4	6	1	6	1	5	4	0
7	4	4	8	2	6	2	6	5	0
8	4	5	9	3	10	5	10	8	1
9	4	6	10	4	11	5	16	15	4
10	5	7	11	7	12	9	24	28	7
11	7	8	13	9	14	19	34	52	17
12*	13	14	19	16	21	37	58	66	40
13	16	17	23	24	26	50	73	83	46
14	19	21	26	30	33	63	85	86	76
15	21	23	28	37	40	75	94	89	86
16	23	25	31	45	46	87	100(4)	100	100(7)
17	28	27	35	53	55	90	100	100	100
18	33	28	38	59	64	94	100	100	100
19	38	31	43	66	71	94	100	100	100
20	46	34	47	73	77	94	100	100	100
21	52	42	52	80	100(18)	100(4)	100	100	100
22	57	48	63	89	100	100	100	100	100
23	64	54	72	92	100	100	100	100	100
24	73	65	81	94					
25	79	69	85	97					
26	86	76	91	98					
27	92	84	96	99					
29	98	93	100	99					
30	100	100	100	100					

*Included 5 males and 5 females from each group excluding T-IV sacrificed
for cytogenic study

() Indicates moribund or sick animals sacrificed.

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TABLE II

TEST MATERIAL: Vinyl Chloride (Ethylene Derived)

Chronic Vapor Inhalation Toxicity Study - Albino Rats,
Albino Mice and Golden Hamsters

Summary of Mortality Data - Albino Mice

Month Number	Cumulative Number of Mortalities at End of Month							T-III M
	UC M	F	T-I M F		T-II M F		F	
1	0	0	0	0	0	0	0	0
2	0	0	0	3	0	0	0	0
3	2	0	6	4	3	0	1	1
4	5	1	19	5	4	1	4	4
5	8	2	26	6	4	2	8	8
6	12	2	30	6	8	3	10	10
7	18	5	31	11	9	12	13	13
8	18	5	32	14	16	30	30	30
9	23	8	38	24	37	58	56	56
10	28	10	42	31	78	82	76	76
11	30	15	54	46	96	94	100	100
12	34	16	57	66	98	95	100	100
13	44	23	70	80	99	97	100	100
14	54	28	79	91	100	100	100	100
15	67	39	88	97	100	100	100	100
16	82	46	100	100	100	100	100	100
17	82	54	100	100	100	100	100	100
18	82	58	100	100	100	100	100	100
19	92	70	100	100	100	100	100	100
20	100	77	100	100	100	100	100	100
21	100	81	100	100	100	100	100	100
22	100	89	100	100	100	100	100	100
23	100	92	100	100	100	100	100	100
24		96						
25		97						
26		100						

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TABLE III

TEST MATERIAL: Vinyl Chloride (Ethylene Derived)

Chronic Vapor Inhalation Toxicity Study - Albino Rats,
Albino Mice and Golden Hamsters

Summary of Mortality Data - Golden Hamsters

Month Number	Cumulative Number of Mortalities at End of Month							
	UC	T-I		T-II		T-III		
	M	F	M	F	M	F	M	F
1	5	4	4	4	7	5	2	5
2	8	11	10	12	9	16	10	11
3	8	13	12	14	12	18	14	13
4	9	15	16	16	15	19	15	13
5	9	18	16	16	16	20	15	15
6	13	18	16	16	20	21	16	16
7	13	28	24	19	22	33	18	26
8	14	28	24	19	23	35	19	28
9	14	29	24	20	25	36	20	28
10	14	30	26	20	26	37	23	29
11	15	31	28	21	26	38	28	36
12	18	36	34	33	37	46	40	45
13	23	40	43	49	46	49	49	58
14	28	46	49	58	54	53	61	69
15	39	61	53	72	66	55	71	84
16	50	78	58	83	74	59	84	100
17	57	81	61	85	76	66	86	100
18	62	85	62	86	77	75	88	100
19	65	90	68	89	79	84	92	100
20	71	96	73	95	86	89	100(5)	100
21	71	100(2)	80	100(3)	100(13)	100(7)	100	100
22	73	100	82	100	100	100	100	100
23	78	100	85	100	100	100	100	100
24	83		88					
25	88		92					
26	90		95					
27	94		98					
28	94		99					
29	99		100					
30	100							

() Indicates moribund or sick animals sacrificed.

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TABLE IV

Vinyl Chloride Inhalation Study - IET NO. 663-03222Projected Incidence of Neoplasms - Males

Species & Organ	Control	Experimental Groups		
		T-I (50 ppm)	T-II (200 ppm)	T-III (2500 ppm)
RATS				
Liver	5/78 (6.4%)	25/81 (30.9%)	56/76 (73.7%)	57/90 (63.3%)
Kidney	0/78	6/81 (7.4%)	0/76	0/90
Skin	19/100(19%)	11/98(11.2%)	5/100(5.0%)	2/100 (2.0%)
Misc.	5/78 (6.4%)	15/81 (18.5%)	8/76 (10.5%)	4/90 (4.4%)
Pituitary				
Brain				
Mesentery				
Adrenals				
Thymus				
Lymph Node				
Genital				
MICE				
Liver	1/55 (1.8%)	17/63 (27%)	74/82 (90.2%)	59/65 (90.8%)
Lung	1/55 (1.8%)	19/63 (30.2%)	55/82 (67.1%)	57/65 (87.7%)
Skin	0/100	0/100	1/100 (1.0%)	4/100 (4.0%)
Misc.	1/55 (1.8%)	7/63 (1.1%)	8/86 (9.3%)	7/65 (10.8%)
Kidney				
Mesentery				
Lymph Node				
Genital				
HAMSTERS				
Liver	0/53	11/46 (23.9%)	7/40 (17.5%)	35/56 (62.5%)
Skin	0/100	5/100 (5.0%)	4/100 (4.0%)	1/94 (1.1%)
Misc.	0/53	7/46 (15.2%)	3/40 (7.5%)	5/56 (8.9%)
Kidney				
Mesentery				
Lymph Node				
Thymus				
Genital				

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TABLE V

Vinyl Chloride Inhalation Study - IBT NO. 663-03222

Projected Incidence of Neoplasms - Females

Species & Organ	Control	Experimental Groups			
		T-I (50 ppm)	T-II (200 ppm)	T-III (2500 ppm)	T-IV (2500 ppm)
RATS					
Liver	4/84 (4.8%)	30/76 (39.5%)	58/76 (76.3%)	62/79(78.5%)	50/73(68.5%)
Kidney	0/84	1/76 (1.3%)	1/76 (1.3%)	2/79 (2.6%)	0/73
Skin	64/100 (64%)	56/100 (56%)	23/100 (23%)	13/99 (13.1%)	25/100 (25%)
Misc.	15/84 (17.9%)	10/76 (13.2%)	7/76 (9.2%)	7/79(8.9%)	5/73(6.8%)
Pituitary					
Brain					
Mesentery					
Adrenals					
Thymus					
Lymph Node					
Genital					
MICE					
Liver	0/47	26/72 (36.1%)	71/86(82.6%)	73/85 (85.9%)	
Lung	2/47 (4.3%)	31/72 (43.1%)	52/86(60.5%)	69/85 (81.2%)	
Skin	2/100 (2.0%)	37/100 (37.0%)	17/100(17.0%)	20/100 (20.0%)	
Misc.	1/47 (2.1%)	28/72 (38.9%)	5/86 (5.8%)	7/85 (8.2%)	
Kidney					
Mesentery					
Lymph Node					
Genital					
HAMSTERS					
Liver	4/35 (11.4%)	6/33 (18.2%)	21/79 (26.6%)	20/33 (60.6%)	
Skin	0/100	2/98 (2.0%)	15/98 (15.3%)	1/99 (1.0%)	
Misc.	4/35 (11.4%)	3/33 (9.1%)	7/79 (8.9%)	2/33 (6.1%)	
Kidney					
Mesentery					
Lymph Node					
Thymus					
Genital					

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* Data Available During Progress

BOR 010688

CYTOGENETIC STUDIES OF BONE MARROW CELLS
FROM RATS EXPOSED TO VINYL CHLORIDE

(5) -1
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Biomedical and Comparative Toxicology Research Laboratory

The Biomedical and Comparative Toxicology Research Laboratory of the Texas Division of Dow Chemical U.S.A. was asked by the Manufacturing Chemists Association to conduct cytogenetic studies on some of the rats which had been exposed to vinyl chloride at Industrial BIO-TEST Laboratories, Decatur, Illinois. The details of the exposure levels and pathological findings will be reported separately. Preliminary reports have appeared in *Chemical Week* (115:30, July 17, 1974) and in the *Annals of the New York Academy of Sciences*, Volume 246, page 219.

The test animals were Charles River CD outbred albino rats which had been exposed to vinyl chloride gas for 7 hours per day, 5 days per week for one year at 0, 50, 200 and 2500 ppm in air. Cytogenetic studies on 5 males and 5 females from each group showed that there was no statistically significant increase in the chromosomal aberration rates in bone marrow cells of the exposed rats.

April 28, 1976

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References for
"Cytogenetic Studies of Bone Marrow Cells From Rats
Exposed to Vinyl Chloride
by Dr. R. V. Johnston et al.

