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A Study of the Toxohormone Content of Several Types of Human Malignant Tumors

Richard I. Delo
Loyola University Chicago

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A STUDY OF THE TOXOHORMONE CONTENT
OF SEVERAL TYPES OF HUMAN MALIGNANT TUMORS

by

Richard I. Delo

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirement for the Degree of
Master of Science

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LOYOLA UNIVERSITY MEDICAL CENTER

LIFE

Richard I. Delo, the oldest of three children, was born September 30, 1933, in Lansing, Michigan.

He was graduated from Carson City High School, Carson City, Michigan, in June, 1951. Following graduation he attended Michigan State University, East Lansing, Michigan, from September, 1951 to June, 1956 when he graduated with a B.A. degree. He taught mathematics and science at the Cherry Creek High School, Englewood, Colorado, from September, 1956 to June, 1958.

In September, 1958 he began his studies at Loyola University School of Dentistry, Chicago, Illinois, and was awarded the degree of Doctor of Dental Surgery in June, 1962. In September, 1962 he began graduate study in the Department of Oral Biology of Loyola University, Chicago, Illinois.

In November, 1963 he was appointed as a resident in Oral Surgery at the Veterans Administration Hospital in Long Beach, California.

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CHAPTER I
INTRODUCTION

Cancer research has been approached from several different points of view by many researchers in recent years. Some investigators have studied the etiologic aspects of the disease, others have investigated the mechanism of metastases, while others have given detailed histologic and histochemical descriptions of all types of malignant tissues. Chemotherapeutic agents, the effect of x-radiation and electron beam, as well as other therapeutic measures have been investigated. The systemic effects of malignant tissues have also been studied extensively during the past fifty years.

The purpose of this investigation was to study the systemic effect of an intraperitoneal injection of an acetone extract of human malignant tissues, specifically the effect upon mouse liver catalase activity. Other investigators have studied the effect of injections of malignant tumor tissue fractions and found the production of anemia, involution of the thymus, decrease in plasma iron concentration, and decrease in liver catalase activity.

An attempt was made in this study to show a quantitative correlation between the decrease in liver catalase activity

and the conventional method of estimation of the degree of malignancy of tumor tissues by histologic evaluation.

CHAPTER II

REVIEW OF LITERATURE

In only one instance in the literature have I observed any studies reported which have correlated quantitatively the amount of depression of liver catalase activity with the type of tumor or the degree of malignancy of the tumor. Numerous investigations have been reported which related to the liver catalase depressing activity of toxohormone. The term toxohormone was coined by Nakahara and Fukuoka (1948). They defined toxohormone as a special toxic substance produced by malignant tissues which is passed into circulation and which acts upon liver cells in some way to reduce the catalase activity.

The early investigators Blumenthal and Brahn (1910), Rosenthal (1912), and Brahn (1914,1916) made the observation that liver catalase activity is markedly reduced in cancer patients and in tumor-bearing animals. The following are some questions raised by these observations and whose answers will be sought in this review: What is the long term effect of a transplanted tumor on liver catalase activity? What effect does the removal of the tumor have on the liver catalase activity? How much is the liver catalase activity decreased in a tumor-bearing animal? What is the mechanism of liver catalase

activity depression?

Greenstein, Jenrette, Mider, and Andervont (1941) felt that a toxic material is produced by the tumor cells which travels to the liver via the blood where it acts to lower the catalase activity. They found that the liver catalase activity in rats was only about one-tenth that of normal following the transplantation of hepatic tumor or Jensen sarcoma. There was a continuous decrease in liver catalase activity during the first four weeks following transplantation. The activity then became constant. The liver catalase activity returned to normal 24 to 48 hours after the removal of the tumor. The effect of starvation upon liver catalase activity was studied and found not to produce an effect.

The influence of residual blood in the liver on the determination of catalase activity was studied by Greenstein and Andervont (1941, 1942, 1943). It was found to contribute a negligible amount to the total catalase activity. It was also shown by these investigators that pregnant mice and mice bearing embryonic implants exhibited no decrease in catalase activity. This led to the conclusion that toxohormone is not produced by benign growths.

Toxohormone was found to be a thermostable, not heat coaguable, water soluble, and alcohol precipitable product of living cancer cells by Nakahara and Fukuoka (1949). The toxo-

hormone concentration was found to be no greater in necrotic than in non-necrotic samples of tumor tissue.

Weill-Malherbe and Schade (1948) varied the protein level of the diet, gave injections of peptone, and gave injections of sheep serum to both normal and tumor-bearing rats. The only change found was in an increase in catalase concentration following injection of sheep serum with regression of tumor in some cases.

Subcutaneous injection of homogenized sarcoma 37 by Adams (1950) produced a depression in liver catalase activity in 24 to 48 hours, and then this enzyme activity decreased again during the growth of new tumor. Similar injections of a variety of normal tissues including whole embryo tissue produced no significant alteration in liver catalase activity. These results provided evidence that tumors release toxic products into the blood which act upon liver catalase.

The effect of the injection of tumor fractions and normal tissue fractions on liver catalase activity of mice was studied by Greenfield (1950, 1951). He found that fractions which were obtained from normal spleen, intestines, and liver produced very little or no depression of liver catalase activity while tumor fractions produced a marked depression. It was determined that the active agent has a molecular weight of approximately 40,000, is effective in both a non-dialyzable and

and dialyzable form, and is stable to heat and strong acid.

Adams (1951) discussed the effect of tumor tissues upon hormonal control mechanisms. Liver catalase activity of normal male mice, which is higher than that of female or castrated male mice, was depressed to a greater extent than is that of the latter by injection of homogenized sarcoma 37. Normal females and castrated males reacted to tumor injection like normal males after treatment with testosterone. Injection of tumor into adrenalectomized females, or castrated and adrenalectomized males, resulted in little or no depression in liver catalase activity, but injection of cortisone into these animals increased the liver catalase activity. The substance in tumors which depressed liver catalase activity appears to act by protecting this liver enzyme from hormonal influences.

A third study of toxohormone was conducted by Fukuoka and Nakahara (1951) in which they examined the effect on the liver catalase activity of adding excess iron to the diet. It was shown that the administration of excess iron in the form of dried liver, blood powder, or ferric chloride nullified the effect of toxohormone upon liver catalase activity. It seems toxohormone does not reduce the ability of the liver cells to synthesize catalase, but rather the ability to store necessary iron. The toxohormone inhibits catalase synthesis by quickly binding iron which is normally available for catalase synthesis.

Begg, Dickinson, and Miller (1953) showed that transplants of benign fibroadenoma produced an 8% decrease in liver catalase activity. Mixed tumor (fibroadenoma-fibrosarcoma) produced a 44% decrease in liver catalase activity. Fibrosarcoma produced a slightly greater decrease in liver catalase enzyme activity.

Seabra and Deutsch (1955) found that aged liver homogenates inhibited catalase activity in vitro and that cystine injected intraperitoneally produced a marked depression in liver catalase enzyme activity. They felt that the cystine was converted by the liver into an active compound. It may be of interest to note that other investigators were unable to show any inhibitions of catalase activity in vitro.

A globulin-like protein which exhibited toxohormone and anemia-inducing activity was isolated by Masamune, et. al., (1958) from cancerous ascitic fluids originating in the stomach. Also, an inactive anemia-inducing toxohormone was isolated from the urine of cancer patients.

Nakahara and Fukuoka (1958) stated that all malignant tumors investigated yielded an active toxohormone fraction and suggested very strongly that the production of toxohormone may be a universal property of malignant tumors. They felt that the action of toxohormone is to suppress the process of catalase synthesis by impairing the utilization of iron.

These investigators indicated that toxohormone has now been isolated from urine of cancer patients, cancer ascites, and gastric juice of gastric cancer patients and is suspected to be present in the blood of cancer patients in very small amounts. They felt that a toxohormone test, as a means of detecting the presence of cancer, will be possible when a method is developed for detecting very low concentrations of toxohormone in the blood.

Many agents were tested by Riley (1959) for their ability to depress liver catalase activity. He found that the following substances had the ability to depress liver catalase activity: homogenates of normal liver, kidney, and spleen, boiled egg yolks, boiled liver, and sodium caseinate. The normal liver homogenate produced depression of liver catalase activity equivalent to that produced by rat hepatoma. These results were not confirmed by other investigators.

