1964

A Comparison of the Toxohormone Content of Human Malignant Tissues

Laurence Lowell McCarthy
Loyola University Chicago

A COMPARISON OF THE TOXOHORMONE CONTENT OF HUMAN MALIGNANT TISSUES

by

LAURENCE LOWELL MCCARTHY

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

Master of Science

JUNE

1964

LIFE

Laurence Lowell McCarthy, the oldest of four children, was born in Glencoe, Illinois on October 12, 1935.

He was graduated from New Trier High School, Winnetka, Illinois in June, 1953. From September, 1953 to January, 1956 he attended the University of Notre Dame, Notre Dame, Indiana.

In September of 1956 he began studies at Loyola University School of Dentistry, Chicago, Illinois and received the degree of Doctor of Dental Surgery in June, 1960. During his senior year he served as president of his class and was elected to membership in the Blue Key National Honor Fraternity.

He entered the United States Army Dental Corps in July, 1960 and served as Chief of Oral Surgery, Dental Clinic #3, Fort Sill, Oklahoma until the termination of his tour of duty in July, 1962.

In September of 1962 he began graduate studies in Oral Biology and clinical studies in Oral Surgery at Loyola University School of Dentistry, Chicago, Illinois.

ACKNOWLEDGEMENTS

To Dr. Gustav W. Rapp, my advisor, I owe the impetus which initiated me into the world of research, the enthusiasm which made it enjoyable, and the direction which made it worthwhile — all of which together have formulated my desire to further continue research. My deepest thanks go to Dr. Rapp, specifically, for his time, constant advice, and generous assistance in the investigation conducted.

I extend to Dr. Patrick D. Toto and to Dr. Maurice V. L'Heureux my most sincere thanks for their constant willingness to help and their constructive criticism and guidance which were invaluable aids in the successful completion of this research.

I extend my sincere gratitude to Miss Aldona Prapuolenis for her aid in my establishing a sound scientific discipline in the laboratory.

To my wife, Jeri-Ann, I owe the years of self-sacrifice and unselfishness she endured to enable me to pursue graduate studies.

In this same vein I wish to thank my parents for their sacrifices which made possible the thought of graduate study.

I would also like to thank Dr. Louis J. Blanchet for serving on my oral examination board in the absence of Dr. Rapp.

TABLE OF CONTENTS

TABLES AND FIGURES

A. TABLES

B. FIGURES

"...it is provided in the essence of things that from any fruition of success, no matter what, shall come forth something to make a greater struggle necessary."

— Walt Whitman

CHAPTER I

INTRODUCTION

Studies of the cachectical conditions such as anorexia, weight loss, and muscular weakness observed in cancer-bearing animals had long suggested that cancer tissues possibly produced some toxic substance. The demonstration of such a substance, however, required an adequate criterion for its assay. The disclosure of depressions of liver catalase activity in all cancer-bearing animals provided a criterion.

By measuring the liver catalase activity in laboratory animals injected with a protein fraction extracted from malignant tissue it was shown that this extract produced a significant decrease in catalase activity. The substance responsible for this effect was called "toxohormone".

Further investigations disclosed that additional systemic changes such as decreased plasma iron, kidney catalase, and hemoglobin present in cancer-bearing animals could be induced in normal animals by the injection of toxohormone-containing tumor fractions. Of these the decrease of blood plasma iron appeared to

be the most sensitive to the effects of toxohormone and therefore a possible valuable aid for further experimentation.

Although research on toxohormone has included attempts at purification and investigations relating to mode of action, no specific research has been directed toward a comparison of the toxohormone content of malignant tissues. Variations in the activities of different samples of similarly prepared toxohormone-containing material have been indirectly demonstrated, but whether the variations are due to differences in the toxohormone content among different types of tumors or cancerous tissues of varying degrees of malignancy are hypotheses which have not been investigated. In an attempt to determine if there is a relation between degree of malignancy and the toxohormone content of malignant tissue, the effect of injected toxohormone, extracted from human malignant neoplasms, on blood plasma iron concentration was studied in mice.

CHAPTER II

REVIEW OF LITERATURE

A. Enzymatic Tumor-Host Relations and Hypothesis of a Cancer Toxin

Blumenthal and Brahn (1910) and Brahn (1916) reported that the liver catalase activity of cancer-bearing patients was "remarkably low" as compared with that of patients with other diseases. Similar results were obtained by Rosenthal (1912) during his studies on tumor-bearing animals, and he hypothesized that some toxic substance might be produced in tumor tissues to decrease the liver catalase activity.

Buckley (1921) stated in his text on cancer that an abnormal hormonal secretion of cancer cells exerted an adverse influence on the body systems because he had observed that the "condition" of the blood was found to be improved temporarily after the surgical removal of a cancerous mass.

It was conclusively demonstrated by Greenstein and co-workers (Greenstein, Jenrette, and White, 1941; Greenstein and Andervont, 1942, 1943) that the liver catalase activity of cancer-bearing animals was significantly lower than that of normal animals and

specifically attributable to the presence of a growing tumor and not to any secondary cause to which the tumor's presence may give rise. In these articles Greenstein et al. reported the following: the activity of catalase was by far the most affected of all the individual enzyme systems studied in the livers of mice and rats with a wide variety of malignant tumors, the decrease in liver catalase activity was progressive with growth of the tumor, the effect of the tumor upon the activity of this liver enzyme was found to be reversible upon removal of the tumor, the decrease was due only to the tumor and not to any growing tissues present in the animal, and the decrease was always present in cancer-bearing animals. These observations subsequently led Greenstein (1947) to state that a tumor may produce the systemic effects noted by giving off some toxic product to the circulation.