1. Kilian, D. J. and Picciano, D. J., Cytogenetic monitoring and vinyl chloride workers, Open Meeting on Evaluation of Systems to Detect Mutagenic Activity of Chemicals, July 16 and 17, 1975, NIH, Bethesda, Maryland.
2. Fleig and Thiess, A. M., Chromosome analysis after vinyl chloride exposure, Arbeitsmedizin, Sozialmedizin, Präventivmedizin 9(12) 260-283 (1974).
3. Johnston, R. V., Mensik, D. J., Pinkerton, M. N., Whorton, E. B., Cytogenetic studies of bone marrow cells from rats exposed to vinyl chloride, TBM-108-1, B16863, (1975).
4. Ducatman, A., Hirschhorn, Kurt, Selikoff, I. J., Vinyl chloride exposure and human chromosome aberrations, Mutation Research 31:163-168 (1975).
5. Rannug, U. et al, The mutagenicity of vinyl chloride after metabolic activation, Ambio 3:194-197 (1974).
6. Funes-Cravioto, F. et al, Chromosome aberrations in workers exposed to vinyl chloride, The Lancet, February 22, 1975, page 459.
7. Purchase, I.F.H. et al, Chromosomal and dominant lethal effects of vinyl chloride, The Lancet, (1975).
8. Bartsch, H., Human, rat and mouse liver-mediated mutagenicity of vinyl chloride in S. Typhimurium strains, Int. J. Cancer 15:429-437 (1975).
9. Malaveille, C., Bartsch, H. et al, Mutagenicity of vinyl chloride, chloroethyleneoxide, chloroacetaldehyde and chloroethanol, Biochemical and Biophysical Research Communications, Vol. 63:363-370 (1975).

4/22/76

ATTENTION: Mr. Milton Freifeld

A Mortality Study of Workers Exposed to Vinyl Chloride

April 22, 1976

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This is a report of study of mortality rates in a population of 10,173 workers who at some time in the past were engaged in the manufacture of vinyl chloride or its polymers. An earlier report on a portion of this population showed that these workers experienced an increased risk of death from cancer of the liver (already found by other investigators) and cancers of the lung, brain and lymphatic system. The larger group represented in this study has permitted a more detailed analysis of the relation between the risk of death from these causes and duration and level of exposures.

The study population was identified by an examination of past and present personnel records in 37 plants which produced either vinyl chloride or polyvinyl chloride. Every man who had been in an exposed job for more than one year was identified and his work history obtained. Men who had worked in the past and had quit or been laid off were traced to determine whether they were still alive as of December 31, 1972, the date chosen as the "closing date" of the study.

Of the 10,173 men selected for study, 95 percent were successfully traced, and 707 deaths were found. The study population included men hired as far back as the late 1930's and included 2,006 men who had worked 15 years or more.

The number of deaths which occurred was compared with the number which would have been expected in a comparable population of United States males for each cause.

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①-2
The total number of deaths was about 16 percent less than expected, and the total number of cancer deaths was about equal to what would be expected.

Some cancers occurred at a lower rate than expected, and these did not appear to be related to the length or level of exposure. Cancer of the urinary organs and the lymphatic system showed some excess deaths (22 percent and 12 percent) but were not related in any systematic way to exposure. There was a 41 percent excess in leukemia deaths, also not related to the circumstances of exposure.

Lung cancer showed an 8 percent excess in the total study group, but in men who had worked for 15 years or more and had begun work at least 20 years before the closing date of the study, there was a 52 percent excess.

Cancers classed as "Other and Unspecified" showed a 44 percent excess in the study population as a whole, but a 230 percent excess in the older group mentioned above. This excess was largely due to an excess of brain cancer, since 38 percent of these cancers were brain, compared with 20 percent for the general population.

There was a slight excess of digestive cancer in men with high exposure, and in men with other than low exposure who had worked at least 15 years beginning at least 20 years before the study date. In these older men 50 percent of the digestive cancers were liver cancer compared with about 9 percent for the general population.

ATTENTION: Mr. Milton Freifeld

② In another analysis, the study population was scored according to the estimated total amount of vinyl chloride to which they were exposed; men were given a score of 1 for each month of low level exposure, 2 for each month at medium exposure, and 3 for each month at high exposure. There was a somewhat irregular increase in mortality from digestive cancer, respiratory cancer and cancer of other and unspecified sites with increasing score. For men with scores of 400 or more (equivalent to 11 years of high exposure or 33 years of low exposure, for example) the excess mortality was 88 percent for digestive cancer, 54 percent for respiratory cancer, and 183 percent for cancers of other and unspecified sites.

The study generally confirms the findings of earlier investigators, but provides additional data for men with long service whose employment began many years ago.

by Dr. W. R. Gaffey

BOR 010693



ITEM 1

UNIVERSITY OF LOUISVILLE
LOUISVILLE, KENTUCKY 40202

April 20, 1976

SCHOOL OF MEDICINE
DEPARTMENT OF MEDICINE
DIGESTIVE, ENDOCRINE AND NUTRITION SECTION

HEALTH SCIENCE CENTER
WALNUT & PRESTON STREETS

Dr. T. R. Torkelson
Dow Chemical Company
Corporate Medical Department
2030 Dow Center
Midland, Michigan 48640

Dear Doctor Torkelson:

May I express our delight in having had members of the Manufacturing Chemists Association Research Committee visit us earlier this month; we hope that their visit was both enjoyable and informative. Our post-lunch meeting helped to clarify a number of points concerning our Manufacturing Chemists Association Grant proposal and I would like to take this opportunity to submit for the members consideration a modification of our original proposal in light of the expressed interests of the members of the research committee.

Since the members expressed interest in supporting proposals A and G, having to do with the study of immunological systems, it seems that these would require no further elaboration or adjustment. My understanding is that the same would be true for proposal C, concerning electron microscopic evaluation of liver tissue. What I would like to do here is (a) amplify on the immediate clinical applicability of some of the other proposals presented, (b) provide a modified budget for these proposals and (c) suggest that if cuts must be made in the program, the cuts that would be least damaging in terms of ongoing efforts and in terms of a cohesiveness of the overall research program, would be the proposals by Dr. Hoffman (H) and Dr. Sigdestad (I) and parts of Drs. Du (B2 B5 B6 B7) and Wong (E2) (see revised budget attached). Firstly, proposal C concerning the glycosaminoglycans in the early detection and etiology of angiosarcoma of the liver presented by Dr. Charles E. Kupchella: this has already produced significant results. Most chemical injury and cancer appear to be associated with "scar" or collagen formation. This collagen formation is associated with the increased production of glycosaminoglycans. Dr. Kupchella's preliminary study indicates that there is a characteristic pattern of glycosaminoglycan urinary excretion among long-term vinyl-chloride-exposed individuals not seen in individuals with alcoholic liver injury, hepatitis, and cancers not directly involving the liver. These urinary excretion patterns appear to change as one develops primary liver cancer such as angiosarcoma. At this point, the findings reported by Dr. Kupchella at the Third

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International Symposium on the Detection and Prevention of Cancer, require additional verification and modifications of this method for use as spot urine testing in large worker population. If future studies continue to verify our present finding, i.e. that this test is indicative of early vinyl chloride injury, it could be applicable throughout the industry where chemically induced fibrosis may occur. This proposal could yield a sensitive, simple and inexpensive means of surveillance for early liver injury. A copy of the paper to be presented in New York is enclosed.

Concerning the proposal by Dr. Wong, and the related proposal by Dr. Streips; I would like to elucidate on the value of this work in determining and directing priorities as to which metabolite should be studied for their potential carcinogenicity. Dr. Wong's ability to synthesize metabolic products of various chemicals allows immediate mutagenic bacterial studies for the identification of those chemicals with greatest carcinogenic capability. In order to control costs and increase benefits for the amount of time and money invested, these mutagenic studies must be performed in order to realistically develop strategies for blocking the etiologic chemical changes leading to the initiation of angiosarcoma. Considering the overall complexity of studies of this sort, we feel that the combined work of Drs. Wong and Streips provides a reasonably straight forward approach to unravelling sequences leading to angiosarcoma.

In addition, Dr. Wong's work will develop a method of detecting trace amounts of chemicals and their metabolic products by use of multivariant analysis in biological tissue. Our present system of storing bloods, urines, and tissue on all workers in the medical surveillance program gives us the equivalent of an immediate clinical trial in a carefully observed worker population with known exposure. This procedure could give us an applicable test system within three years.

Finally, Dr. Du's work will make use of animal studies in determining the most specific and earliest enzymatic and biochemical alterations produced by chemical exposure. Although some have expressed the view that biochemical alterations have all been identified and worked out, our clinical experience indicates the need for studies which will objectively determine which screening methods should be applied and to which of the exposed industrial population. The animal studies proposed by Dr. Du will allow us to apply these methods under controlled conditions of exposure, dose and duration, providing useful information in just a few years. This will be far more efficient means of determining the best screening methods to employ based on hard scientific data rather than clinical opinion.

Simultaneously, Dr. Wong will utilize these same animal tissues for verifying the sensitivity and specificity of the multivariant analysis system for trace organic element detection in biological tissue. The detection of a trace product does not by itself prove a causal relationship nor indicate recent or past exposure. Therefore, it is vital that these studies be done (simultaneously with the studies for sensitivity and specificity) for verification of the relationship of detection to cause of injury. This information will greatly help in the interpretation of our findings from our stored human blood, urine and tissue samples.