Kampschmidt, Adams, and McCoy (1959) administered an extract of Walker carcinoma 256 and found that it produced changes in normal rats similar to those found in rats bearing the Walker tumor. Single injections of toxohormone induced anemia, decreased plasma iron, and thymus involution. Plasma iron was 250-500 times as sensitive as liver catalase to toxohormone.

Delmon and Biraben (1959) injected a crude toxohor-

mone polypeptide preparation into rats bearing grafted Guerin epithelioma. It appeared that this injection retarded growth of the tumor and almost completely prevented metastases.

The toxohormone fractions of human hepatoma, rhodamine sarcoma, and normal rat liver were isolated by Fujii, et. al., (1960). It was found that on injection they all produced a depression in liver catalase activity. However, it required 2 to 10 times more of the normal liver than of the tumor tissues to produce any measurable depression of the liver catalase activity while the tumor tissue produced a marked depression.

Nakahara (1960) supported the concept that systemic effects in the tumor-bearing host involve the alteration of the enzyme level of normal tissues toward that of tumor tissues. Alteration of other properties in the tumor-bearing host, such as involution of the thymus and decrease in liver ferritin, plasma iron decrease, and diphosphopyridine nucleotide synthesis, were produced by administration of toxohormone. These studies provide a chemical basis for further study of the effects of tumors on the host.

A substance from biochemical mutants of yeast and staphylococci with impaired respiration was isolated by Callao and Montoya (1961) which significantly depressed liver catalase activity when injected intraperitoneally into mice. The produc-

tion of toxohormone-like substances by respiration deficient mutants of microorganisms suggests that these mutants may be equivalents of cancer cells. These observations led to the conclusion that some respiration deficient mutants of microorganisms produce a toxohormone-like factor which has the same effect as substances produced by cancer cells.

The effect of crude toxohormone fractions of human malignant and non-malignant tissues on mouse liver catalase activity was studied by Yunoki and Griffin (1960, 1961). They found that injections of 10 mg. of the malignant tissue fractions produced a decrease in liver catalase activity. Equivalent doses of non-malignant tissue fractions were not effective in reducing liver catalase activity. However, there was some indication that human spleen may possess some toxohormone activity.

Amberlite XE-64 column chromatography showed that there are three toxohormone fractions in all malignant tissues studied thus far. These toxohormone fractions were designated as TH₁, TH₂, and TH₃. The TH₂ fraction was the most active of the three fractions. It was found to be 10,000 to 50,000 times more active than a crude toxohormone preparation. It was of interest to note that the TH₂ fraction could not be separated from a crude toxohormone fraction of any non-malignant tissue investigated.

An analysis of the TH₂ fraction indicated that 80% of it could be accounted for as polypeptide, with leucine, alanine, glutamic acid, and aspartic acid being present in the highest concentrations. Also found to be present were: glycine, serine, proline, arginine, valine, threonine, phenylalanine, tryptophan, lysine, isoleucine, cystine, methionine, and tyrosine. The remaining 20% consisted largely of phospholipids.

From this study it was concluded that toxohormone activity requires the presence of both polypeptide and lipid components. Acid hydrolysis almost completely destroyed the toxohormone activity, indicating that the polypeptide is essential. Conclusive evidence as to the absolute necessity of the lipid component for toxohormone activity has not been obtained.

Greenfield and Meister (1951) by paper chromatography showed the presence of the following amino acids in toxohormone: alanine, glycine, serine, proline, aspartic acid, arginine, valine, threonine, phenylalanine, hydroxyproline, alpha-amino-butyric acid, tryptophan, lysine, leucine, isoleucine, and glutamic acid.

Similar amino acid composition was established by Okushima (1952) with the exceptions of isoleucine, hydroxyproline, alpha-amino-butyric acid, phenylalanine, and tryptophan.

Fukuoka and Nakahara (1953) found that the following

eight amino acids may be the major components of toxohormone polypeptide: alanine, proline, aspartic acid, arginine, phenylalanine, lysine, leucine, and glutamic acid.

Nakahara and Fukuoka (1954) determined that only three specific amino acids need to be included in the reaction mixture for the synthesis of toxohormone in vitro: arginine, phenylalanine, and leucine.

The following amino acids were identified by Ono, et. al., (1955) as being the components of toxohormone: alanine, glycine, serine, proline, aspartic acid, arginine, valine, threonine, phenylalanine, hydroxyproline, lysine, leucine, isoleucine, and glutamic acid.

Ohashi and Ono (1959) isolated alanine, glycine, arginine, valine, leucine, glutamic acid, and cystine from a toxohormone fraction.

The following amino acids have been isolated from the toxohormone fraction by at least half of the investigators just mentioned: alanine, glycine, proline, aspartic acid, arginine, valine, threonine, phenylalanine, lysine, leucine, and glutamic acid. These results are summarized in Table I. The exact chemical composition of toxohormone is not known at the present time.

The mechanism of action of toxohormone is not known. There has been only speculation up to this point. Many have

said that toxohormone acts on liver cells to inhibit the synthesis of catalase, but no work has been done to show how this is accomplished.

Table I

AMINO ACID COMPOSITION OF TOXOHORMONE

	Green- field (1951)	Okushima (1952)	Fukuoka & Nakahara (1953)	Ono et al. (1955)	Ohashi & Ono (1959)	Yunoki & Griffin (1961)
alanine	x	x	x	x	x	x
glycine	x	x	-	x	x	x
serine	x	x	-	x	-	x
proline	x	x	x	x	-	x
aspartic acid	x	x	x	x	-	x
arginine	x	x	x	x	x	x
valine	x	x	-	x	x	x
threonine	x	x	-	x	-	x
phenylalanine	x	-	x	x	-	x
hydroxyproline	x	-	-	x	-	-
alpha-amino- butyric acid	x	-	-	-	-	-
tryptophan	x	-	-	-	-	-
lysine	x	x	x	x	-	x
leucine	x	x	x	x	x	x
isoleucine	x	-	-	x	-	x
glutamic acid	x	x	x	x	x	x
cystine	-	-	-	-	x	x
methionine	-	-	-	-	-	x
tyrosine	-	-	-	-	-	x

CHAPTER III
MATERIALS AND METHODS

It was decided that mice would be used as the experimental animal in this study. Mice were selected because they readily show a depression in liver catalase enzyme activity when relatively small amounts of the toxohormone preparation are injected intraperitoneally. A marked depression in liver catalase enzyme activity twenty-four hours after injection has been demonstrated by other investigators. For purpose of uniformity, male mice of Swiss Webster strain weighing 25 to 28 grams were used. Male mice were chosen because other investigators have shown that the liver catalase enzyme activity of normal male mice is depressed to a greater extent than is that of normal female mice following the injection of extracts of tumor tissue.

Frozen samples of various human malignant tissues were obtained from Cook County Hospital, Chicago, Illinois. All of the tissues were accompanied by the pathologist's diagnosis and representative histologic sections. Photomicrographs were taken of these histologic sections and are included in this thesis for further scrutiny by other observers who may in the future be interested in this or related problems.

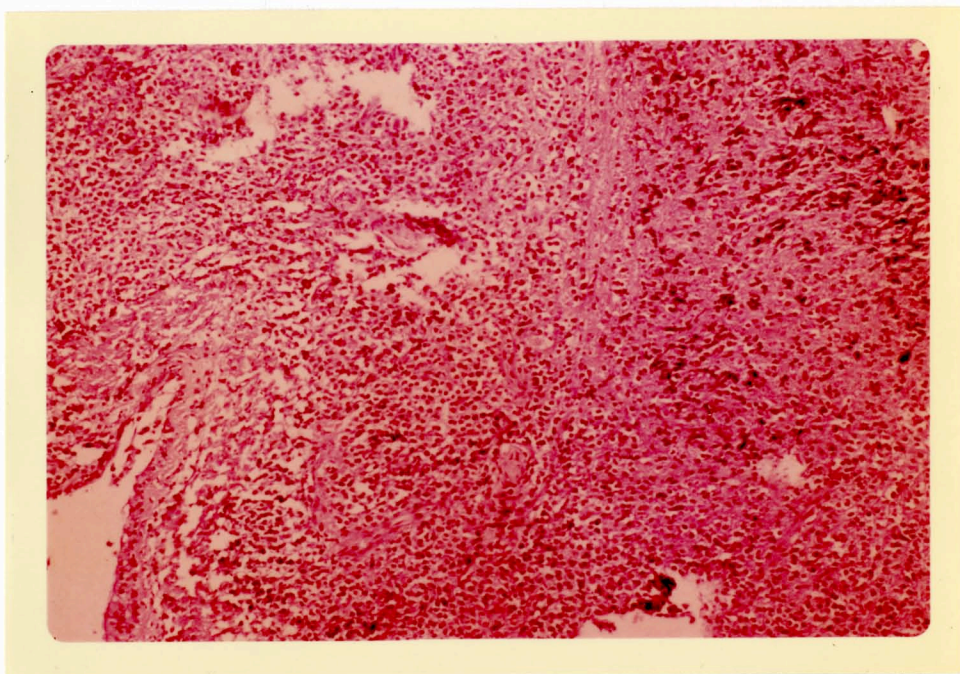


Fig. 1
Anaplastic Carcinoma, Bronchiogenic (279-63)
Hematoxylin and Eosine Staining. 50X.