B. Toxohormone

1. Discovery and Fundamental Characteristics

In 1948 Nakahara and Fukuoka, using liver catalase activity as a criterion for bio-assay, demonstrated that a substance which was extracted from human cancerous tissue with water and then precipitated

with alcohol after removing heat-coagulable material was capable of significantly decreasing liver catalase activity beyond the normal limits of variability after twenty-four hours when injected into mice. Injections of fifty to one hundred milligrams of the substance produced an average depressed liver catalase activity, expressed in cubic centimeters of oxygen liberated from hydrogen peroxide, of 4.3. Normal mice were found to have an average liver catalase activity of 8.0. Positive results were obtained from all malignant tissues used, carcinoma as well as sarcoma, primary as well as secondary or metastatic, and surgical as well as autopsy materials; it was clearly distinguishable from similarly prepared fractions of normal tissues and normal tissue necrosates which had no special depressing effect on liver catalase activity in vivo (the average activity was found to be between 7.2 and 7.5 cubic centimeters of oxygen after injections of these fractions); and it exhibited no inhibiting action on crystalline catalase in vitro. The substance was called "toxohormone" by the investigators to express its supposed biological status of being a cell-produced substance which is released into circulation and which produces a clearly definable

biochemical lesion in a target organ. They conceived of it as being the pathological counterpart of hormones. Variations demonstrated in the activities of different samples of the extracted material led Nakahara to hypothesize that there might be a relation between histological character and toxohormone content of malignant tissue, but he was unable to demonstrate this supposition.

The original work done by Nakahara and Fukuoka was confirmed and substantiated by Greenfield and Meister (1951). It was also shown by these authors that fractions from the necrotic and non-necrotic areas of a malignancy produced reductions of liver catalase activity of about the same magnitude in mice (between a 40 and a 44 per cent reduction from normal).

Endo et al. (1955) demonstrated that toxohormone and a substance extracted from malignant tissue by Hargreaves and Deutsch (1952) which inhibited liver catalase activity in vitro but not in vivo (the Kochsaft factor) were entirely different entities since toxohormone inhibited liver catalase activity in vivo but not in vitro.

It has been demonstrated by investigators that

"toxohormone fractions" prepared from samples of normal tissues such as liver, spleen, kidney, and whole skinned animal homogenates produce very little if any decrease in liver catalase activity (Greenfield and Meister, 1951; Nakagawa, Kosuge, and Tokunaka, 1955; Kampschmidt, Adams, and McCoy, 1959; Fujii, Kawachi, Okuda, Haga, and Yamamura, 1960).

As stated by Nakahara and Fukuoka (1958), in a review of related literature, all the malignant tumors which have been tested, human and animal, have yielded a liver catalase-depressing fraction (toxohormone). This is in direct accord with the fact that significantly decreased liver catalase activity has been found in all animals bearing malignant tumors. The following is a list of the tumors which have been investigated: human carcinomas of the stomach, rectum, colon, bladder, and breast; human lymphosarcoma; human mammary fibrosarcoma; mouse mammary sarcoma; rat fibrosarcoma; Brown-Pearce carcinoma; chicken sarcoma; human hepatoma; rhodamine sarcoma; and Walker carcinosarcoma.

Paralleling experimentation on toxohormone, Adams (1950, 1951) found that injections of whole homogenates of both mouse sarcoma and carcinoma pro-

duced depressions of liver catalase in mice, and he inferred that the depression was due to the release of toxic material from the injected tumor homogenates. He also demonstrated that the injection of homogenates of a variety of normal tissues produced no significant catalase depressions. Lucké et al. (1952), using parabiotic rats, showed that the liver catalase activity in the non-tumor-bearing partner in the parabiotic union was reduced to the level of single rats bearing the tumor, and he inferred that a humoral transmission of some agent from the tumor was responsible.

Further evidence in support of the validity of the liver catalase-depressing factor of Nakahara and Fukuoka has been demonstrated by other investigators. Kampschmidt et al. (1959) reported that injections of one hundred milligrams of an alcohol-precipitable extract of Walker carcinosarcoma 256 depressed the liver catalase activity of rats after twenty-four hours from a normal level of 5400 catalase units to 3150 catalase units. Fujii et al. (1960) reported that injections of twenty milligrams of a picric acid-hydrochloric acid-alcohol-Precipitable extract of both human hepatoma and rhodamine sarcoma

reduced the liver catalase activity of mice approximately fifty and sixty per cent respectively.

2. Purification

Although some research has been done on the purification of toxohormone (Nakahara and Fukuoka, 1950, 1954; Fukuoka and Nakahara, 1953; Nakagawa, Kosuge, and Tokunaka, 1955; Ono, Sugimura, and Umeda, 1955, 1956), the precise chemical nature of toxohormone has not yet been elucidated. Nakahara and Fukuoka (1958), in a summation of the present concepts, state that in its elementary form it is probably a polypeptide of rather small molecular size. It probably occurs in cancerous tissue either as aggregates of the elementary form or in close association with some other substance, possibly nucleic acid; it occurs in both a dialyzable and non-dialyzable form, the former being derived from the latter when digested with papain or pepsin. The enzymatic action which yields the dialyzable form may simply dissociate the nucleic acid from the elementary form.

3. Mode of Action

The mode of action of toxohormone has also come under the investigation of researchers (Fukuoka and Nakahara, 1951, 1953; Ono, Umeda, and Sugimura, 1956;

Ono, Ohashi, and Yago, 1960). Nakahara (1960) has stated that the outstanding changes observed in cancer-bearing animals seem to be specifically related to disturbances in iron metabolism, and these changes have been elucidated in normal animals by the injection of toxohormone-containing tumor fractions (Nakahara and Fukuoka, 1949; Greenfield and Meister, 1951; Ono, Umeda, and Sugimura, 1956; Hoshizima, 1957; Kampschmidt, Adams, and McCoy, 1959; Fujii, Kawachi, Okuda, Haga, and Yamamura, 1960). He suggests that the production of decreased liver catalase activity, increased liver protoporphyrin, and decreased liver ferritin and blood plasma iron by injections of toxohormone as described in these articles are closely related phenomena and as such speak strongly for the implication of toxohormone in the disturbed iron metabolism of cancer-bearing animals.

Other changes present in cancer-bearing animals and brought about by injections of the active tumor fraction (toxohormone) but not related to iron metabolism have been observed. Fukuoka (1952) demonstrated thymus involution in toxohormone-injected animals, and Ono (1959) demonstrated the depression of diphosphopyridine nucleotide synthesis in the liver of injected

animals.