Dr. T.R. Torkelson
page -3-

We would like to again point out the importance of our being able to continue to pursue this multidisciplinary research approach in the study of the vinyl chloride problem. By addressing the vinyl chloride problem as a model and in this systematic manner we feel that this unique combination of investigations will go far toward providing understanding chemical carcinogenesis in general and the etiology of angiosarcoma. Our program can be left essentially intact with the attached budget. The work proposed, we feel, is clearly defined and should produce positive results well in excess of investment. In addition, we would also like to point out that funding of such a program will sharpen our overall skills and further develop this approach applicability to other chemicals and in other situations. I believe that the budget proposed is well within the desirability and capability of the Manufacturing Chemists Association's constituency. In essence, we are asking the vinyl chloride industry to support the program at somewhat less than a level that has been supported by the B.F. Goodrich Company alone for the past two years.

Thank you for your serious consideration.

Sincerely yours,



Carlo H. Tamburro, M.D.
Associate Professor of Medicine
Chief, Digestive Diseases & Nutrition
Section

CHT:mmm
Enclosures

cc: Dr. Zeb G. Bell, Jr.
Dr. Walter D. Harris
Dr. Maury Johnson
Mr. Howard L. Kusnetz
Dr. W.E. Rinehart
Dr. W. Mayo Smith
Mr. R.N. Wheeler

BOR 010696

REVISED BUDGET
UNIVERSITY OF LOUISVILLE
RESEARCH PROPOSAL

RESEARCH TECHNIQUES AND METHODS FOR DETECTION
AND PREVENTION OF CARCINOGENESIS IN INDUSTRIAL WORKERS

This revised budget is for proposals A1-4, G, D, C, E1, B3-4, and F, all of which have the most clinical applicability. We have excluded proposals B1, B2, B5, B6, B7, E2, H, and I.

INDIVIDUAL BUDGETS

Proposal	Title	Investigator	Personnel Budget	Supplies	Total
A 1-4	Immunological Systems for the Detection of Vinyl Chloride and Other Chemical Injury	P. Fortwengler	34,100	16,795	50,895
G	Tissue Antigenic Systems of Detection	E. Espinosa	9,000	5,000	14,000
D	Electron Microscopic Evaluation of Liver Tissue from Chemical Workers	R. Schrodt		7,000	7,000
C	Tissue and Urinary Acid Mucopolysaccharide Changes Related to Vinyl Chloride Injury: Use In Early Detection and Diagnosis	C. Kupchella	8,000	7,500	15,500
E 1	Multivariant Analysis of Biological End Products and Biochemicals to Document Chemical Exposure for Early Diagnosis	J. Wong	10,000	15,000	25,000
B 3 & 4	Biochemical Enzymatic Systems for Detection of Vinyl Chloride and Other Chemicals	J. Du	27,000	7,332	34,332
F	Assays for Identifi- cation of the Carcino- genic Potential of Industrial Chemicals	U. Streips	12,000	11,000	23,000
		Subtotals	100,100	69,627	169,727
		Total	169,727		

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A. SUMMARY OF BUDGETS

Salaries:	100,100	
Supplies:	69,627	
Indirect Cost:	65,065	
65%		TOTAL: 234,792

B. POSSIBLE ALTERNATE FUNDING SUGGESTION:

Salaries:	100,100	
Supplies:	69,627	
Indirect Cost:	30,035	
30%		TOTAL: 199,757

URINARY AND TISSUE GLYCOSAMINOGLYCAN PATTERNS IN HEPATIC ANGIOSARCOMA

Charles E. Kupchella and Carlo H. Tamburro

Cancer Center and Department of Medicine
University of Louisville School of Medicine
Louisville, Kentucky 40201

I. INTRODUCTION

The recent discovery of a relationship between vinyl chloride and angiosarcoma of the liver has received much attention (1-3). Although there are now systematic detection programs for vinyl chloride workers (3,4), there is as yet no specific chemical abnormality that serves as a good indicator of early, vinyl-chloride-induced liver injury and angiosarcoma. Alpha fetoprotein has been a relatively valuable serological marker for hepatocellular carcinoma (5), but it has not as yet proven useful in the detection of angiosarcoma (6). New leads are needed if more specific tests are to be developed for angiosarcoma.

The literature suggests that the glycosaminoglycans in the urine and/or blood should be evaluated as a possible aid in early detection. The production of sulfated glycosaminoglycans is characteristic of malignant vascular tumors of the skin and some pathologists use this feature as a diagnostic aid (7). Barr and Bonin (8) observed a strong positive alcian-blue, glycosaminoglycan staining reaction in human angiosarcoma tissue and suggested that an attempt be made to qualitate and quantitate the production of glycosaminoglycans in the neoplasms, serum, and urine of those at risk. They pointed out that the urinary glycosaminoglycans may have diagnostic significance in angiosarcoma and, if so, a glycosaminoglycan spot test might easily be employed as a gross screening test of vinyl chloride production workers.

A number of other observations place the glycosaminoglycans in a relevant position with regard to angiosarcoma. Angiosarcoma is accompanied by connective tissue abnormalities (2,9) and changes in tissue, urinary, and blood glycosaminoglycans have been found to occur in many connective-tissue disorders -- including connective tissue disorders of the liver (10-14) -- as well as in hepatic cancer (15-17).

Supported in part by grants from the B. F. Goodrich Company and the American Cancer Society (IN-111) and a contract with the National Cancer Institute (N01-CN-55212).

The purpose of this study was to make a preliminary determination of the glycosaminoglycan patterns in tissue and urine associated with angiosarcoma of the liver and with vinyl-chloride-induced liver injury other than angiosarcoma and to compare these patterns with those in normal controls and those associated with other liver disease. Our goal was to evaluate the use of glycosaminoglycan patterns in the early detection of vinyl-chloride-induced liver injury and angiosarcoma and to explore the role of the glycosaminoglycans in the etiology of vinyl chloride injury.

II. PROCEDURES AND MATERIALS USED

Urine specimens were collected as occasional samples from: 9 normal controls; 9 individuals with histories of occupational exposure to vinyl chloride and having abnormal, liver, biochemical studies; 6 with "other" cancers prior to surgery; 3 with angiosarcoma; 8 with active viral hepatitis; 6 with cirrhosis; 2 with lung-liver metastases; and 4 with metabolic disorders of the liver (congenital and indirect hyperbilirubinemia).

In one case of angiosarcoma, 24-hr urines were collected on alternate days beginning 2 weeks prior to death.

Urine samples were collected without preservative and frozen at -76° until analysis. Specimens were divided into two 25 ml samples and one 5 ml sample. Urinary creatinine was measured on the 5 ml sample using a Technicon Autoanalyzer. The degree of urinary glycosaminoglycan polymerization was estimated by dialyzing one 25 ml sample for 24 hours in tap water; the sample was then treated identically to an undialyzed sample by the method of DiFerrante (18) using cetylpyridinium chloride as a precipitant. After resolubilization of the glycosaminoglycans in water, duplicate samples were assayed for total uronic acid by the modified carbozole reaction of Bitter and Muir (19). The remaining glycosaminoglycans were reprecipitated with cetylpyridinium chloride and separated into the wash, hyaluronic acid, chondroitin sulfate, and heparin fractions as described by Schiller et. al. (20). Each of the fractions was assayed for uronic acid (μ g per mg of creatinine).

Autopsy tissue was obtained in 2 cases of hepatic angiosarcoma (tumor tissue and non-tumor tissue adjacent to tumor), 2 cases of cirrhosis, and in 3 control cases (gun-shot wound victims without liver pathology) and analyzed for glycosaminoglycans by a previously reported modification (21) of the method of Schiller et. al. (20). Uronic acid was determined in each of the hyaluronic acid, chondroitin sulfate, and heparin fractions.

Pieces of tissue were subjected to alcian-blue-periodic-acid-Schiff staining with and without hyaluronidase and diastase pretreatment. These procedures were carried out according to the methods described by Mowry (22).

Ascitic fluid was also obtained at autopsy in one case of angiosarcoma and analyzed for glycosaminoglycans. The fluid was centrifuged

GLYCOSAMINOGLYCANS IN ANGIOSARCOMA

and the sediment analyzed as tissue above. The supernatant was treated by the method for urine described above.

III. RESULTS

A summary of the urinary glycosaminoglycan measurement is given in Table I. Normal controls had the least urinary glycosaminoglycans (measured as uronic acid) of all groups. All other groups showed some elevation. The levels in angiosarcoma, hepatitis, cirrhosis, and liver metastases were significantly elevated ($P < .05$) over normal controls. The cirrhotic group exhibited the greatest variance in urinary glycosaminoglycans. No significant differences were found in urinary creatinine levels between groups.

There were no significant differences between groups in either the percentage of the total glycosaminoglycans that was dialyzable (Table I) or in the percentage of the unfractionated total that appeared in the hyaluronic acid, chondroitin sulfate, and heparin fractions.

Seven of 9 vinyl-chloride-exposed individuals other than those with angiosarcoma had positive chondroitin sulfate fractions with negative hyaluronic acid and heparin fractions. This was true in only 3 of 32 other urines evaluated in this same manner.