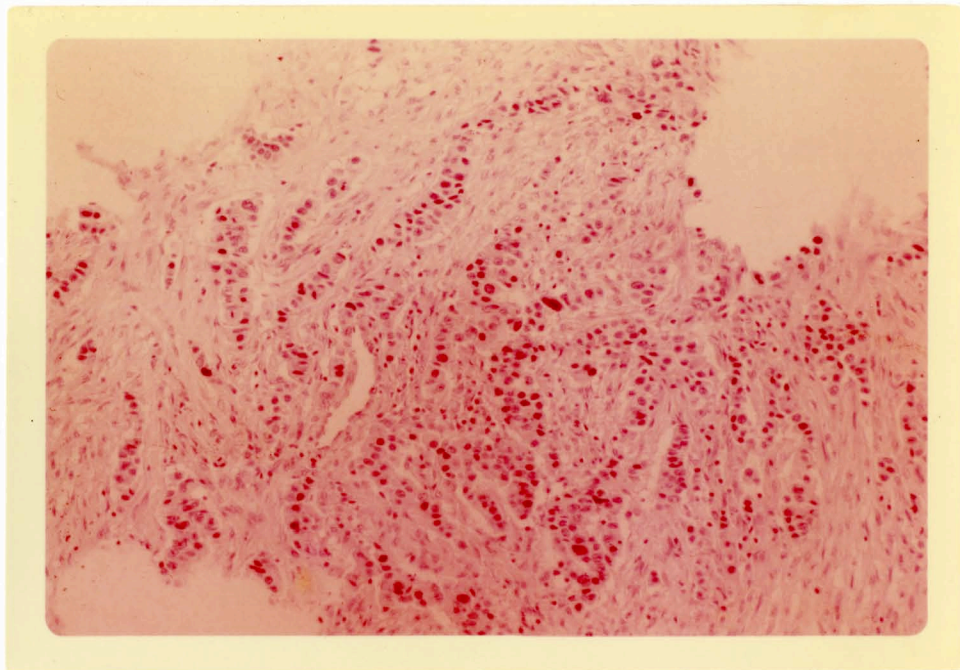


Fig. 2 Adenocarcinoma of Breast (6339-63)
Hematoxylin and Eosine Staining. 50X

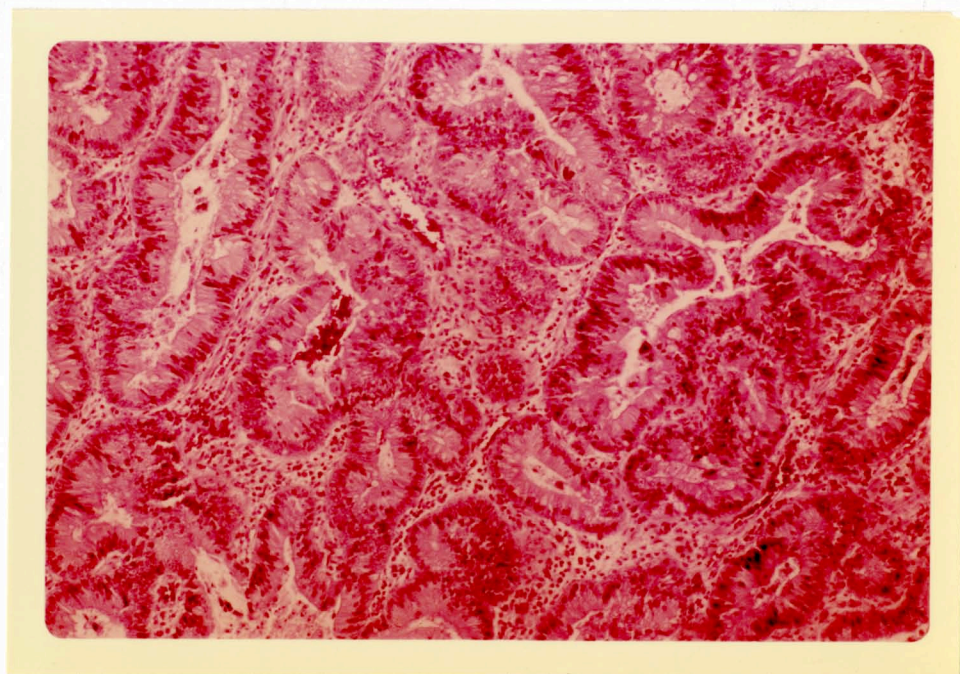


Fig. 3 Adenocarcinoma of Colon (14187-63)
Hematoxylin and Eosine Staining. 50X.

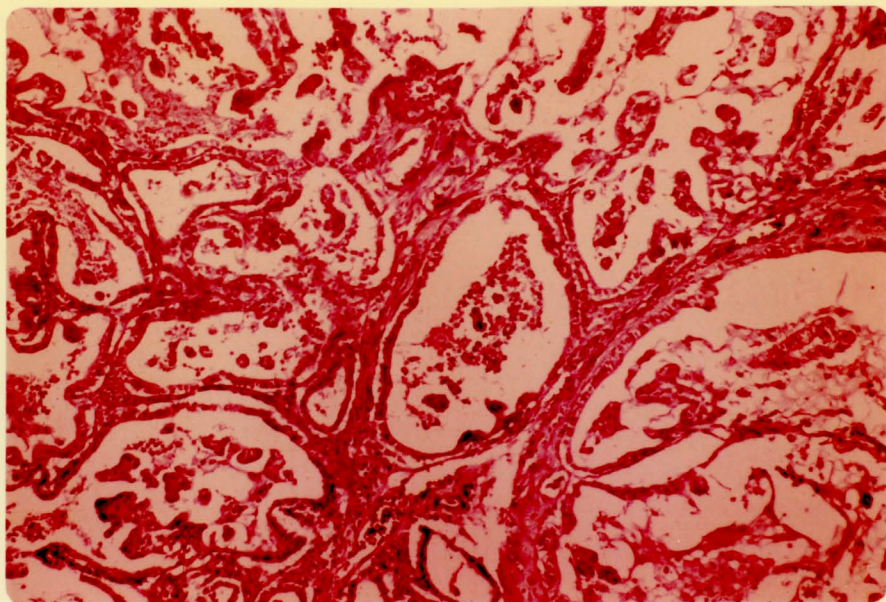


Fig. 4 Adenocarcinoma of Colon (9741-63)
Hematoxylin and Eosine Staining. 50X.