C. Tumors, Toxohormone, and Blood Plasma Iron Relations

One of the first reports in the literature concerning the relationship of tumors and blood plasma iron concentration was that of Heilmeyer and Plötner (1937) who reported that the plasma iron level in cancer-bearing animals was "low".

Postiglione (1946), Yanagisawa (1955), and Iijima (1956) all reported that the plasma iron concentration in cancer patients was considerably decreased (Sugimura, 1957).

Sugimura (1957), investigating the distribution of radioactive iron injected into tumor-bearing rats, noted that the concentration of iron in the plasma of these animals was decreased approximately 30 per cent.

Kampschmidt et al. (1959) reported that injections of toxohormone-containing material produced a pathological alteration in the blood plasma iron concentration of rats. The iron level was reduced from an average normal concentration of 281 micrograms per one hundred milliliters to an average of eighty-four micrograms per one hundred milliliters eight hours after single injections of 0.10 milligrams of an

alcohol-precipitable extract of Walker carcinosarcoma 256 prepared after the method of Nakagawa et al. (1955). Injections of one hundred milligrams of the same material produced a reduction from an average plasma iron concentration of 294 micrograms per one hundred milliliters to sixty-two micrograms per one hundred milliliters after twenty-four hours. This same study revealed plasma iron to be 250-500 times as sensitive to toxohormone as liver catalase.

Fujii et al. (1960) showed that the plasma iron level of rats was significantly reduced from an average of ninety-three micrograms per one hundred milliliters to an average of approximately fifty micrograms per one hundred milliliters within twelve hours following intraperitoneal injections of five milligrams of toxohormone-containing material (picric acid-hydrochloric acid-alcohol precipitate) extracted from human hepatoma.

CHAPTER III

MATERIALS AND METHODS

A. Preparation and Injection of Tumor Fractions

Frozen biopsy specimens of seven malignant neoplasms were obtained from Cook County Hospital, Chicago, Illinois. They consisted of the following tumors as determined through histological examination by members of the Department of Pathology of that hospital: one adenocarcinoma, rectum; four adenocarcinomas, colon; one adenocarcinoma, breast; and one anaplastic carcinoma, bronchiogenic. The tumor samples were maintained in a frozen state until they were homogenized.

Homogenization and extraction of fat from each of the seven tumor specimens was accomplished similarly to the method of Fujii et al. (1960). No attempt was made to further purify the remaining material by reprecipitation since it had been demonstrated by other investigators that plasma iron concentration is very sensitive to small amounts of toxohormone, and it was felt that even small amounts of this crude toxohormone-containing material would depress mouse

plasma iron concentration enough so that adequate comparisons could be made. The method used is outlined as follows:

1) Approximately two grams of frozen tumor tissue was homogenized with approximately ten volumes of acetone in a Potter-Elvehjem homogenizer suspended in an ice-water bath to minimize frictional heating of the sample.
2) The suspension obtained was centrifuged at five thousand R.P.M. for ten minutes and the fat-containing supernatant decanted and discarded.
3) The protein-containing precipitate was washed with acetone, dried in air, and preserved in a freezer until needed for injection.

Experimental animals employed in all instances were young male albino mice of a homogeneous strain weighing approximately twenty to twenty-five grams. Upon receipt they were transferred to standard mouse cages and maintained on a diet of Wayne Lab-Blox and adequate water supply. In most instances the animals were allowed to remain at least two and not more than

ten days in their cages before experimentation. Their weight was found not to vary more than plus or minus three grams during their repository period.

At the time of injection, one hundred milligrams of the dried tumor fraction, containing the total protein component, were mixed with 2.5 milliliters of 0.9 per cent sodium chloride solution. Employing a two cubic centimeter tuberculin syringe armed with a twenty-five gauge needle, 0.5 of a milliliter of the saline-tumor fraction suspension, containing twenty milligrams of the tumor fraction, was injected intraperitoneally into each of five mice.

The injected mice were maintained in a normal environment on the pre-injection diet and, by pooling plasma, two determinations of the collective blood plasma iron concentration made twenty-four hours after injection (Table VII).

All seven tumor specimens were tested utilizing seven groups of five mice per group.

B. Blood Plasma Iron Determination

The method employed for the determination of blood plasma iron concentration was derived from the method of Kitzes, Elvehjem, and Schuette (1944). Neither their method nor any other method reviewed

(Barkan and Walker, 1940; Peters, Giovanniello, Apt, and Ross, 1956) could in entirety be used for plasma iron determination in mice since the volume of plasma required in these procedures (2.5 ml.) could not be obtained from any one mouse, the initially intended number to be used per determination, or consistently from groups of five mice, the number finally employed in this investigation. The amount of plasma that could be obtained from any one mouse varied from 0.2 to 0.7 of a milliliter. Volumes below 0.6 of a milliliter were obtained 80 per cent of the time.

Initially, as mentioned above, plasma iron determinations were attempted on single normal mice, but sufficient plasma volumes necessary for the adopted method could not be consistently obtained. Furthermore, the results showed a fluctuation in the normal range of values so extensive as to prevent any suitable comparisons among single mice (Table V). Because of these reasons, plasma was pooled in equal volumes from each of five mice for every determination of blood plasma iron, thereby insuring a sufficient plasma volume and decreasing the standard deviation. No more than five mice per group could be used because of the limited amount of tumor tissue available. The

procedure is outlined as follows:

1) By means of cardiac puncture, approximately one ml. of blood was obtained from each of five mice, lightly anesthetized with ether. A twenty-five gauge needle was used on a ten cubic centimeter glass syringe coated with a solution of 0.1 molar sodium oxalate. The blood samples were transferred to five five-milliliter semi-micro centrifuge tubes, respectively.

2) The oxalated blood samples were centrifuged at 1550 R.P.M. for ten minutes.

3) The plasma was separated from the blood cells by means of a dropper pipette. One-tenth of a ml. was obtained from each of the five plasma samples and transferred to another five ml. semi-micro centrifuge tube.

4) Two-tenths of a ml. of distilled water was added to the 0.5 of a ml. of pooled plasma and the tube was placed in boiling water for two minutes.