The pattern of daily glycosaminoglycan excretion prior to death due to angiosarcoma in one individual is given in Figure 1.

Total tissue glycosaminoglycan levels for angiosarcoma tumors, fibrotic tissue adjacent to tumors, cirrhotic liver tissue and normal liver tissue are shown in Figure 2. Fractional hyaluronic acid, chondroitin sulfate, and heparin levels are given in Figure 3.

Histochemically, angiosarcomatous tissue exhibited a strong alcian-blue positive staining reaction. Alcian-blue staining was only slightly less in "non-tumor" tissue adjacent to tumor masses. The staining reaction in tissue from normal liver was very weak and only slightly stronger in cirrhotic liver tissue. The strong alcian-blue reaction in angiosarcomatous tissue did not occur if sections were pretreated with hyaluronidase.

Ascitic fluid sediment was uronic-acid-positive in only the hyaluronic acid fraction -- 112 μ g uronic acid per gram of dry, defatted sediment; ascitic fluid supernatant contained 1.7, 1.2, and 0.2 μ g uronic acid per ml in the hyaluronic acid, chondroitin sulfate, and heparin fractions, respectively.

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IV. DISCUSSION

The literature indicates that normal male creatinine excretion is 1.5 g per 24 hours (23). Thus, our normal mean (Table I) of $3.2 \pm .4$ μ g cetylpyridinium chloride-precipitable uronic acid per mg creatinine falls in the middle of the normal ranges reported by Varma et. al. (24),

2.6 - 4.7 μ g/mg; DiFerrante and Rich (25), 2.9 - 4.8 μ g/mg; and Kao and Leslie (26), 1.8 - 4.9 μ g/mg.

Although our study was not controlled for age, Goldberg and Cotlier (27) have shown that urinary glycosaminoglycan excretion is constant from ages 20-70. Manley et. al. (28) have shown that the proportion of urinary glycosaminoglycans in the chondroitin sulfate fraction is constant from ages 20-70. Manley et. al. also reported that the chondroitin sulfate fraction is highest at birth and that it gradually drops until age 20, suggesting that urinary chondroitin sulfate reflects tissue growth.

The fact that we found no differences between groups in the creatinine concentration is significant in that it indicates that occasional samples do reflect 24-hour excretion when normalized to creatinine. Precedent for expressing glycosaminoglycan measurements as a function of creatinine content in occasional urine samples has been established by DiFerrante and Rich (25) and Pennock (29). Manley et. al. (28) have shown that the creatinine/uronic acid ratio is steady from ages 20-70.

Our results indicate that the liver diseases evaluated are accompanied by elevated urinary glycosaminoglycan excretion. Our tissue data suggests that this reflects liver-tissue glycosaminoglycan elevation and conforms to the reports by others that both hepatic connective tissue disorders (10-14) and hepatic cancer (15) result in increased hepatic glycosaminoglycan levels. It may be significant that the angiosarcoma patients had half the urinary glycosaminoglycan excretion of patients with liver metastases and that our analysis of angiosarcomatous tumor tissue exhibited half the glycosaminoglycan content reported by Kojima et. al. (15) for hepatocellular carcinoma.

While our data suggest that liver disease results in a decrease in the proportion of highly polymerized glycosaminoglycans, variance was large within each group and none of the differences between groups were statistically significant.

Although we have not completed the characterization of isolated glycosaminoglycan fractions, our data indicate: 1) that the chondroitin sulfates are the primary urinary glycosaminoglycans in both normal controls and in disease states; 2) that the chondroitin sulfates and heparin are the dominant glycosaminoglycans in normal and cirrhotic livers (Figure 3). Chondroitin sulfate is elevated in the fibrotic, non-tumor, portions of angiosarcomatous livers while heparin is the predominant glycosaminoglycan in tumor tissue. Hyaluronic acid is also apparently elevated relative to chondroitin sulfate in angiosarcomatous tumors (Figure 3); and 3) that hyaluronic acid is the sole glycosaminoglycan in ascites fluid sediment.

These qualitative data are in general agreement with those reported by others. Goldberg and Cotlier (27), Douglas et. al. (30), and Varma et. al. (24) have reported that the chondroitin sulfates are the predominant urinary glycosaminoglycans. Varma et. al. reported that 2/3 of urinary glycosaminoglycans are chondroitin-4-and chondroitin-6-sulfate and this agrees with our data on normal controls and on those with liver disease.

Kojima et. al. (15) reported that in hepatocellular carcinoma

GLYCOSAMINOGLYCANS IN ANGIOSARCOMA

tissue, chondroitin sulfates and hyaluronic acid were increased 33 and 10 times, respectively, over amounts found in healthy livers; the heparin and heparan sulfate proportions dropped. This contrasts with our data on angiosarcoma tissue, i.e. heparin and hyaluronic acid increased 5 and 10 times, respectively, over normal tissue; chondroitin sulfate levels rose but fell in proportion to other glycosaminoglycans. Galambos and Shapira (10) reported that the chondroitin sulfates are dominant in normal livers and in hepatic fibrosis, but Kojima et. al. (15) report that chondroitinase-resistant and hyaluronidase-resistant glycosaminoglycans are dominant. Kuroda et. al. (31) also reported that heparan sulfate is the dominant glycosaminoglycan in the normal liver. Our histochemical observation that nearly all of the increased alcian-blue positive material in angiosarcomatous livers was susceptible to hyaluronidase digestion suggests that the observed chondroitin sulfate elevation is due to chondroitin-4- and/or chondroitin-6-sulfate.

The increases in liver and urinary glycosaminoglycans may well reflect an important role of these substances in the process of fibrogenesis and in tumor growth. Galambos and Shapira (10) reported that hyaluronic acid was elevated during hepatic fibrogenesis. If a similar fibrotic process is operative in angiosarcoma, it may be that the observed tumor-tissue heparin increase is reflective of tumor growth. We did observe a four- to six-fold greater heparin level in tumor tissue than in adjacent, non-tumor tissue.

The observation that the chondroitin sulfates tend to be the exclusive uronic-acid-positive constituents in the urine of individuals is paradoxical in that those glycosaminoglycan fractions that are most elevated in angiosarcomatous tissue are those that are absent from the urine of individuals who may well have early, vinyl-chloride-induced liver injury. This pattern may be due to the selective action of lysosomal, glycolytic enzymes in the liver and/or may reflect the role of the chondroitin sulfates in early fibrotic changes in the liver. Certainly the potential usefulness of this pattern in early detection warrants the more complete evaluation now ongoing in our laboratory.

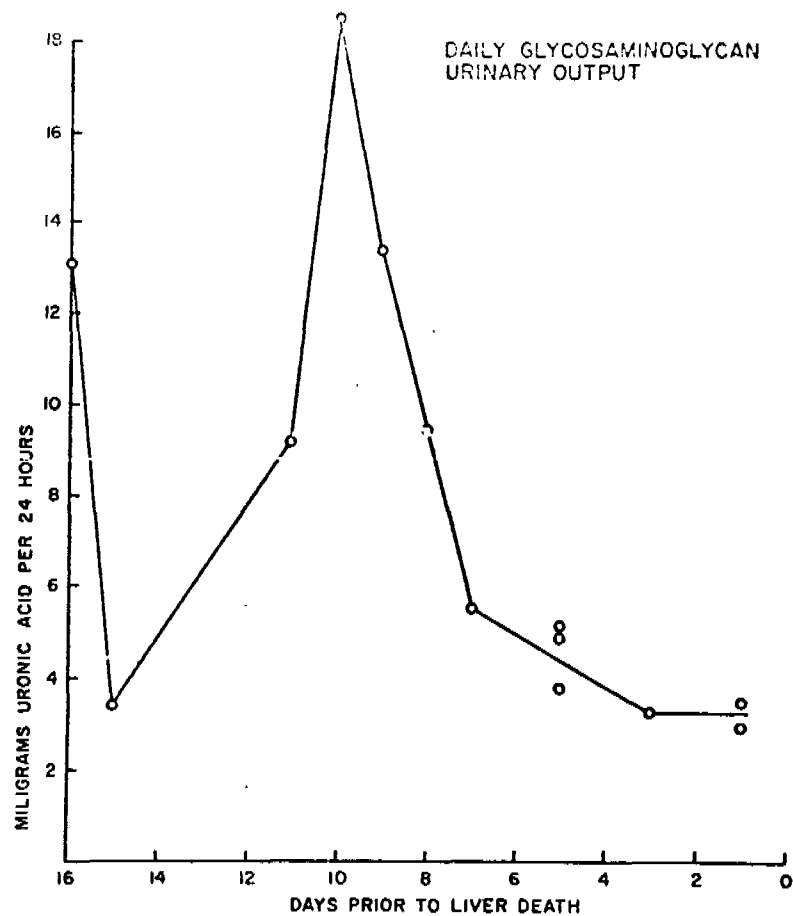
V. SUMMARY

Glycosaminoglycans were measured in urine and tissue of patients with hepatic fibrosis and hepatic cancer including vinyl-chloride-exposure-associated liver injury and angiosarcoma. Angiosarcoma, hepatitis, cirrhosis, and liver metastatic patients exhibited significantly elevated glycosaminoglycan excretion. Angiosarcoma tissue exhibited elevated glycosaminoglycan levels with the greatest increases in the heparin fraction. Histochemically, angiosarcomatous tissue gave a strong alcian-blue staining reaction which could be prevented by pretreatment with hyaluronidase. Although vinyl-chloride-exposure-associated liver injury other than angiosarcoma was not accompanied by a significantly elevated glycosaminoglycan excretion, this condition tended to be associated with a urinary glycosaminoglycan excretion pattern in which the chondroitin sulfate fraction was the only uronic-acid-positive fraction.