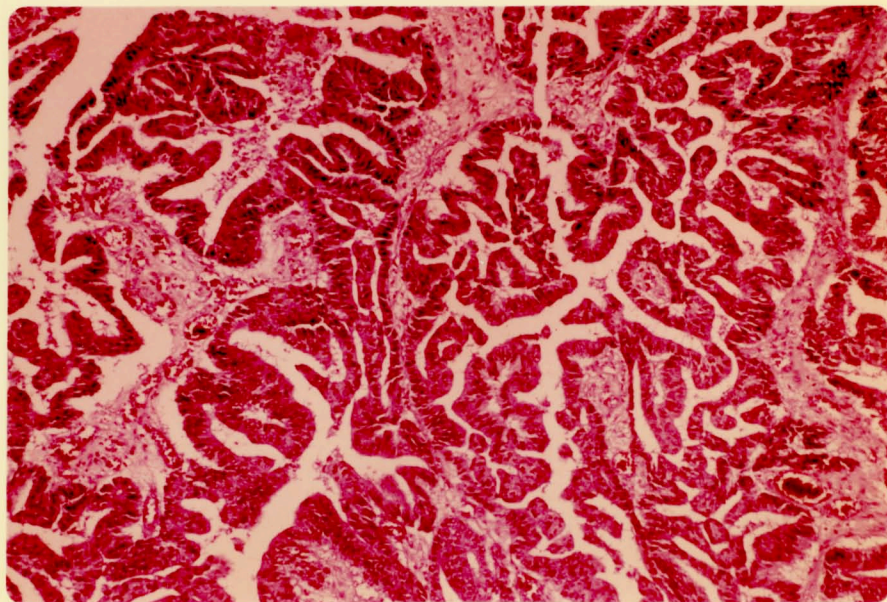
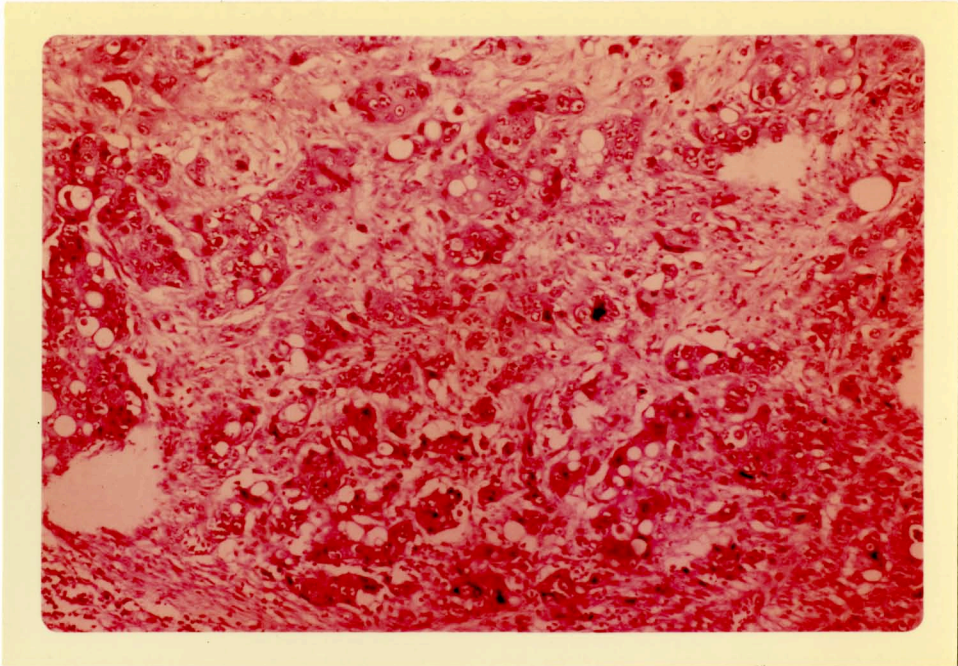
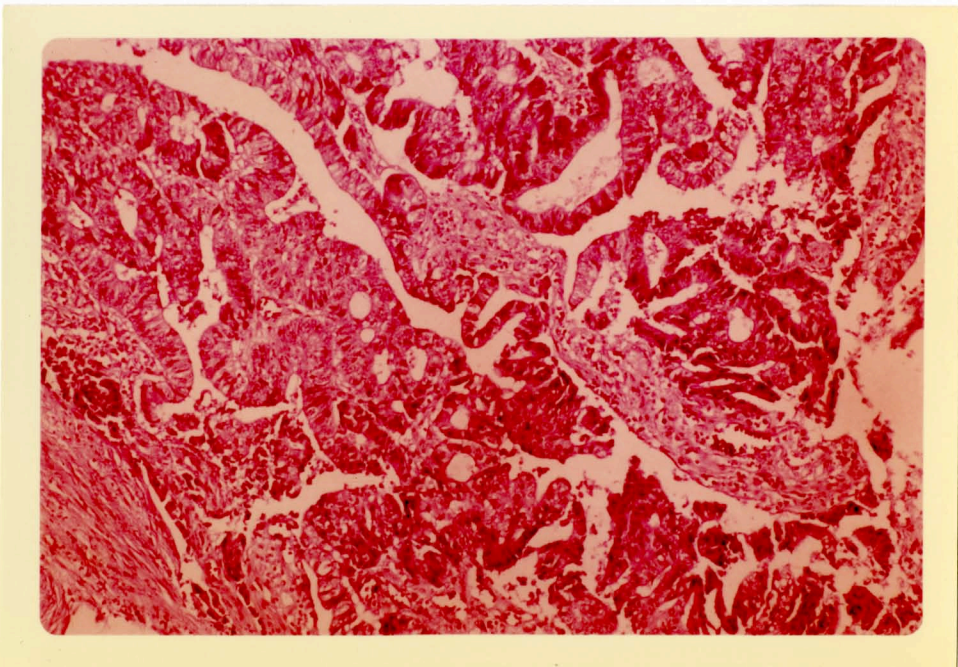


Fig. 5 Adenocarcinoma of Colon (8719-63)
Hematoxylin and Eosine Staining. 50X



**Fig. 6 Adenocarcinoma of Colon (282-63)
Hematoxylin and Eosine Staining. 50X**



**Fig. 7 Adenocarcinoma of Rectum (6673-63)
Hematoxylin and Eosine Staining. 50X**

The toxohormone was prepared by the homogenization of the human malignant tissue in a Potter homogenizer with acetone of approximately three times the volume of the tissue. The heavy strands of fibrous tissue which remained were removed to facilitate complete homogenization of the tissue sample. Following complete homogenization, the material was centrifuged for 10 minutes, after which the supernatant was decanted and discarded. The remaining portion was air dried at room temperature and was used as the toxohormone preparation.

The dried toxohormone preparation was weighed and suspended in normal saline to give a concentration of 40 mg. per milliliter. An intraperitoneal injection of 0.5 ml. of this suspension was administered to each of two mice for each sample of toxohormone. It has been shown by earlier work in this field that 20 mg. injections give the most reproduceable results.

Twenty-four hours after the injection of the toxohormone preparation the animals were anesthetized with ether and the liver immediately removed for assay.

The liver was immediately homogenized for one minute in ice cold distilled water in a high speed electric blender. The volume of distilled water used was approximately twice the volume of the liver to be assayed. This homogenate was then diluted to 100 ml. with ice cold distilled water and shaken vigorously so as to give a uniform distribution of the very

small particles of liver. From this point on, this dilution of the liver homogenate will be referred to as the "initial dilution". Two aliquots of this homogenate, each containing 2 ml., were pipetted into suitable containers and diluted to 50 ml. with ice cold distilled water. These preparations were shaken vigorously as above.

This final dilution was then assayed for the catalase activity using a photometric procedure which is a modification of the method of Goldblith and Proctor (1950). This procedure is based upon the measurement of the amount of hydrogen peroxide broken down by the catalase enzyme in a ten minute period at ice water temperature.

One milliliter of this final dilution was placed in each of two reaction tubes. The first reaction tube contained 1 ml. H_2O , 10 ml. of 0.075% H_2O_2 in phosphate buffer at pH 6.9, and 4 ml. 10% trichloroacetic acid (TCA). This tube gave the zero minute catalase activity since the proteins were precipitated immediately upon contact with the TCA and the enzymic reaction was stopped.

The second reaction tube contained 1 ml. H_2O and 10 ml. of 0.075% H_2O_2 in phosphate butter at pH 6.9. This tube was allowed to incubate for 10 minutes in an ice water bath.

From the first reaction tube 1.0 ml. aliquots were pipetted into each of two other reaction tubes containing 4 ml.

H₂O. Ten milliliters of 0.005 N KMnO₄ were added to each of these two tubes and the optical density was determined within one minute on a Coleman Jr. Photoelectric Colorimeter at a wavelength of 515 millimicrons.

After the 10 minute incubation period 4 ml. 10% TCA were added to stop the reaction in the second reaction tube. After thoroughly mixing, 5 ml. aliquots were pipetted into two other reaction tubes. Ten milliliters of 0.005 N KMnO₄ were added to each tube as described before. The optical density of each solution was determined.

A blank was prepared which contained 12 ml. H₂O and 4 ml. 10% TCA. After thoroughly mixing, 5 ml. aliquots were pipetted into two other reaction tubes and allowed to stand in an ice water bath. Ten milliliters of 0.005 N KMnO₄ were added to each tube as had been done for the tubes containing the material for assay. The optical density of the blank solution was measured and the values of the samples assayed were corrected for the inherent color of the reagents.