5) Two-tenths of a ml. of 25 per cent trichloroacetic acid was then added, the mixture stirred thoroughly and centrifuged

at 1550 R.P.M. for ten minutes.

6) The clear protein-free supernatant was decanted into a five ml. graduated test tube and the volume was noted.

7) Distilled water was added to the precipitate in an amount equal to the difference between the noted volume of the supernatant and 1.2 ml. The precipitate was broken up, stirred well, and the tube was centrifuged at 1550 R.P.M. for ten minutes.

8) The resultant supernatant was decanted into the previous supernatant and distilled water added, if necessary, to make 1.2 ml.

9) The following were added to the tube containing the clear supernatants:

   a) 0.05 of a ml. of undiluted thioglycolic acid

   b) 0.05 of a ml. of 5 per cent a,a'-dipyridyl

   c) 0.2 of a ml. of 25 per cent sodium citrate

   The contents were mixed.

10) The resultant 1.5 ml. pink solution was transferred to a microcuvette, and after fifteen minutes its absorbance at 508 mμ was read on a Beckman Junior Spectrophotometer

which had been adjusted to the zero reading with distilled water.

11) The concentration of blood plasma iron was calculated by using a previously prepared standard curve which plotted concentration of iron against absorbance (Figure 2). The actual value as obtained from the graph was multiplied by a factor of two since only 0.5 of a ml. of plasma was used, and the graph was based on values obtained from one ml. of standard iron solutions.

Two determinations were run on each sample of pooled plasma.

The reagents were tested for each group of determinations made by performing a determination on a known iron concentration and comparing the result with an originally obtained value.

Co-ordinates used in the preparation of the standard iron curve were obtained by averaging the absorbance readings obtained from three determinations performed on an iron-free solution and each of the following six iron concentrations derived from a standard iron solution: 30, 60, 90, 120, 180, and 240 micrograms per one hundred milliliters (Table I).

The concentration range was adopted from normal rat plasma iron levels as reported by Kampschmidt et al. (1959). Determinations were run in the following manner:

1) Two-tenths of a ml. of 25 per cent trichloroacetic acid, 0.05 of a ml. of undiluted thioglycolic acid, 0.05 of a ml. of 5 per cent a,a'-dipyridyl, and 0.2 of a ml. of 25 per cent sodium citrate were added to one ml. of distilled water containing a known concentration of iron.
2) The resultant 1.5 ml. solution was transferred to a microcuvette and after fifteen minutes its absorbance at 508 mμ was read on a Beckman Junior Spectrophotometer which had been adjusted to the zero reading with distilled water.

The equation for the linear curve used in plotting concentration against absorbance was calculated from the statistical method of "least squares" (Batson, 1960) (Figure 1).

Normal blood plasma iron concentration and standard deviation were obtained by performing the aforementioned plasma iron determination on seven

groups of five mice per group, averaging the results, and applying a standard statistical method for determining standard deviation (Page and Culver, 1961) (Table VI).

The precision and accuracy of the adopted method was determined and evaluated for the standard iron solutions and plasma by recovery experiments and by experiments dealing with reproducibility of results (Tables II, III, and IV).

C. Determination of Degree of Malignancy

Histological sections prepared from the tumor specimens used were obtained from Cook County Hospital, and a comparison of the degree of malignancy of each of the seven tumors was made using the histopathological characteristics of: anaplasia, increased number of mitotic figures, nuclear hyperchromatism and increased nucleocytoplasm ratio, and loss of cell polarity. Each of the four histopathological characteristics were assigned values from one to three; three being the number used to indicate the greatest relative anaplasia, nuclear hyperchromatism and size, number of mitotic figures, or loss of polarity in relation to the other neoplasms. The mitotic index was determined by counting the number of mitotic

figures in ten high power fields. The tumors which had the greatest sum total of values were considered to be the most malignant (Boyd, 1953) (Table VIII).

CHAPTER IV

FINDINGS

The findings have been placed in chronological order as an aid to understanding the investigative process used to arrive at the conclusions presented.

The yield of dried precipitates obtained from the extraction of the protein component of approximately two grams of each of the seven tumors employed varied in weight from 115 to 200 milligrams.

Table I lists the average and individual absorbance figures obtained from colorimetric determinations performed on known iron concentrations and an iron-free solution. The range of absorbance values, .018 through .180, was of necessity low and narrow since the related iron concentration range desired, zero through 240 micrograms per one hundred milliliters, was similarly characterized. However, even in this range it was found that readings could be made quite accurately on the Beckman Junior Spectrophotometer.

The co-ordinates used in the preparation of the standard iron curve were obtained from the

TABLE I

ABSORBANCE READINGS OF KNOWN IRON CONCENTRATIONS

TABLE I

mcg./100 ml.	A_1	A_2	A_3	$A_{Average}$
0	.017	.019	.018	.018
30	.043	.041	.044	.043
60	.061	.065	.060	.062
90	.083	.080	.077	.080
120*				.099
180	.139	.138	.140	.139
240	.180	.175	.185	.180

*See Table II

figures presented in Table I: the $\underline{x}$ component was given absorbance values, and the $\underline{y}$ component was given concentration values. The plotted points indicated a linear curve, and the statistical equation shown in figure 1 was used to calculate the straight line. Figure 2 is a representation of the standard iron curve used. The curve does not originate at zero since the spectrophotometer was adjusted to zero with distilled water and the first reading made with distilled water plus reagents.