TABLE I

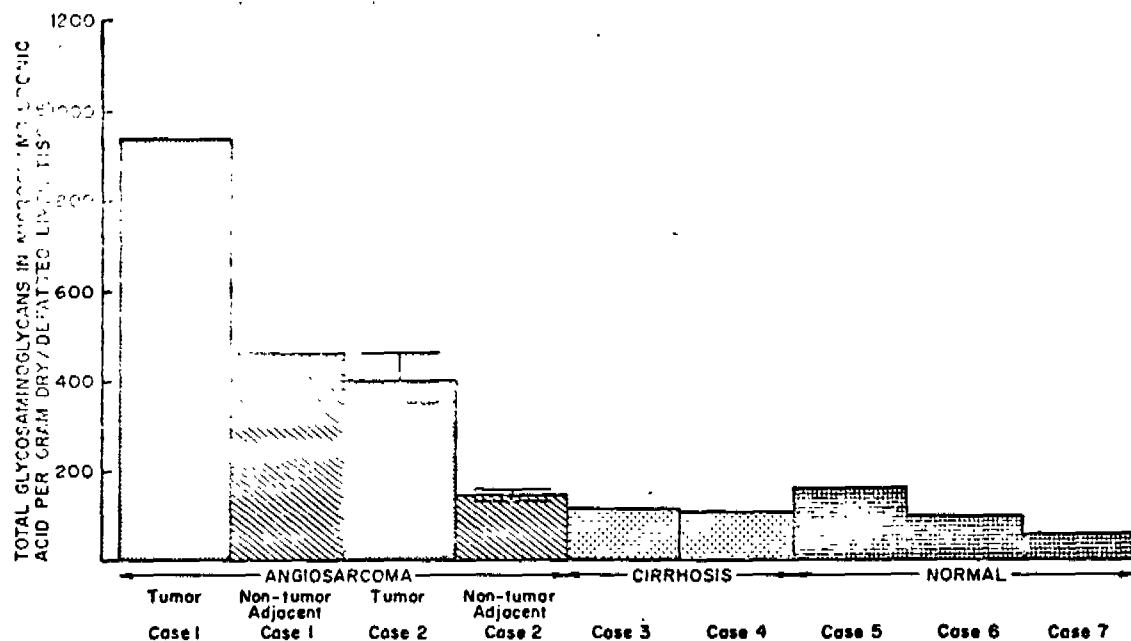
Urinary Glycosaminoglycan Levels in μ g Uronic Acid Per mg Creatinine
by Liver Diseases Category

<u>Patient group</u>	<u>Cases</u>	<u>ug uronic acid per mg creatinine ($\pm$ 1 S.E.)</u>	<u>% uronic acid not dialyzable ($\pm$ 1 S.E.)</u>
normal control	9	3.2 $\pm$.4	65 $\pm$ 5
vinyl chloride exposed	9	4.1 $\pm$.4	39 $\pm$ 6
other cancer	6	4.5 $\pm$ 1.5	39 $\pm$ 6
other liver disease	4	5.1 $\pm$ 0.8	37 $\pm$ 13
angiosarcoma	3	7.6 $\pm$ 1.6	41 $\pm$ 22
hepatitis	8	8.5 $\pm$ 1.8	52 $\pm$ 14
cirrhosis	6	12.7 $\pm$ 3	53 $\pm$ 9
liver metastasis	2	13.8 $\pm$.9	42

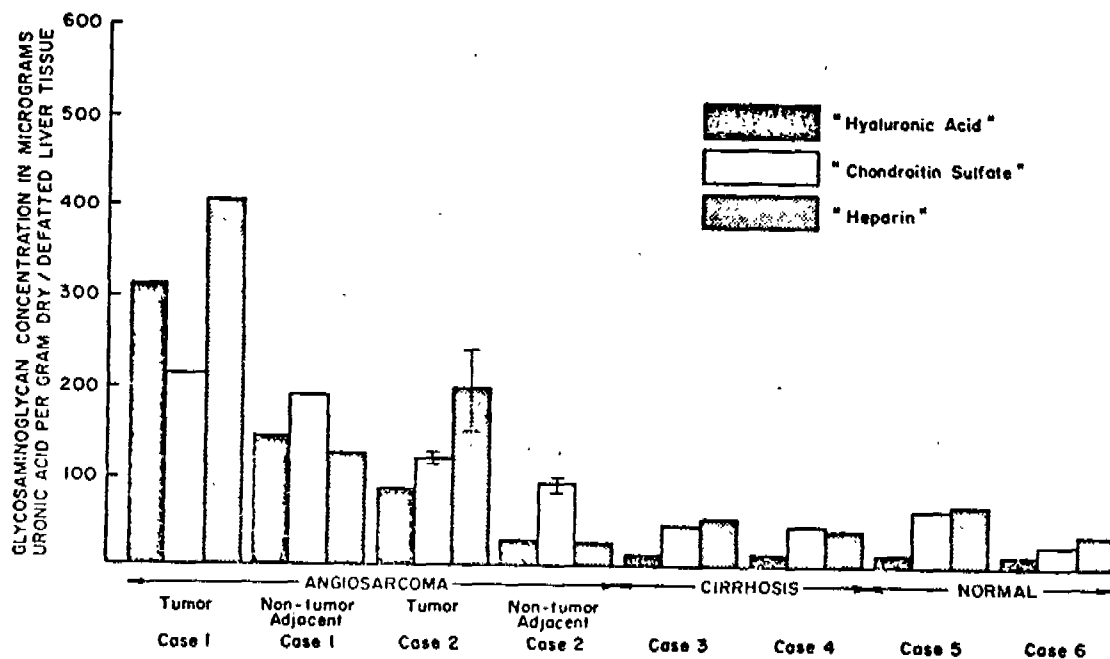


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GLYCOSAMINOGLYCAN CONCENTRATION IN ANGIOSARCOMA,
CIRRHOSIS, AND NORMAL LIVER TISSUE



CONCENTRATION OF INDIVIDUAL GLYCOSAMINOGLYCAN FRACTIONS
ISOLATED FROM HEPATIC ANGIOSARCOMA, CIRRHOSIS,
AND NORMAL LIVER TISSUE



- Figure 1 Urinary glycosaminoglycan output in one angio-sarcoma patient during the 16-day period prior to death.
- Figure 2 Glycosaminoglycan concentration in angiosarcomatous, cirrhotic and normal human liver tissue.
- Figure 3 Fractional concentrations of glycosaminoglycans in angiosarcomatous, cirrhotic and normal human liver tissue.

VI. REFERENCES

1. Creech, J. L. and Johnson, M. H. Angiosarcoma of the Liver in the Manufacture of Polyvinyl Chloride. *J. Occup. Med.* 16: 150-151, 1974.
2. Falk, H., Creech, J. L., Peckh, D. W., Johnson, M. N., and Key, M. M. Hepatic Disease Among Workers at a Vinyl Chloride Polymerization Plant. *JAMA* 230: 59-63, 1974.
3. Makk, L., Creech, J. L., Whelan, J. G., and Johnson, M. N. Liver Damage and Angiosarcoma in Vinyl Chloride Workers: A Systematic Detection Program. *JAMA* 230: 64-68, 1974.
4. Creech, J. L., Makk, L., Whelan, J. and Tamburro, C. H. Hepatotoxicity Among Polyvinyl Chloride Production Workers During First Year of Surveillance Program. *Gastroenterology* 67: 786, 1974.
5. Kohn, J. and Weaver, P. C. Serum Alpha Fetoprotein in Hepatocellular Carcinoma. *Lancet* 2: 334-336, 1974.
6. Tamburro, C. H., Makk, L. and Creech, J. L. Unpublished observation.
7. Girard, D., Johnston, W. C., and Graham, J. H. Cutaneous Angiosarcoma. *Cancer* 25: 868-883, 1970.
8. Barr, R. and Bonin, M. "Letters." *JAMA* 231(9): 914, 1975.
9. Popper, H., and Thomas, L. B. Alterations of Liver and Spleen Among Workers Exposed to Vinyl Chloride. *Ann. NY Acad. Sci.* 246: 172-194, 1975.
10. Galambos, J. T., and Shapira, R. Natural History of Hepatitis: IV Glycosaminoglycuronans and Collagen in the Hepatic Connective Tissue. *J. Clin. Invest.* 52(11): 2952-2962, 1973.
11. Koizumi, T., Nakamura, N., and Abe, H. Changes in Acid Mucopolysaccharide in the Liver in Hepatic Fibrosis. *Biochim. Biophys. Acta.* 148: 749-756, 1967.
12. Kojima, J. Studies on the Metabolism of Hepatic Connective Tissue in Fibrosis of the Liver. *Med. J. Osaka Univ.* 16: 419-429, 1964.
13. Rubin, E. Autoradiographic Characterization of Sulfated Acid Mucopolysaccharides in Experimental Cirrhosis. *J. Histochem. Cytochem.* 14: 688-689, 1966.
14. Patrick, R. S. and Kennedy, J. S. The Synthesis of Sulfated Mucopolysaccharide at Sites of Hepatic Fibrosis Induced by Carbon Tetrachloride, Amyloidosis, and the Implantation of Catgut. *J. Pathol. Bacteriol.* 88: 549-555, 1964.
15. Kojima, J., Nakamura, N., Kanatani, M. and Ohmori, K. The Glycosaminoglycans in Human Hepatic Cancer. *Cancer Res.* 35(3): 542-547, 1975.