Since this procedure requires rapid transfer of the entire liver to ice water bath conditions, it is not practical to weigh the liver. Adams (1950) stated that liver catalase activity should be based on total liver protein. Since the non-protein nitrogen accounts for less than 10% of the total nitrogen in the liver and appears to be relatively constant, protein

nitrogen is employed as a standard. A total nitrogen determination was carried out so as to express the catalase activity in terms of mg. H_2O_2 decomposed/mg. nitrogen. It has been shown by other investigators (Day, Gabrielson, and Lipkind) that a greater uniformity among the liver catalase activities from one group of animals to the next is found when the catalase activity is based upon milligrams of nitrogen.

The "initial dilution" of the homogenate was assayed for its nitrogen content by the Koch-McMeekin micro-Kjeldahl method. One milliliter aliquots from the "initial dilution" of liver homogenate were pipetted into each of two Nesslerization tubes. A glass bead was added to minimize "bumping". One milliliter of 9 M H_2SO_4 was added and the sample was heated and digested until dense white fumes filled the tube and the liquid became charred. The tube was cooled at room temperature for 30 seconds before adding one drop of 30% H_2O_2 . Gentle boiling for 2 to 5 minutes usually cleared the solution. However, if the charring persisted or returned, the treatment with 30% H_2O_2 was repeated. The solution was cooled to room temperature and diluted to 35 ml. with distilled water. A blank was prepared containing 1 ml. of the acid digestion solution, 34 ml. distilled water. The tubes were then placed in an ice water bath and cooled to 11° C. Fifteen ml. of Nessler-Folin reagent were then added and the contents of the tubes were mixed well. The

tubes were then allowed to stand at room temperature for 10 minutes.

During the next 10 minutes the samples were read against the blank which was set at zero optical density at a wave length of 520 millimicrons on the Coleman Jr. Photoelectric Colorimeter.

Standard curves were prepared for milligrams of hydrogen peroxide per sample and milligrams of nitrogen per sample. The standard curves were used to convert from optical density of the unknown sample to either milligrams of hydrogen peroxide or milligrams of nitrogen respectively.

The catalase activity standard curve was expressed in milligrams of H_2O_2 per sample. Ten milliliters of 3% H_2O_2 were diluted to 100.0 ml. with distilled water. Into six 100 ml. volumetric flasks were pipetted 6.0, 5.0, 4.0, 3.0, 2.0, and 1.0 ml. of the peroxide solution and each was diluted to 100.0 ml. with phosphate buffer. Five milliliters of each dilution was added to a reaction tube containing 2 ml. of 10% TCA.

The colorimeter was adjusted to zero optical density at 515 millimicrons with a reference tube containing 2 ml. of 10% TCA and 15 ml. of distilled water. Ten milliliters of 0.005 N $KMnO_4$ were added to one reaction tube. It was shaken vigorously, and quickly read at 515 millimicrons. This procedure was repeated for each tube. A blank was prepared which contained

2 ml. of 10% TCA, 5 ml. of distilled water, and 10 ml. of 0.005 N KMnO_4 . The blank was shaken and read as the other tubes. The results of this determination appear in Table II. They were recorded on Graph I to arrive at the standard curve.

Values for the determination of the nitrogen standard curve were obtained by placing the number of milliliters of the standard $(\text{NH}_4)_2\text{SO}_4$ (5 ml. = 0.15 mg. N) solution containing 0.21, 0.24, 0.30, 0.36, 0.45, 0.54, 0.60, and 0.69 milligrams of nitrogen serially into test tubes graduated at 35 and 50 milliliters. One milliliter of 9M H_2SO_4 acid digestion solution was added to each tube and diluted to 35.0 ml. with distilled water.

A blank was prepared by placing 1 ml. of the acid digestion solution into a test tube graduated at 35 and 50 ml. and diluted to 35 ml. with distilled water. All the tubes were then placed in an ice water bath and allowed to reach a temperature of 11° C.

Fifteen milliliters of Nessler-Folin reagent were added to each tube. The solutions were mixed by inversion. The tubes were allowed to stand for 10 minutes at room temperature and the optical density was read within the next 10 minutes. The colorimeter had been set at zero optical density with the blank and the values were read at 520 millimicrons. The values thus obtained are shown in Table III and on Graph II to arrive at the nitrogen standard curve.

Table II
DETERMINATION OF
CATALASE ACTIVITY STANDARD CURVE

tube no.	1	2	3	4	5	6	7	8
ml. 3% H ₂ O ₂ /100 ml.	6	5	4	3	2	1	0	0
mg. H ₂ O ₂ /sample	1.019	0.849	0.679	0.509	0.339	0.169	0	0
ml. H ₂ O ₂	5	5	5	5	5	5	0	0
ml. H ₂ O	0	0	0	0	0	0	5	15
ml. KMnO ₄	10	10	10	10	10	10	10	0
ml. TCA	2	2	2	2	2	2	2	2
total volume	17	17	17	17	17	17	17	17
optical density								
A	.010	.065	.330	.640	.925	1.20	1.50	0
B	.008	.055	.320	.650	.850	1.20	1.50	0
C	.010	.065	.320	.650	.900	1.10	1.49	0
D	.015	.065	.320	.630	.890	1.14	1.50	0
E	.005	.070	.320	.620	.880	1.14	1.50	0
average	.010	.640	.320	.638	.889	1.16	1.50	0