Since the method used to determine blood plasma iron concentration was a new one, tests were run to determine its precision and accuracy. The precision of a method depends upon reproducibility of results, and in general, the standard deviation of a set of values from a set of determinations upon the same material is a measurement of the reproducibility of a measurement (Page and Culver, 1961). The method used to determine the standard deviation of results obtained from a standard iron solution was the performance of absorbance measurements on several aliquots from that solution and solving the statistical equation, $\sigma = \sqrt{\left[\frac{\sum(x - \bar{x})^2}{n - 1}\right]}$. The standard deviation was found to be plus or minus .0028 units

EQUATION: $\hat{X} = \bar{X} + b(Y - \bar{Y})$ where:

$\hat{X}$ is the expected value of X at a given value of Y,

$\bar{X}$ is the mean of the observed values of X,

b is the slope of the line computed as: $\frac{\Sigma xy}{\Sigma y^2}$ where:

$$\Sigma xy = \Sigma XY - \frac{(\Sigma X)(\Sigma Y)}{n}$$

$$\Sigma y^2 = \Sigma Y^2 - \frac{(\Sigma Y)^2}{n}$$

$\bar{Y}$ is the mean of the given values of Y

$$\hat{X} = 21.0 + 0.66Y$$

FIGURE 1.

STRAIGHT LINE EQUATION

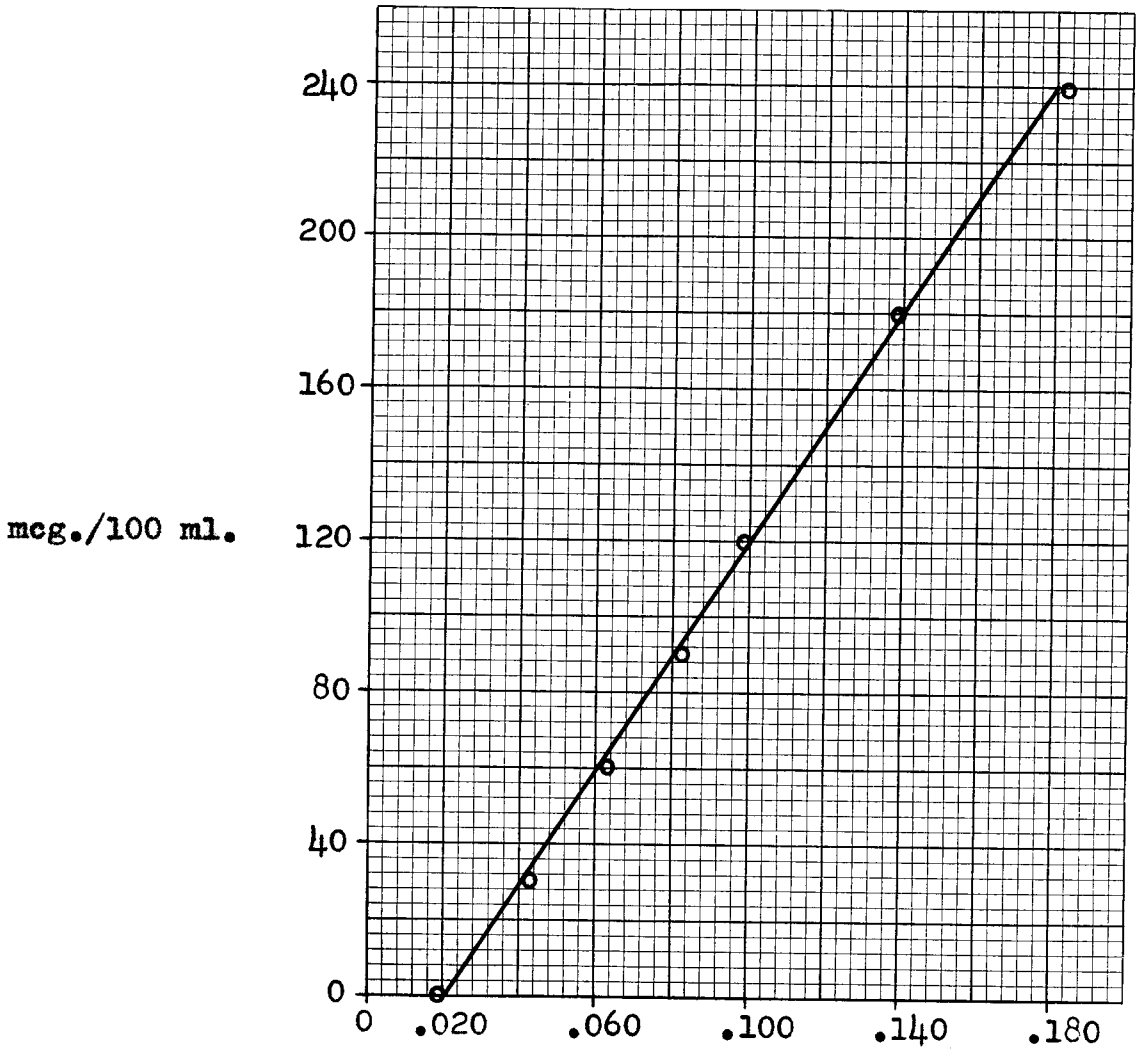


FIGURE 2.

STANDARD IRON CURVE

(2.8 adjusted). The coefficient of variation was 2.8% (Table II). The standard deviation of measurements on plasma was found to be plus or minus 3.46 and was determined by performing duplicate iron concentration measurements on each one of a number of plasma specimens and solving the equation, $\sigma = \sqrt{\frac{\sum d^2}{2n}}$ (Table III). In general, the permitted variations for clinical chemistry tests are limits or errors based on plus or minus 3σ or upon a coefficient of variation of plus or minus 2-3% (Page and Culver, 1961). The accuracy of the adopted method was determined for plasma by the performance of recovery experiments. A known amount of iron was added to one-half of a divided plasma sample and the divided samples quantitatively analyzed for iron. In this manner the percentage of added iron recovered could be determined and used to assess the accuracy of the method. The average amount recovered was 96.8% as shown in Table IV.

As indicated in Chapter III, determinations of plasma iron concentration were attempted on single mice, but sufficient plasma volumes could not be consistently obtained. Furthermore, the dispersion of normal values, as measured by standard deviation,

TABLE II

REPRODUCIBILITY OF RESULTS USING AN IRON SOLUTION

TABLE II

Concentration of Iron Solution
120 mcg./100 ml.