GLYCOSAMINOGLYCANS IN ANGIOSARCOMA

16. Anghileri, L. J. Metabolism of Acid Mucopolysaccharides in Hepatoma and in Normal Liver. *Oncology* 30: 304-317, 1974.
17. Yamamoto, K., and Teryama, H. Comparison of Cell Coat Acid Mucopolysaccharides of Normal Liver and Various Ascites Hepatoma Cells. *Cancer Res.* 30: 2257-2261, 1970.
18. DiFerrante, N. M. The Measurement of Urinary Mucopolysaccharides. *Anal. Biochem.* 21: 98-106, 1967.
19. Bitter, T., and Muir, H. A Modified Uronic Acid Carbazole Reaction. *Anal. Biochem.* 4: 330-334, 1962.
20. Schiller, S., Slover, G. A., and Dorfman, A. A Method for the Separation of Acid Mucopolysaccharides: Its Application to the Isolation of Heparin from the Skin of Rats. *J. Biol. Chem.* 236(4): 983-987, 1961.
21. Kupchella, C., and Steggerda, F. The Distribution of Acid Mucopolysaccharides in the Canine Gastrointestinal Mucosa. *Trans. NY Acad. Sci.* 34: 351-360, 1971.
22. Mowry, R. W. Alcian Blue Techniques for the Histochemical Study of Acidic Carbohydrates. *J. Histochem. and Cytochem.* 4: 407, 1956.
23. Sunderman, F. W. and Boerner, F. Normal Values in Clinical Medicine. W. B. Saunders. Philadelphia, p. 353, 1949.
24. Varma, R. S., Varma, R., Allen, W. S., and Wardi, A. H. Urinary Excretion of Acid Mucopolysaccharides in Schizophrenia. *Biochem. Med.* 11(4): 358-369, 1974.
25. DiFerrante, N. and Rich, C. The Determination of Acid Aminopolysaccharide in Urine. *J. Lab. Clin. Med.* 48: 491-494, 1956.
26. Kao, K. and Leslie J. Micro Fractionation and Determination of Urinary Glycosaminoglycans. *Biochem. Med.* 9(4): 317-326, 1974.
27. Goldberg, J. and Cotlier, E. Specific Isolation and Analysis of Mucopolysaccharides (Glycosaminoglycans) from Human Urine. *Clin. Chim. Acta.* 41: 19-27, 1972.
28. Manley, G., Severn, M. and Hawksworth, J. Excretion Patterns of Glycosaminoglycans and Glycoproteins in Normal Human Urine. *J. Clin. Pathol.* 21: 339-345, 1968.
29. Pennock, C. A. A Modified Screening Test for Glycosaminoglycan Excretion. *J. Clin. Pathol.* 22: 310, 1969.

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30. Douglas, C., Nowak, J. and Danes, B. Mucopolysaccharides in Urine During Normal Human Development. *Pediatr. Res.* 7: 724-727, 1973.
31. Kuroda, J., Saito, S., Seno, N., Nagase, S., and Anno, K. Isolation and Chemical Characterization of Mucopolysaccharides from Rat Tumors. *Cancer Res.* 34(2): 300-312, 1974.

IMMUNOPATHOLOGIC OBSERVATIONS IN LIVER ANGIOSARCOMA

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I. INTRODUCTION

In hepatic fibrosis and angiosarcoma associated with vinyl chloride exposure of industrial workers, manifestations of the disease could not be detected in most cases until the process was far advanced (1). Normal values of liver function tests were reported in a case with significant vinyl chloride hepatic fibrosis (2), and only a small percentage of workers of a plant unit where seven cases of liver angiosarcoma were diagnosed had abnormal blood screening tests (3). Thus, conventional liver function tests do not appear to be sensitive indicators of vinyl chloride liver disease. Development of more sensitive methods for detecting the disease in early stages would be of great importance. An approach to this may be provided by immunologic studies. In such a study the question arises whether the fibrotic and angiosarcomatous livers contain antigens that are different from those present in normal tissue and whether such changes could stimulate an immunologic response. The purpose of this study was to search for antigenic changes in the angiosarcomatous tissue and to test for possible presence of an antibody response in the host.

II. PROCEDURES AND MATERIALS USED

A. Patients' Sera and Tissue Specimens

Serum samples from B.F. Goodrich Co. workers with histories of vinyl chloride exposure of several years included samples from two individuals with liver angiosarcoma, ten with liver dysfunction with fibrosis and ten with normal liver function tests. The patients with angiosarcoma died and the diagnosis was confirmed at autopsy and portions of tumor and neighboring liver tissues were obtained at autopsy. Patients with liver dysfunction with fibrosis included individuals with

abnormalities in liver function tests and fibrosis detected at biopsy. Tissues were also obtained from coroner's autopsies of healthy individuals a few hours after death by gunshot wounds.

B. Tissue Extracts and Antisera

Liver angiosarcoma and adjacent liver tissue and post-mortem tissues considered to be normal were frozen and stored at -70°C until used. Portions were cut, thawed and homogenized in 2-3 volumes of distilled water in a Potter-Elvehjem grinder in an ice bath until a smooth suspension was obtained. After centrifugation at $20,000 \times G$ for 30 min, the supernatant fluid containing the aqueous extract was lyophilized. Albino rabbits were immunized with the tumor or normal liver extracts in Freund's complete adjuvant and sera collected and stored following procedures detailed elsewhere (4). Reaction of these immune sera with human serum or plasma was eliminated by absorption with 100 mg of lyophilized, pooled normal human serum/ml antiserum. Antisera were routinely absorbed in this manner prior to use. Additional absorption with tissue extracts was carried out with 100 mg lyophilized extract/ml antiserum. Absorptions followed a procedure described previously (5).

C. Treatment of Tissue Extracts

Enzymatic treatment of tissue extracts was carried out with Pronase and trypsin as previously described (6). Periodate oxidation was done according to Rajam *et al.* (7). Ammonium sulfate and cold ethanol fractionations were carried out as detailed (8).

D. Immunodiffusion and Immunofluorescence

Double immunodiffusion was carried out in 0.8% agarose in phosphate-buffered saline pH 7.2 (PBS) containing 0.1% sodium azide. Circular wells, 2 mm in diameter, 3 mm apart were used. Immunoelectrophoresis was performed according to Scheidegger (9) using 0.8% agarose in 0.025 M Veronal buffer at pH 8.2.

In immunofluorescent studies cryostat sections of rat kidney, stomach or intestine and liver (4 microns) were used as substrate for antimitochondrial, antismooth muscle and antinuclear antibodies. Liver sections from rats exposed 4, 8 and 14 days to 1-2% vinyl chloride for 4 hr/day were also used. The sections were covered with dilutions of patients' sera for 45 min at room temperature, washed twice in PBS for 10 min and then covered with fluorescein conjugated IgG fraction of rabbit anti-human immunoglobulins serum (Cappel Laboratories, Inc.) for 45 min and washed as before prior to examination. Cryostat sections of liver angiosarcoma and liver tissue considered to be normal (4 microns) were washed twice in PBS for 10 min to wash off nonfixed immunoglobulins. After drying,

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sections were stained with fluorescein conjugated IgG fractions of rabbit anti-human IgG serum and goat anti-human IgM serum (Cappel Laboratories, Inc.). Sections were washed as above and examined under the fluorescent microscope. Representative frozen sections were stained with hematoxylin and eosin to allow correlation between immunofluorescence and the histological findings. The localization of the tissues was indicated by the demonstration of their staining by anti-nuclear factor according to the indirect immunofluorescent procedure.

E. Elution of Tumor-bound IgG

The tumor and liver tissues were extracted five times with PBS to wash off nonfixed immunoglobulins and then extracted at pH 2.5 to release bound IgG according to a procedure applied in the elution of renal-bound antibody (10).

F. Circulating Tissue Antigens

Liver-specific antigen LSA (8), tissue antigens of wide organ distribution (4) and bile antigens (11) were tested in the patients' sera by immunodiffusion as described previously.