Graph I

CATALASE ACTIVITY STANDARD CURVE

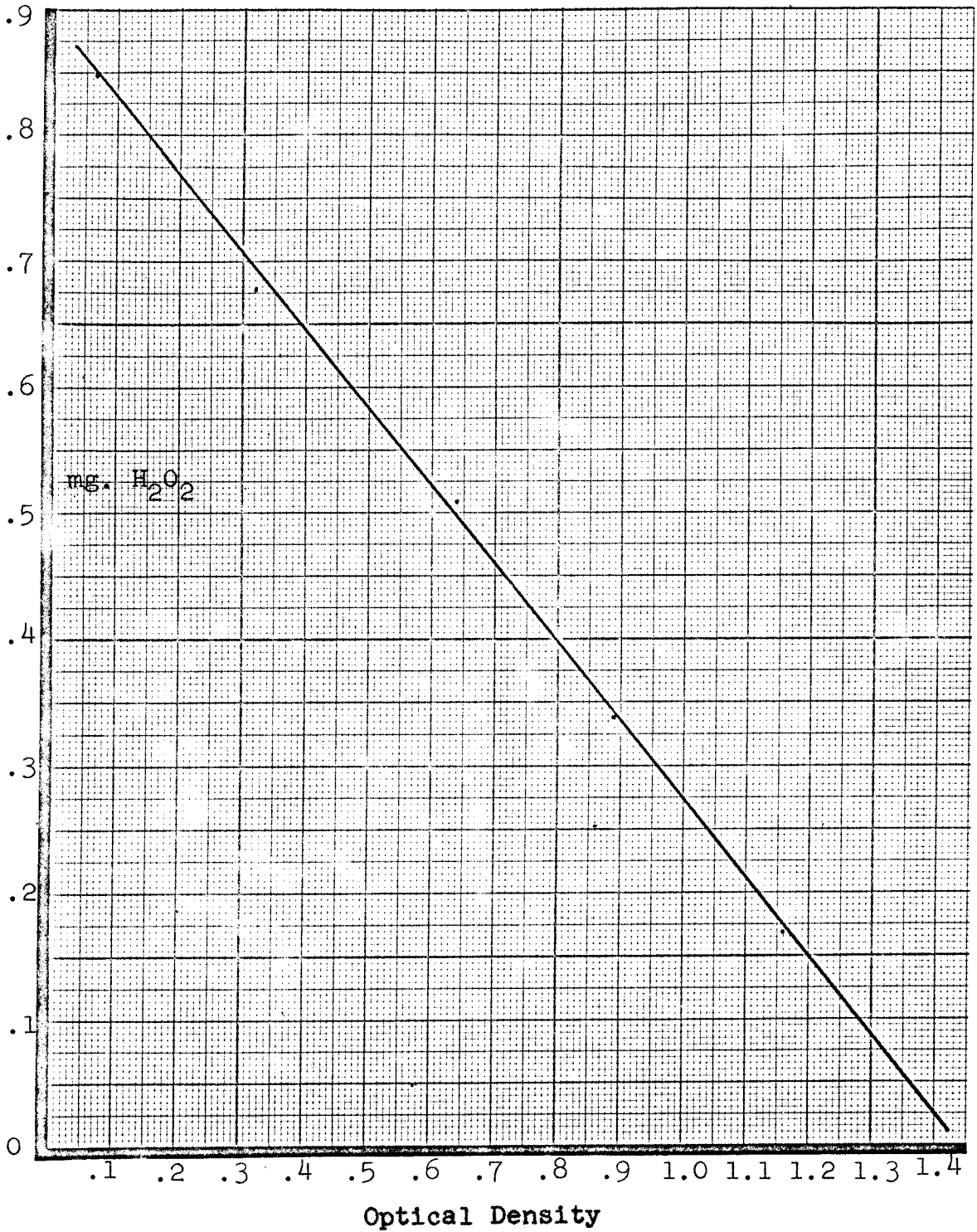
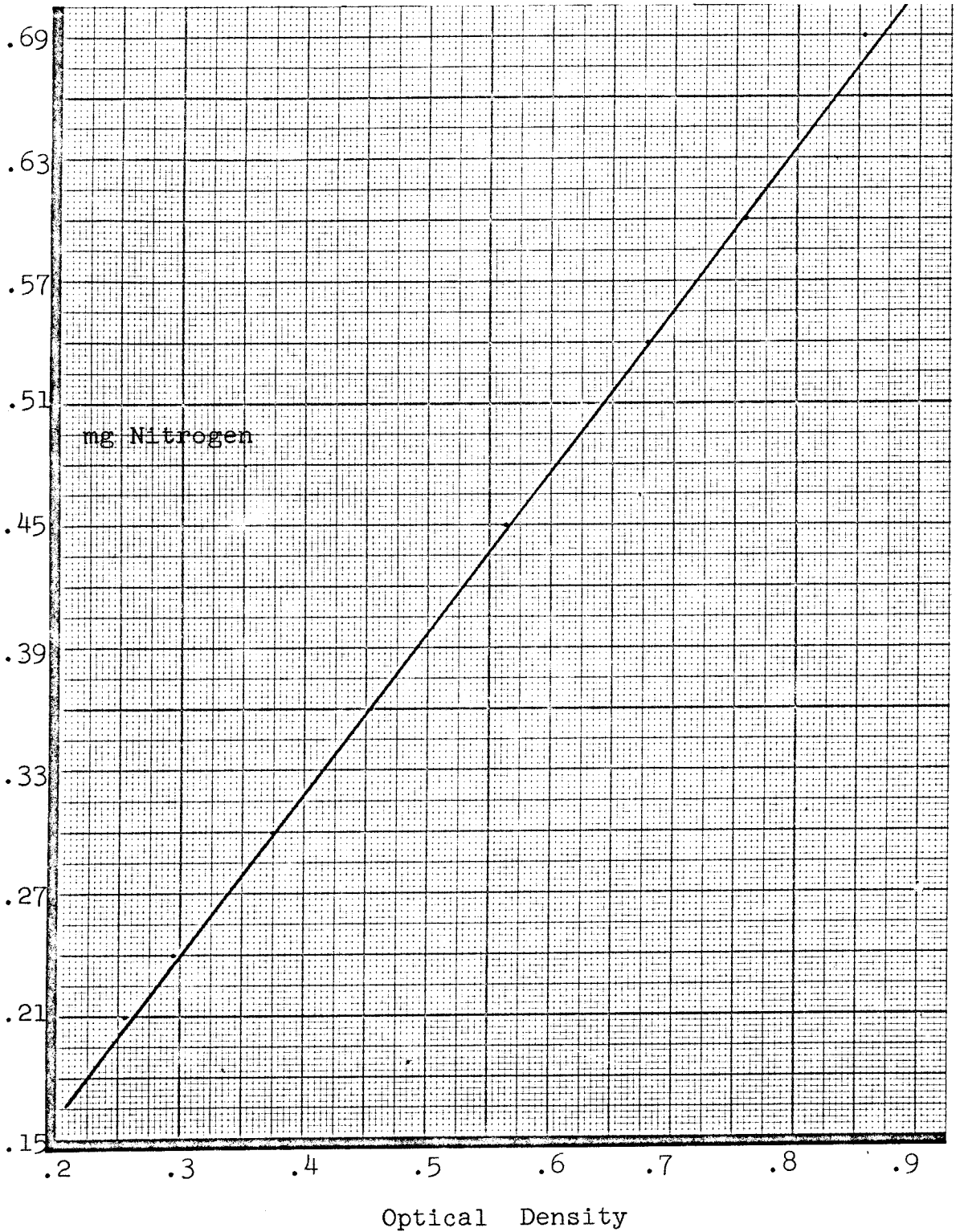


Table III
 DETERMINATION OF
 NITROGEN STANDARD CURVE

tube no.	1	2	3	4	5	6	7	8	9
ml. std. $(\text{NH}_4)_2\text{SO}_4$	7	8	10	12	15	18	20	23	0
mg. N	.21	.24	.30	.36	.45	.54	.60	.69	0
ml. H_2SO_4	1	1	1	1	1	1	1	1	1
ml. Nessler-Folin reagent	15	15	15	15	15	15	15	15	15
optical density									
A	.260	.295	.389	.470	.585	.732	.780	.870	0
B	.250	.292	.359	.435	.572	.650	.755	.840	0
C				.431	.522	.650	.721	.820	0
D				.470	.570	.681	.774	.880	0
average	.255	.294	.374	.452	.562	.678	.758	.853	0

NITROGEN STANDARD CURVE



CHAPTER IV

FINDINGS

The results obtained by the procedure described previously have shown without exception that the injection of toxo-hormone produced depression of the liver catalase activity.

The liver of a mouse was removed under ether anesthesia and was immediately homogenized in a high speed electric blender in approximately twice its own volume of ice cold distilled water for one minute. The homogenate thus obtained was then diluted to 100 ml. with ice cold distilled water and vigorously shaken. Two milliliters of this homogenate were placed in each of two 50.0 ml. volumetric containers, diluted to the mark with ice cold distilled water, and shaken vigorously.

One milliliter of this dilution was placed in each of two reaction tubes. The first reaction tube contained 1 ml. of sample, 1 ml. of water, 10 ml. of 0.075% H_2O_2 in phosphate buffer at pH 6.9, and 4 ml. of 10% TCA. The second reaction tube contained 1 ml. of sample, 1 ml. of water, and 10 ml. of 0.075% H_2O_2 in phosphate buffer at pH 6.9. The second tube was allowed to incubate for 10 minutes in an ice water bath.

From the first reaction tube 1 ml. portions were pipetted into each of two tubes, each containing 4 ml. of dis-

tilled water. Ten milliliters of 0.005 N KMnO_4 were added to each tube and the optical density determined on the photoelectric colorimeter at 515 millimicrons within one minute.

For example, if the average of these two optical density readings was 0.90 and this value was used to calculate the milligrams of H_2O_2 present, from the standard curve it was found that this optical density is equivalent to 0.335 mg. H_2O_2 . Since, however, only 1 ml. was taken for assay from the original reaction tube this value must be multiplied by 5 to make it equivalent to the 5 ml. portions taken from the original reaction tube of the 10 minute incubation sample. This procedure gave the result of 1.675 mg. H_2O_2 present in the zero minute sample. Following the 10 minute incubation in the ice water bath, 4 ml. of 10% TCA were added to the second reaction tube. The tube was shaken vigorously and 5 ml. portions were placed in each of two other tubes. Ten milliliters of 0.005 N KMnO_4 were added to each tube and the optical density was determined. The average optical density was 0.85, which from the standard curve is equivalent to 0.365 mg. H_2O_2 .