ALIQUOTS(n)	ABSORBANCE(x)
#1	.097
#2	.102
#3	.094
#4	.097
#5	.100
#6	.102
#7	.100
#8	.101
#9	.096
#10	.102
#11	.097

MEAN($\bar{x}$) = .099

$$\text{STANDARD DEVIATION}(\sigma) = \sqrt{\left[\frac{\sum(x - \bar{x})^2}{n - 1}\right]}$$

$$\sigma = \pm .0028$$

$$\text{COEFFICIENT OF VARIATION(C.V.)} = \frac{\sigma}{\bar{x}} 100\%$$

$$\text{C.V.} = 2.8\%$$

TABLE III

REPRODUCIBILITY OF RESULTS USING PLASMA

TABLE III

PLASMA SAMPLES(n)	PLASMA IRON CONCENTRATION OF DIVIDED SAMPLES mcg./100 ml. (a)	(b)
1	258	254
2	262	258
3	234	238
4	228	222
5	240	246

STANDARD DEVIATION(σ) $= \sqrt{\frac{\sum d^2}{2n}}$ where: d = difference between duplicates

$\sigma = \pm 3.46$

TABLE IV

RECOVERY OF ADDED IRON

TABLE IV

DIVIDED PLASMA SAMPLES	Fe ADDED mcg.	Fe FOUND mcg.	ADDED Fe RECOVERED - %
#1 Human a)	0	0.40	
b)	0.9	1.34	104
#2 Human a)	0	0.58	
b)	0.9	1.44	96
#3 Mouse a)	0	1.05	
b)	0.9	1.90	94
#4 Mouse a)	0	1.18	
b)	0.9	2.02	93
#5 Mouse a)	0	1.42	
b)	0.9	2.29	97
			Average = 96.8%

was considered too extensive to permit an adequate comparison of toxohormone activity utilizing the criterion of plasma iron concentration. The mean plasma iron level for single normal mice was found to be 246 micrograms per one hundred milliliters with a standard deviation of plus or minus 21.0 micrograms (Table V). The standard deviation and mean iron concentration of plasma pooled from seven groups of five normal mice per group were then determined. The mean was 249 micrograms per one hundred milliliters, and the standard deviation was plus or minus 10.63 micrograms which was considered acceptable for making adequate comparisons (Table VI).

The injection of twenty milligrams of the toxohormone-containing tumor fractions produced no apparent visual effects in the mice, nor did any of them die prior to the determinations of blood plasma iron concentration. As mentioned previously, two determinations were made on each sample of pooled plasma obtained from five similarly injected mice, and the results were averaged. Table VII shows the tumors from which the injectable materials were obtained and the average resultant plasma iron concentrations which ranged from 125 to 212 micrograms per

TABLE V

VALUES, MEAN, AND STANDARD DEVIATION OF PLASMA IRON CONCENTRATION AMONG SINGLE NORMAL MICE

TABLE V

MOUSE(n)	PLASMA IRON CONCENTRATION(x) mcg./100 ml.
#1	256
#2	262
#3	236
#4	225
#5	244
#6	210
#7	236
#8	284
#9	270
#10	230
#11	266
#12	228
#13	250

MEAN($\bar{x}$) = 246 mcg./100 ml.

$$\text{STANDARD DEVIATION}(\sigma) = \sqrt{\left[\frac{\sum (x - \bar{x})^2}{n - 1}\right]}$$

$$\sigma = \pm 21.0$$

$$\text{COEFFICIENT OF VARIATION}(\text{C.V.}) = \frac{\sigma}{\bar{x}} 100\%$$

$$\text{C.V.} = 8.5\%$$

TABLE VI

VALUES, MEAN, AND STANDARD DEVIATION OF THE IRON CONCENTRATION OF PLASMA POOLED FROM GROUPS OF FIVE NORMAL MICE

TABLE VI

GROUPS(n)	PLASMA IRON CONCENTRATION(x) mcg./100 ml.
1	236
2	252
3	240
4	264
5	244
6	248
7	262

MEAN($\bar{x}$) = 249 mcg./ 100 ml.

STANDARD DEVIATION(σ) = $\sqrt{\left[\frac{\sum (x - \bar{x})^2}{n - 1}\right]}$

$\sigma = \pm 10.63$

COEFFICIENT OF VARIATION(C.V.) = $\frac{\sigma}{\bar{x}}$ 100%

C.V. = 4.2%

TABLE VII

POOLED BLOOD PLASMA IRON CONCENTRATIONS OF GROUPS OF FIVE MICE INJECTED WITH TUMOR FRACTIONS

TABLE VII

TUMOR	AVERAGE PLASMA IRON CONCENTRATION mcg./100 ml. (a)	(b)	Average
1) Anaplastic carcinoma, bronchiogenic (279-63)	172	176	174
2) Adenocarcinoma, breast (6339-63)	200	204	202
3) Adenocarcinoma, colon (14187-63)	128	122	125
4) Adenocarcinoma, colon (9741-63)	164	170	167
5) Adenocarcinoma, colon (8719-63)	208	216	212
6) Adenocarcinoma, rectum (6673-63)	180	174	177
7) Adenocarcinoma, colon (282-63)	212	208	210

one hundred milliliters.

Periodic testing of the reagents revealed them to be stable throughout the investigation and capable of giving consistent results.

To compare the toxohormone activity of the seven malignant tumors employed, a graph was prepared which showed the resultant blood plasma iron concentrations of seven groups of five mice per group injected with the toxohormone-containing tumor fractions. The plus or minus 10.63 standard deviation of plasma iron concentration obtained from the groups of five normal mice was utilized in the preparation of the graph. This measure of dispersion includes both the normal spread of plasma iron values and the inherent laboratory errors. The graph depicts three distinct and separate groups into which the resultant plasma iron concentrations may be divided. The normal plasma iron concentration was also included for comparison (Figure 3).