III. RESULTS

A. Angiosarcoma-related Antigen

To test for presence of new antigens appearing in liver angiosarcoma, antiangiosarcoma serum was absorbed with human serum and liver extract and tested by immunodiffusion with extracts of both normal liver and angiosarcoma tumor at varying concentrations. This absorption eliminated all reactivity with liver extracts prepared from five normal individuals but not with the angiosarcoma extracts where one line of precipitation remained (Fig. 1). This line of precipitation could still be seen after additional absorption of the antiserum with kidney extract. In contrast, absorption with the tumor extracts eliminated completely the angiosarcoma-related line of precipitation. Absorption with spleen and lung extracts also eliminated this line of precipitation. Thus, the antigen appeared to be of restricted tissue distribution and not angiosarcoma specific. The antigen was inactivated by trypsin and Pronase and thus appeared to be a protein or closely associated to protein. Incubation of the tumor extracts for 1 hr at 4°C in citrate buffer, pH 2.5, resulted in inactivation of the antigen whereas incubation in phosphate buffer, pH 5.0, neutral or alkaline pH up to pH 10.0, did not affect it. The antigen was shown to be relatively thermolabile. Incubation of the tumor extracts for 30 min in PBS at 25° and 56°C did not affect the antigen whereas incubation at 70°C and higher

completely inactivated it. The antigen precipitated mainly at 20-30% saturated ammonium sulfate and at ethanol concentrations of 30-70% (Table I).

B. Absence of a Normal Tissue Antigen in Angiosarcoma

Antiliver serum absorbed with human serum gave several arcs of precipitation in immunoelectrophoresis with extracts of normal liver and angiosarcoma tissue (Fig. 2a). These lines could not be seen following additional absorption of the anti-serum with normal liver. In contrast, absorption with liver angiosarcoma extract failed to eliminate one of the arcs of precipitation (Fig. 2b). Thus, the tissue antigen related to this arc of precipitation appeared to be absent in the angiosarcomatous tissue whereas the antigens corresponding to the other arcs were present. The absent antigen in angiosarcoma was shown to be present in kidney and lung extracts in addition to liver by absorption and direct immunodiffusion tests. Physicochemical characterization studies indicated this antigen to be unaffected by Pronase and trypsin and inactivated by periodate treatment. The antigen was relatively thermostable withstanding incubation at 70°C for 30 min in PBS. The antigen was destroyed following incubation of the liver extract in citrate buffer at pH 2.5 or lower for 1 hr at 4°C; incubation at pH 5.0 or higher (up to pH 10.0) did not affect it. This antigen precipitated over a wide range of ammonium sulfate and ethanol concentrations (Table I).

C. Angiosarcoma-bound IgG

IgG fluorescent staining appeared in a linear pattern in the peripheral portion of the tumor cells suggesting in vivo binding by the tumor of the immunoglobulin. This staining is illustrated in Fig. 3. Fig. 4 shows the angiosarcomatous cells surrounding irregular vascular spaces. Relatively coarse, linear fluorescence was also present along some hepatic cords and strands of connective tissue. There was no evidence of IgM. Control post-mortem liver tissue did not show any significant fluorescence of bound IgG. Staining for IgG of the tumor sections did not change after several washings at pH 7.2 indicating that the IgG was firmly bound to the tumor. In contrast, sections showed marked diminution of staining after washing at pH 2.5. Elution of bound IgG from saline-extracted tumor homogenates was thus attempted at acid pH. With the five successive saline extractions the amount of saline soluble IgG gradually diminished to nondetectable levels; and at acid pH bound IgG was released from the homogenate. Similar treatment of liver homogenates did not demonstrate presence of bound IgG (Table II).

D. Circulating Autoantibodies and Tissue Antigens

Serum autoantibodies to nuclei, mitochondria and smooth

muscle were negative in all patients examined. In addition, serum from these patients did not show reactivity with liver from rats exposed to vinyl chloride. Liver-specific antigen LSA (8), bile antigens (11) and other tissue antigens (4) associated with liver damage were not detected in these patients.

IV. DISCUSSION

The immunologic classification of cancer have been under intense investigation during recent years, and antigenic differences between normal and malignant tissue are considered to be fundamental factors in the immunologic approach to cancer therapy and diagnosis. Liver angiosarcomatous tissue was thus analyzed in this work for presence of neoantigens, normal tissue antigens and tumor-bound immunoglobulins. Several normal tissue antigens were found by immunodiffusion to be present in the tumor, but one antigen of rather wide organ distribution was not detected. These findings are in agreement with observations in other tumors indicating that tumor cells contain many of the antigens of their original hosts and lack some normal tissue antigens. For example, immunohistochemical studies have shown the loss of kidney antigens in stilbestrol- and x-ray-induced kidney tumors (12), of skin antigen in 3-methylcholanthrene-induced mouse squamous cell carcinoma (13) and of certain muscle antigens in 20-methylcholanthrene-induced rat rhabdomyosarcoma (14). By far the most extensively studied class of tumors are the chemically induced hepatomata where deletion of liver antigens have been shown in tumors induced with 4-dimethylaminoazobenzene (15, 16), diethylnitrosamine (15) and 2-acetamidofluorene in the rat (15, 17) and o-aminoazotoluene in the mouse (18). In human carcinoma, loss of antigens have been reported in squamous cell carcinoma (12, 19), loss of the ABH blood group isoantigens in some solid tumors (20, 21) and of HL-A isoantigen in lymphoma (22). In addition, it has been well documented that as cells transform from a normal state to malignancy they may gain new antigenic specificities. Tumor-specific transplantation antigens have been demonstrated in a number of experimentally induced tumors (23-26) as well as in spontaneous tumors in man (27-29). In the present report immunodiffusion analyses of liver angiosarcoma and other human tissues with rabbit antiangiosarcoma serum did not indicate the presence of a tumor-specific antigen but rather of an antigen found in lung and spleen but not in liver and kidney. This antigen is being further characterized in our laboratory.

Of particular interest is the demonstration of tumor-bound IgG by immunofluorescence and elution experiments. This finding must however be interpreted with caution and should be confirmed in biopsy specimens. The tumor-bound IgG may represent specific antitumor antibody, antibody fixed by the tumor tissue "nonspecifically" or part of both. Further speculation is premature until it has been shown that the staining pattern

is due to the deposition of a specific antibody, that the eluted antibody is specific or until the relevant antigen has been identified. Work is in progress to determine the precise significance of the finding of IgG in the tumor.

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V. SUMMARY

Immunodiffusion analyses of human liver angiosarcoma associated with vinyl chloride exposure indicated presence in the tumor of an antigen not detected in normal liver and kidney but found to be present in lung and spleen. This antigen was shown to be a protein, inactivated by Pronase and trypsin, relatively susceptible to heating and to acid pH and precipitated mainly at 20-30% saturated ammonium sulfate and at 30-70% ethanol concentrations. The tumor was shown to contain several antigenic constituents of normal tissue but one normal tissue antigen was not detected. This antigen was characterized as a substance unaffected by Pronase and trypsin and inactivated by periodate. It was relatively thermostable, affected by acid pH and precipitated over a wide range of ammonium sulfate and ethanol concentrations. Tumor specimens obtained at autopsy contained bound IgG as shown by immunofluorescence and elution experiments suggesting possible in vivo binding of IgG to the tumor.

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TABLE I

Angiosarcoma-related Antigen and Antigen Absent from
the Tumor in Ammonium Sulfate and Ethanol Fractions

Fraction tested	Presence of ^a	
	Angiosarcoma-related ^b antigen	Antigen absent from ^c Angiosarcoma
Ammonium sulfate:		
0-20% saturation	+	-
20-30% saturation	++	+
30-50% saturation	-	+++
50-70% saturation	-	++
Ethanol:		
0-20%	-	++
20-30%	-	+++
30-50%	++	+++
50-70%	++	++
^d SN	-	+

^a
+++, ++, + indicate strength of double diffusion reaction
in dilution assay.

^b
Detected in angiosarcoma fractions.

^c
Detected in liver fractions.

^d
SN = supernate of the 70% ethanol precipitation, dialyzed
and lyophilized.

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TABLE II

IgG in Saline and Acid Extracts of
Angiosarcoma and Liver Tissues

Weight solid extracted from		
Preparation tested	1 gm (wet weight) tissue	Presence of IgG ^a
	(mg)	
Angiosarcoma:		
Saline extract 1	29.3	+++
Saline extract 2	8.6	++
Saline extract 3	6.2	+
Saline extract 4	5.6	-
Saline extract 5	6.1	-
Acid extract	6.1	++
Liver:		
Saline extract 1	47.4	+++
Saline extract 2	14.5	+++
Saline extract 3	8.6	+
Saline extract 4	7.4	-
Saline extract 5	6.4	-
Acid extract	9.0	-

^a Tested by immunodiffusion at concentrations of the eluates ranging up to 2%. Present at concentrations 0.05-0.1% (+++); 0.2-0.5% (++); 1-2% (+); negative at 2% (-).

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LEGENDS OF FIGURES

- Fig. 1 Demonstration of angiosarcoma-related antigen. Peripheral wells have 2-fold serial dilutions of liver angiosarcoma extract (a) and normal liver extract (b). Dilutions are placed in end point at 100 ng/ml in the upper right well. In each plate contain rabbit antiangiosarcoma serum absorbed with normal human serum and liver extract.
- Fig. 2 Demonstration of antigen absent in angiosarcoma. (a) Trough contains rabbit antihuman liver serum absorbed with normal human serum. (b) Trough contains the antiliver serum additionally absorbed with angiosarcoma extract. In both plates top wells have 10% solution of liver extract and lower wells angiosarcoma extract. Anode is to the right.
- Fig. 3 Immunofluorescent staining of liver angiosarcoma by fluorescein conjugated IgG fraction of rabbit anti-human IgG serum (x 400).
- Fig. 4 Cryostat section of angiosarcoma tumor stained with hematoxylin-eosin (x 400).