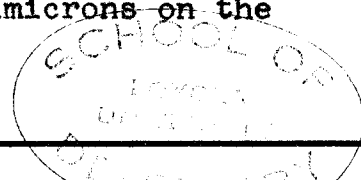
The difference between the milligrams of H_2O_2 present at zero minutes and that present at ten minutes is equal to the milligrams of H_2O_2 destroyed by the catalase enzyme. It was necessary to multiply the difference between the zero and ten minute values by 25 to correct for the dilution of 2 ml. of the

original homogenate to 50 ml. The total nitrogen was determined on the initial homogenate after dilution to 100 ml. while the milligrams of H_2O_2 was determined on a dilution of 2 ml. of this dilution to 50 ml. The calculation of milligrams of H_2O_2 decomposed was carried out as follows:

(zero min. value - 10 min. value) X 25 = milligrams H_2O_2 decomposed
(1.68 - 0.37) X 25 = 32.75 milligrams H_2O_2 decomposed

The total nitrogen content of the original dilution of the homogenate was assayed. One milliliter of the homogenate was placed in each of two Nesslerization tubes and a glass bead was added to prevent "bumping". One ml. 9 M H_2SO_4 was added and then heated to digest until dense white fumes filled the tube and the liquid became charred. After 30 seconds cooling at room temperature, one drop of 30% H_2O_2 was added and the solution gently heated. The solution usually cleared, however if the charring persisted or returned, the H_2O_2 treatment was repeated. The solution was cooled to room temperature and diluted to 35 ml. with distilled water. A blank was prepared which contained 1 ml. of the acid digestion solution and 34 ml. of distilled water. The tubes were placed in an ice water bath and cooled to 11° C. Fifteen milliliters of Nessler-Folin reagent were added to each tube, mixed well, and allowed to stand for 10 minutes.

The samples were read against the reagent blank which was set at zero optical density at 520 millimicrons on the



photoelectric colorimeter. The average of the two optical densities was 0.354 which is equivalent to 0.279 mg. nitrogen on the standard curve.

Calculation of liver catalase activity:

$$\frac{32.75 \text{ mg. H}_2\text{O}_2 \text{ decomposed}}{0.279 \text{ mg. N}} = 117.98 \text{ mg. H}_2\text{O}_2 \text{ decomposed /mg. N}$$

The remaining normal animals and the animals injected with the acetone precipitated extracts of the various human malignant tissues were assayed for their liver catalase activity in the same manner as described above.

The range of catalase activity for the animals injected with the extracts of human malignant tissues was from 63.45 to 99.51 mg H_2O_2 decomposed per mg. N. These values may be compared to that of the normal which is 117.98. These values converted to percent decrease in catalase activity were 46.22% and 15.66% respectively. It is of interest to note that the tumor producing the greatest percent depression of liver catalase activity was an adenocarcinoma of the colon which was judged by histologic evaluation to possess the least degree of malignancy of any of the tumors tested. All of the above data is compiled in Table IV.

Table V was included to show how the histologic evaluation of the degree of malignancy was arrived at by the pathologist.

The histologic estimation of the degree of malignancy of the tissues investigated is based upon relative values of 1, 2, and 3. The most anaplastic tissue investigated was assigned a relative value of 3 and the least anaplastic, a relative value of 1. The other tissues were given relative values between these end points.

The mitotic index (M.I.) was determined by counting the number of mitotic figures in ten high power (x450) microscopic fields. The highest M.I. was assigned a relative value of 3 and the lowest, a relative value of 1. These relative values were placed under M (mitotic figures).

In the same manner as described above relative values were assigned for nuclear hyperchromatism and nucleus to cytoplasm ratios and for loss of polarity.

The relative values for each tissue were added. The tissue with the highest total relative value was considered the most malignant and the lowest total relative value the least malignant.

Table IV

DATA

sample	zero min. mg. H ₂ O ₂	10 min. mg. H ₂ O ₂	mg. H ₂ O ₂ used	mg. N ml.	mg. H ₂ O ₂ mg. N	mg. H ₂ O ₂ mg. N avg.	% de- crease
normal	41.88	9.13	32.75	.279	117.38		
normal	53.50	6.58	46.93	.381	123.18	117.98	
normal	48.75	1.13	47.63	.420	113.40		
279-63	51.25	4.13	47.13	.494	99.39		
279-63	52.50	6.88	45.63	.458	99.63	99.51	15.66
6339-63	48.13	9.88	38.25	.450	85.00		
6339-63	46.88	6.58	40.30	.572	70.45	77.73	34.12
14187-62	51.88	9.25	42.63	.561	75.98		
14187-62	55.63	10.25	45.38	.389	116.65	96.31	18.37
9741-63	46.00	6.13	39.88	.650	61.35		
9741-63	35.50	6.63	26.88	.410	65.55	63.45	46.22
8719-63	46.25	18.38	27.88	.444	62.78		
8719-63	41.25	2.75	38.50	.579	66.48	64.63	45.37
282-63	46.88	3.13	43.25	.450	97.23		
282-63	44.13	2.75	41.38	.456	90.73	93.98	20.35
6673-63	46.88	11.88	35.00	.404	86.63		
6673-63	52.50	4.63	47.88	.468	102.30	94.46	19.94

Table V

tumor sample	A	M.I.	M	NH&S	P	total	Catalase activity mg.H ₂ O ₂ /mg. N
adenocarcinoma Breast(6339-63)	3	12	3	3	3	12	77.725
adenocarcinoma Colon(282-63)	3	13	3	2	3	11	93.979
anaplastic ca. Bronchiogenic (279-63)	3	5	1	2	3	9	99.509
adenocarcinoma Colon(8719-63)	2	5	1	2	2	7	64.625
adenocarcinoma Rectum(6673-63)	2	6	1	2	2	7	94.462
adenocarcinoma Colon(14187-63)	1	6	1	2	1	5	96.313
adenocarcinoma Colon(9741-63)	2	4	1	1	1	5	63.450

A anaplasia
 M.I. mitotic index
 M. mitotic figures
 NH&S nuclear hyperchromatism & nucleus to cytoplasm
 P loss of polarity ratio

CHAPTER V

DISCUSSION

The classical criteria for the estimation of the degree of malignancy are based entirely upon histologic evaluation. These methods have been used for many years and the criteria seem to be based upon individual interpretation. This may account for the difficulty encountered in finding a pathologist who would make an attempt to classify the tumor tissues used as to their degree of malignancy.

The determination of liver catalase activity is based upon an established procedure which gives results which can be duplicated by various investigators. It is not based upon individual judgment but rather upon basic accepted biochemical assay techniques. A biochemical assay does not take into consideration individual differences in interpretation.

Catalase is a heme-protein enzyme which catalyzes the decomposition of H_2O_2 . It is present in animal tissues in liver and red blood cells, which may indicate the undesirability of H_2O_2 in tissues, although there is no direct evidence for the presence of such peroxide. In animal tissues there are some enzymes which yield, in vitro, H_2O_2 as an end product. It is thought that hydrogen peroxide has not been isolated from ani-

mal tissues because of the presence of catalase enzyme which quickly destroys it. This is a necessary physiologic reaction because the peroxide is a toxic product which if allowed to exist would lead to the formation of methemoglobin.

It is not possible to show a correlation between liver catalase activity and a histologic estimation of the degree of malignancy from this study since a functional value is being compared to a judgment value.

It would seem from a review of the literature, that the depression of liver catalase activity is a reliable determination of the degree of malignancy. Many investigators have shown that non-malignant tissues from various sites including liver, kidney, spleen, and embryonic tissue produce comparatively little or no reduction in liver catalase activity. In one study, Begg, Dickinson, and Miller (1953) transplanted tumor tissue of common origin which spontaneously changed from fibroadenoma to mixed fibroadenoma-fibrosarcoma and then to fibrosarcoma. They found that the benign tumor produced an 8% decrease in liver catalase activity, the mixed tumor produced a 44% decrease in liver catalase activity, and that the malignant tumor produced a slightly greater decrease in liver catalase activity. It has been shown by many investigators using malignant tumors of both animal and human origin that a marked depression in liver catalase activity results from the transplantation of these tissues

or from the injection of an extract of these tissues.

The effect of a tumor upon the host seems to be a more reliable measure of the degree of malignancy than the histologic appearance of the tissue. The information to be gained from a histologic evaluation of a tissue specimen is that it is or is not malignant and that a certain clinical course usually is expected of this particular type of malignancy. However, if we are to search for more reliable therapeutic measures, more must be known. We must be able to assess its effect upon the physiology of the host if we expect to counteract this effect or eliminate the disease. It is possible that cancer cells are normal cells which have had their chemistry altered in such a way as to make them behave abnormally. If this is true it may be possible to artificially alter their chemistry by chemotherapeutic agents so as to return them to normal.

It is felt by this investigator that the depression of liver catalase activity is a much more reliable measure of the degree of malignancy than an empirical evaluation of the histologic appearance of a tissue specimen.