A comparison of the degree of malignancy of each of the seven tumors was made. As indicated in Chapter III, values from one to three were assigned the four histopathological characteristics utilized; three indicating the greatest relative degree of

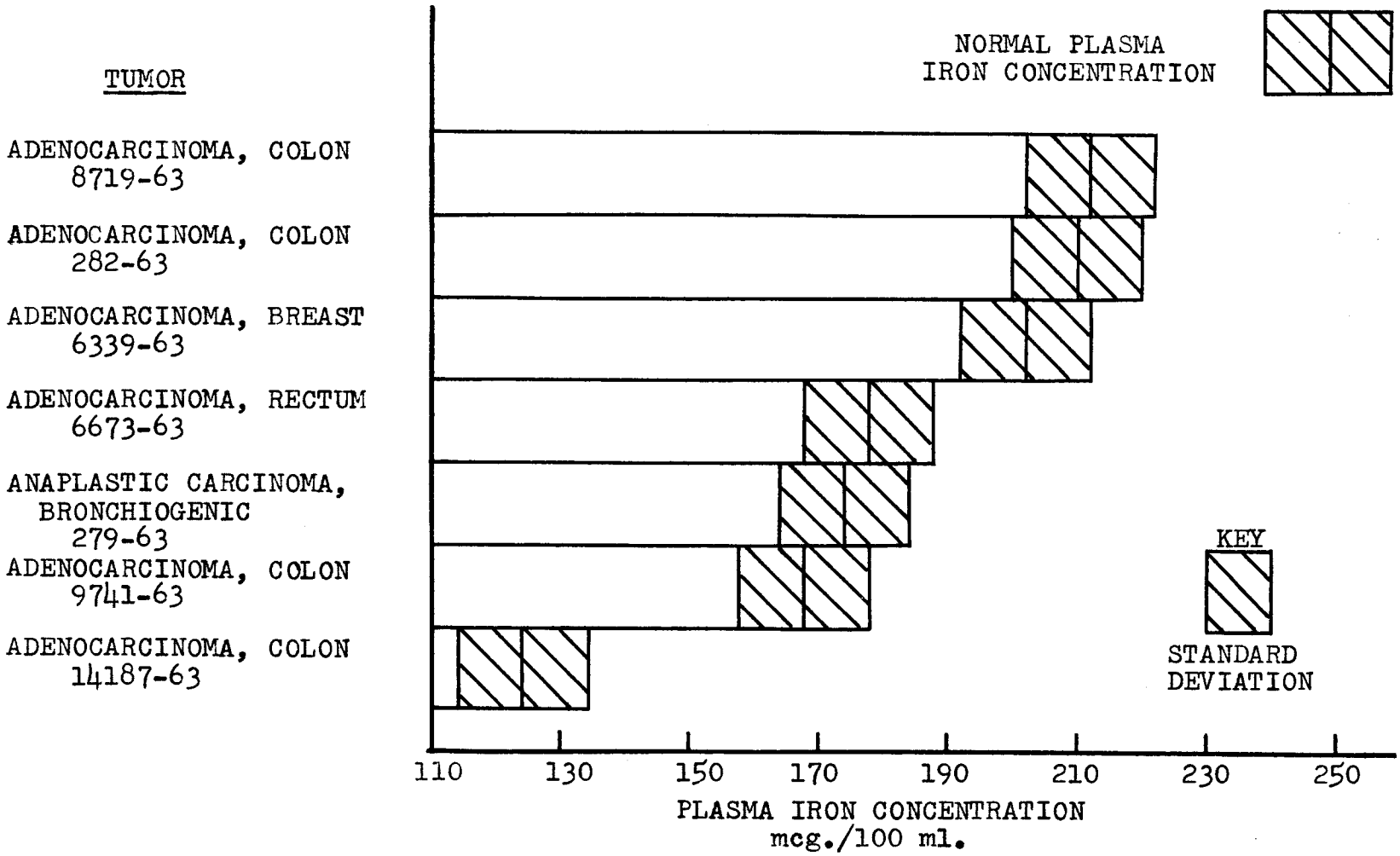


FIGURE 3.

TUMOR : PLASMA IRON CONCENTRATION

malignancy. The sum total of the histopathological values was then calculated for each tumor and the comparison was made. The adenocarcinoma of the breast (6639-63) and the adenocarcinoma of the colon (282-63) were found to be the most malignant. Two adenocarcinomas of the colon (14187-63 and 9741-63) were found to be the least malignant (Table VIII). As mentioned in Chapter III, tumors with the greatest sum total of histopathological values were considered to be the most malignant, and conversely, tumors with the least sum total of values were considered to be the least malignant.

TABLE VIII

COMPARISON OF DEGREE OF MALIGNANCY

TABLE VIII

TUMOR	A.	(M.I.)	M.	N.H.&S.	P.	TOTAL
Adenocarcinoma, breast(6339-63)	3	(12)	3	3	3	12
Adenocarcinoma, colon(282-63)	3	(13)	3	2	3	11
Anaplastic carcinoma, bronchiogenic(279-63)	3	(5)	1	2	3	9
Adenocarcinoma, colon(8719-63)	2	(5)	1	2	2	7
Adenocarcinoma, rectum(6673-63)	2	(6)	1	2	2	7
Adenocarcinoma, colon(14187-63)	1	(6)	1	2	1	5
Adenocarcinoma, colon(9741-63)	2	(4)	1	1	1	5

*A. - Anaplasia
(M.I.) - Mitotic Index
M. - Number of Mitotic Figures
N.H.&S. - Nuclear Hyperchromatism and Nucleocytoplasm Ratio
P. - Loss of Polarity

CHAPTER V

DISCUSSION

The method adopted for the quantitative measurement of iron in small volumes of blood plasma was found to be precise, accurate, and reliable for the investigation conducted.

Although the range or dispersion of normal plasma iron levels was reduced by pooling blood from groups of five twenty-five gram male mice, it is suggested that even larger groups of mice of this weight be used to further reduce the standard deviation if very precise comparisons of plasma iron values are to be made. For this investigation, however, the range obtained was suitable for adequate comparisons of the plasma iron levels produced by injectable fractions extracted from different tumors.