REFERENCES

1. Heath, C.W., Jr., Falk, R., and Cressh, J.L. Characteristics Of Cases Of Angiosarcoma Of The Liver Among Vinyl Chloride Workers In The United States. *Ann. N.Y. Acad. Sci.*, 246:231-236, 1975.
2. Berk, P.D., Martin, J.J., and Waggoner, J.G. Persistence Of Viral Chlamydia In Liver Tissue After Cessation Of Exposure. *Ann. N.Y. Acad. Sci.*, 246:70-77, 1975.
3. Wyand, R.H., Martin, J.J., Falk, R., D.L., Buchanan, J., Gaudreau, C.J., Grady, A., and Doll, A.M. Antigenic Analysis Of Liver Tissue Screening Tests And Illness History Among Chemical Workers Involved In The Manufacture Of Polyvinyl Chloride. *Ann. N.Y. Acad. Sci.*, 246:65-67, 1975.
4. Espinosa, E. Circulating Tissue Antigens. I. Tissue Antigens In Serum Of Patients With Diseases Involving Injury Of The Liver And Of Other Organs. *Clin. Exp. Immunol.*, 16:152-162, 1974.
5. Espinosa, E., and Kaplan, M.H. Antigenic Analysis Of Human Heart Tissue. Identification Of Antigens With Specificity Restricted To Heart And Skeletal Muscle In Acid Extracts Of Myocardium. *J. Immunol.*, 100:1020-1031, 1968.
6. Espinosa, E., and Kaplan, M.H. Antigenic Analysis Of Human Heart Tissue. Antigens With Restricted Organ Distribution In Acid Extracts Of Human Myocardium. *J. Immunol.*, 105:416-425, 1970.
7. Rajam, P.C., Gaudreau, C.J., Grady, A., and Rundlett, S.T. Preparation, Derivation And Partial Characterization Of Organ-Specific Antigens From Human Brain. *Immunology*, 17:367-385, 1969.
8. Espinosa, E. Circulating Tissue Antigens. II. Studies On An Organ-Specific Antigen Of Human Liver. *Lab. Invest.*, 29:556-561, 1973.
9. Scheidegger, J.J. Une Micro-Méthode De L'Immuno-Électrophorèse. *Internat. Arch. Allergy, Basel*, 7:103-110, 1955.
10. Krishnan, C., and Kaplan, M.H. Immunopathologic Studies Of Systemic Lupus Erythematosus. II. Antinuclear Reaction Of γ -Globulin Eluted From Homogenates And Isolated Glomeruli Of Kidney From Patients With Lupus Nephritis. *J. Clin. Invest.*, 46:569-579, 1967.
11. Espinosa, E. Circulating Tissue Antigens. III. Identification And Characterization Of Antigens Of Limited And Of Wide Body Distribution In Human Gallbladder Bile. Presence In Serum Of Patients With Acute Hepatitis. Submitted for publication.
12. Nairn, R.C., Richmond, H.G., McEntegart, M.G., and Fothergill, J.E. Immunological Differences Between Normal And Malignant Cells. *Br. Med. J.*, 2:1335-1340, 1960.
13. Carruthers, C., and Baumler, A. Immunochemical Staining With Fluorescein-Labeled Antibodies As An Aid In The Study Of Skin Cancer Formation. *J. Natl. Cancer Inst.*, 34:191-200, 1965.

14. Fel, V.J., and Tsikarishvili, T.M. Reduction Of Normal Muscle Antigens In Rat Tumors Of Muscle Origin Induced By Intramuscular Injections Of 20-Methylcholanthrene. *Cancer Res.*, 24:1675-1677, 1964.
15. Baldwin, R.W., and Barker, C.R. Antigenic Deletions In Carcinogen-Induced Rat Hepatoma. *Nature*, 214:292-293, 1967.
16. Kalnins, V.I., and Stick, H.F. Loss Of Liver Cell Antigens In Azo-Dye Induced Hepatomas Of Rats. *Nature*, 200:109-110, 1963.
17. Hiramoto, R., Bernecky, J., Jurandowski, J., and Pressman, D. Immunohistochemical Staining Properties Of The N-2-Nitro Rat Hepatoma. *Cancer Res.*, 21:1372-1376, 1961.
18. Abramov, G.I., Kuznetsova, N.I., and Postnikova, S.A. The Antigenic Structure Of Mouse Hepatomas. I. Organ-Specific Antigens Of The Liver And Immuno-electrophoretic Study Of Their Occurrence In Hepatomas. *Neoplasma*, 9:120-130, 1962.
19. Hillemans, H.G. Serological And Immunological Studies On The Pathogenesis Of Cervical Cancer. *Z. Naturforsch.*, 17B:240-261, 1962.
20. Kay, H.E.M. A And B Antigens In Normal And Malignant Cells. *Br. J. Cancer*, 11:409-414, 1957.
21. Davidsohn, I., and Ni, L.Y. Loss Of Isoantigens A, B And H In Carcinoma Of The Lung. *Am. J. Pathol.*, 57:307-314, 1969.
22. Seigler, H.F., Kremer, W.B., Metzgar, R.S., Ward, F.E., Haung, A.T., and Amos, D.B. HL-A Antigenic Loss In Malignant Transformation. *J. Natl. Cancer Inst.*, 46:577-583, 1971.
23. Cryan, W.S., Hide, R.M., and Garb, S. Demonstration By Gel Diffusion Of Antigen In Spontaneous Mouse Tumors. *Cancer Res.*, 26:1458-1465, 1966.
24. Heppner, G.H., and Pierce, G. In Vitro Demonstration Of Tumor-Specific Antigens In Spontaneous Mammary Tumors In Mice. *Internat. J. Cancer*, 4:212-218, 1969.
25. Isojima, S., Yagi, Y., and Pressman, D. Antigens Common To Rat Hepatoma Induced With 2-Acetylaminofluorene. *Cancer Res.*, 29:140-144, 1969.
26. Kahan, B.D., Holmes, E.C., Reisfeld, R.A., and Morton, D.L. Water Soluble Guinea Pig Transplantation Antigen From Carcinogen-Induced Sarcomas. *J. Immunol.*, 102:28-36, 1969.
27. Hughes, L.E., and Litton, B. Antigenic Properties Of Human Tumors: Delayed Cutaneous Hypersensitivity Reactions. *Br. Med. J.* 1:209-212, 1964.
28. Itakura, K. Studies On Human Cancer Antigens By Gel Diffusion Methods. *Gann*, 54:93-104, 1963.
29. McKenna, J.M., Sanderson, R.P., and Blakemore, W.S. Extraction Of Distinct Antigens From Neoplastic Tissue. *Science*, 135:370-371, 1962.

Fig 1

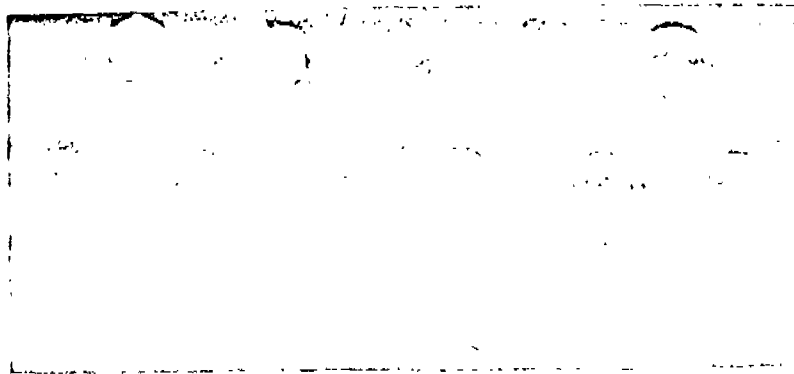
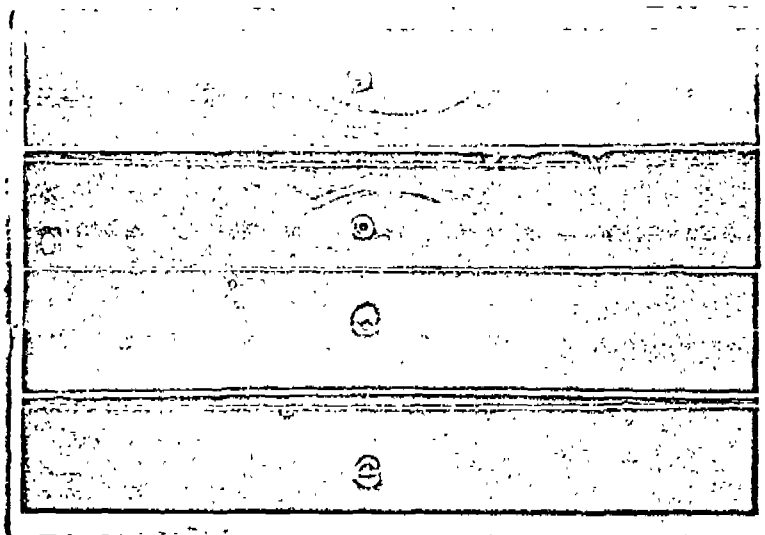


Fig 2



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