This study brings to light further studies which must be carried out to give more insight into this problem. A technique must be perfected which will enable the detection of toxohormone in the blood of patients with cancer. It is very possible that a sample of acetone precipitated fraction of blood

might be injected intraperitoneally into a mouse and the liver catalase activity determined. A depression of liver catalase activity would be indicative of the presence of cancer. It may be that subclinical cases of cancer could be detected and chemotherapy instituted to destroy the disease in its very early state. This technique may also be employed as a continuous assay of the status of malignant disease during or following chemotherapy, surgery, or x-radiation.

An attempt has been made to show the merits of a new diagnostic procedure. It will not replace histologic examination of tissues but could go several steps further than the histologic method of arriving at the diagnosis, prognosis, and a judgment of the effectiveness of therapy.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The purpose of this study was to investigate the effect of an intraperitoneal injection of an extract of human malignant tissues upon mouse liver catalase activity. An attempt was made to determine whether any quantitative correlation exists between the depression in liver catalase activity and the estimation of the degree of malignancy of tumor tissues as judged by histologic evaluation.

The procedure used involved the intraperitoneal injection of an acetone extract of human malignant tissues into mice. Twenty-four hours following the injection, the liver was removed and assayed for its catalase activity. Colorimetric methods of analysis were used and are fully described in Chapter III. The catalase activity was expressed on the basis of milligrams of H_2O_2 destroyed per milligram of nitrogen.

Acetone extracts of the human malignant tissues investigated all produced a decrease in liver catalase activity, whereas those of normal tissues have not been shown to produce such changes. This decrease varied from 15.66% to 46.22%. The malignant tissue whose extract produced the greatest depression of liver catalase activity had been judged to be the least

malignant, using the histologic estimation of the degree of malignancy as described in Chapter IV. The other samples similarly showed lack of order comparison.

From this study it is not possible to show a positive correlation between liver catalase activity depression produced by an extract of a given malignant tissue and the degree of malignancy of that tissue as estimated by the histologic evaluation as previously described. However, since previous work by other investigators show an increase in toxohormone activity with the development of malignancy in a tumor, it seems justified to conclude that the effect of the tumor upon the host appears to be a more reliable measure of the degree of malignancy than does the histologic appearance of the tissues.

BIBLIOGRAPHY

- Adams, D.H.: The mechanism of the liver catalase depressing action of tumors in mice. *British Journal of Cancer*, 4: 183-195 (1950).
- Adams, D.H.: Mouse liver catalase. The antagonistic effect of tumor tissue upon the hormonal control mechanisms. *British Journal of Cancer*, 5: 409-416 (1951).
- Begg, R.W.; Dickinson, T.E.; and Miller, J: The effect of benign and malignant tumors on liver catalase activity. *Canadian Journal of Medical Science*, 31: 315-319 (1953).
- Blumenthal, F. and Brahn: Die Katalasewirkung in normaler und in carcinomatöser leber. *Z. Krebsforsch*, 8: 436 (1910).
- Busch, Harris: "Biochemistry of the Cancer Cell", Academic Press (1962).
- Callao, V. and Montoya, E.: Toxohormone-like factor from microorganisms with impaired respiration. *Science*, 134: 2041-2042 (1961).
- Day, F., Gabrielson, F.C., and Lipkind, J.B.: Depressions in the activity of liver catalase in mice injected with homogenates of normal mouse spleen. *Journal of The National Cancer Institute*, 15: 239-252 (1954).
- Delmon, G. and Biraben, J.: Action of injection of toxohormone extracts on growth and development of transplantable animal tumors. *Comptes rendu du societie biologique*, 153: 1334-1336 (1959).
- Fujii, S.; Kawachi, T.; Okuda, H.; Haga, B.; and Yamamura, Y.: The isolation of a basic protein having toxohormone activity from tumor tissues. *Gann*, 51: 223-229 (1960).
- Fukuoka, F. and Nakahara, W.: Mode of action of toxohormone. A third study of toxohormone, a characteristic toxic substance produced by cancer tissue. *Gann*, 42: 55-67 (1951).

- Goldblith, S.A. and Proctor, B.E.: Photometric determination of catalase activity. *Journal of Biological Chemistry*, 187: 705-709 (1950).
- Greenfield, R.E. and Meister, A.: Effect of injections of tumor fractions on liver catalase activity of mice. *Journal of the National Cancer Institute*, 11: 997-1005 (1950-1951).
- Greenstein, J.P.: "Biochemistry of Cancer", 2nd. ed., Academic Press, New York (1954).
- Greenstein, J.P. and Andervont, H.B.: Note on the liver catalase activity of pregnant mice and of mice bearing embryonic implants. *Journal of the National Cancer Institute*, 4: 283-284 (1943).
- Greenstein, J.P.; Jenrette, W.V.; Mider, G.B.; and Andervont, H.B.: The relative enzymatic activity of certain mouse tumors and normal control tissues. *Journal of the National Cancer Institute*, 2: 293-299 (1941).
- Harrow, B. and Mazur, A.: "Textbook of Biochemistry", 7th.ed., W.B. Saunders Company, Philadelphia (1960).
- Hepler, O.E.: "Manual of Clinical Laboratory Methods", 4th.ed. rev., Charles C. Thomas, Springfield, Illinois (1951).
- Jujii, S.; Kawachi, T.; Okuda, H.; Haga, B.; and Yamamura, U: The isolation of a basic protein having toxohormone activity from tumor tissues. *Gann*, 51: 223-229 (1960).
- Kampschmidt, R.F.; Adams, M.E.; and McCoy, T.A.: Some systemic effects of toxohormone. *Cancer Research*, 19: 236-239 (1959).
- Koch, F.C. and Hanke, M.E.: "Practical Methods in Biochemistry", Baltimore, The Williams and Wilkins Company (1953).
- Masamune, H.; Kaketa, H.; and Abe, S.: Chemical nature of toxohormone (Nakahara), V. KIK-inactive toxohormones from urine of cancer patients with original tumor in different organs. *Tohoku Journal of Experimental Medicine*, 68: 321-324 (1958).

- Masamune, H.; Tsuiki, S.; Kamiyama, S.; Abe, S.; Haga, M.; Kawasaki, H.; Kaketa, H.; and Abe, S.: Chemical nature of toxohormone (Nakahara)--globulin-like toxohormone from cancerous ascitic fluid. *Tohoku Journal of Experimental Medicine*, 67: 309-318 (1958).
- Nakahara, W.: Chemical basis for tumor host relations. *Journal of the National Cancer Institute*, 24: 77-87 (1960).
- Nakahara, W. and Fukuoka, F.: A toxic cancer tissue constituent as evidenced by its effect on liver catalase activity. *Japanese Medical Journal*, 1: 271-277 (1948).
- Nakahara, W. and Fukuoka, F.: Toxohormone. A characteristic toxic substance produced by cancer tissue. *Gann*, 40: 45-69 (1949).
- Nakahara, W. and Fukuoka, F.: The newer concept of cancer toxin. *Advances in Cancer Research*, 5: 157-177 (1958).
- Nakahara, W. and Fukuoka, F.: "Chemistry of Cancer Toxin; Toxohormone", Springfield, Illinois; Charles C. Thomas (1961).
- Ono, T.; Umeda, M.; and Sugimura, T.: Purification of toxohormone. *Gann*, 48: 91-100 (1957).
- Ono, T.; Sugimura, T.; and Umeda, M.: Preparation of potent concentrates of toxohormone free from nucleic acid. *Gann*, 46: 617-630 (1955).
- Riley, E.F.: Depression of liver catalase by various agents. *Cancer Research*, 19: 285-299 (1959).
- Seabra, A., and Deutsch, H.F.: Studies on catalase inhibition as related to tumors. *Journal of Biological Chemistry*, 214: 447-454 (1955).
- Weil-Malherbe, H. and Schade, R.: Studies on the liver catalase of normal and cancerous rats. *Biochemical Journal*, 43: 118-125 (1948).
- Yunoki, K. and Griffin, C.A.: Purification of toxohormone by column chromatography. *Cancer Research*, 20: 533-540 (1960).
- Yunoki, K. and Griffin, A.C.: Concentration and properties of a highly purified toxohormone preparation. *Cancer Research*, 21: 537-544 (1961).

APPROVAL SHEET

The thesis submitted by Dr. Richard I. DeLo has been read and approved by four members of the Department of Oral Biology.

The final copies have been examined by the Director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

April 15, 1964
Date

John Rapp, Ph.D.
Signature of Adviser