From results obtained it may be stated that intraperitoneal injections of twenty milligrams of the similarly prepared total protein-containing fractions extracted from each of the seven human malignant tumors tested produced definite decreases in the blood plasma iron concentrations of young male

mice after twenty-four hours. This is in agreement with the results of other investigators who have studied the effects of protein-containing tumor fractions on blood plasma iron concentration (Kampschmidt et al., 1959; Fujii et al., 1960; Ono et al., 1960). Also in accord with results obtained by these investigators is the fact that plasma iron appears to be quite sensitive to the substance causing its depression since the injection of only twenty milligrams of the crude protein-containing fraction used in this investigation produced substantial depressions; as much as 50 per cent in one instance, although the average was a 30 per cent decrease. However, this research problem was not specifically undertaken to show that a fraction of malignant tissue produces unique systemic changes when injected into laboratory animals. It has been well demonstrated by other investigators that a characteristic, toxic, protein or protein-like substance, called"toxohormone", is produced by malignant tissue, and is responsible for certain systemic changes, including that of decreased blood plasma iron concentration, when injected into laboratory animals. This knowledge has been used as a premise upon which this investigation

was based, and the findings presented which demonstrate a decrease in the normal plasma iron concentration after injections with malignant tumor fractions serve only to help elucidate this knowledge without attempting to prove its veracity.

Interpretation of the findings, in view of the purpose of this research, seem to justify the following conclusions. If the graph presented in figure 3 is analyzed, it is apparent that the resultant decreased plasma iron concentrations, induced in mice by similar injections of the protein-containing fraction of malignant tumors, can be divided into three distinct groups which are not connected by any limits of the standard deviations. It is therefore possible to divide the seven human malignant neoplasms investigated into three unrelated groups in reference to their effect on plasma iron concentration and to conclude that various individual tumors among the seven produce varying amounts of the substance which causes this effect, presumably toxohormone, or that the substance produced may be more systemically toxic in some instances. Furthermore, if the relative degree of malignancy of each of the tumors is compared with the related plasma iron depressions induced by

them, it generally appears that of the seven human malignant neoplasms tested, the most malignant tumors produced the least depression of plasma iron, and conversely, the least malignant tumors produced the greatest depression of plasma iron,(Table IX). If the bronchiogenic anaplastic carcinoma is excluded, being a different type of tumor than the other six, the picture is made even clearer. Therefore it would seem that the more malignant a tissue is the less toxohormone it produces. If this hypothesis is viewed in light of Greenstein's (1955) idea that a substance which is a toxin in a tumor due to abnormal production may only be a normal regulator of metabolic processes in normal tissues, it is conceivable that a tumor which more closely resembles normal tissue, although possessing an exaggerated metabolism, may produce greater amounts of this "regulator" (toxohormone) than the tumor with a high degree of malignancy which may be mostly adapted to growth and as such has lost or altered its biochemical mechanism to produce this "regulator" in large amounts. However, before this hypothesis could be stated as a theory for malignant tissue, greater numbers of the same type of malignancy from the same organ would

TABLE IX

COMPARISON OF RELATIVE DEGREE OF MALIGNANCY
WITH RELATED PLASMA IRON DEPRESSIONS

TABLE IX

TUMORS (Grouped According To Graph In Fig. 3)	AVERAGE PLASMA IRON CONC. mcg./100ml.	VALUES DENOTING RELATIVE DEGREE OF MALIGNANCY (From Table VIII)
Adenocarcinoma, breast(6339-63)	202	12(most malignant)
Adenocarcinoma, colon(282-63)	210	11
Adenocarcinoma, colon(8719-63)	212	7
(Anaplastic carcinoma, bronchiogenic)(279-63)	(174)	(9)
Adenocarcinoma, rectum(6673-63)	177	7
Adenocarcinoma, colon(9741-63)	167	5
Adenocarcinoma, colon(14187-63)	125	5(least malignant)

have to be compared. In addition, serial sections of the tumors should be made for histopathological examination since it is possible that a single section may not be representative of the whole. In this project only six tumors were of the same type of malignancy (adenocarcinoma) and only four of these were from the same organ (colon). The results obtained from these four alone demonstrate minimal but good evidence for the hypothesis presented.

No attempt was made to determine the total protein content of the various samples of malignancies employed since this was considered unnecessary for this particular investigation in view of the fact that the representative tumor samples were similarly prepared and equal amounts of the dried fractions injected, and it was not the purpose of this investigation to compare toxohormone content with total protein content.

In relation to this investigation, two other questions remain to be answered; is toxohormone characterized by different degrees of toxicity, and do different types of malignancies produce varying amounts of toxohormone or toxohormone of varying degrees of toxicity?

CHAPTER VI

SUMMARY AND CONCLUSIONS

An investigation was undertaken to compare the toxohormone content of human malignant tissues.

The following seven human malignant tumors were tested: four adenocarcinomas, colon; one adenocarcinoma, breast; one adenocarcinoma, rectum; and one anaplastic carcinoma, bronchiogenic.

The measurement of blood plasma iron concentration in mice after injections of similarly prepared fractions of these tumors served as the means by which comparisons were made.

Tumor fractions were prepared by homogenizing two-gram frozen tumor samples with acetone, centrifuging the suspension, and drying the resultant precipitate in air after decanting and discarding the supernatant.

For each of the seven malignancies tested, twenty milligrams of the dried protein-containing fraction were injected intraperitoneally into each of five twenty-five gram male albino mice and, by pooling plasma, two spectrophotometric determinations

of the collective blood plasma iron concentration made twenty-four hours after injection. Groups of five mice were utilized to decrease the normal dispersion of plasma iron levels and to obtain sufficient plasma volumes for the determinations.

A new spectrophotometric method of determining blood plasma iron concentration was developed for this study since other methods reviewed required larger volumes of plasma than could be obtained.

Normal blood plasma iron concentration and standard deviation were calculated for plasma pooled from five mice by performing the adopted spectrophotometric determination on seven groups of five normal mice per group, averaging the results, and applying a statistical method for determining standard deviation.

A comparison of the degree of malignancy of each of the seven tumors was made using the histopathological characteristics of: anaplasia, increased number of mitotic figures, nuclear hyperchromatism, and loss of cell polarity.

The relative degree of malignancy of each of the tumors was compared with the related plasma iron depressions induced by their injected fractions.

The following conclusions seem to be justified on the basis of the results of this study:

1) The adopted spectrophotometric method of blood plasma iron determination appears to be precise, accurate, and reliable for utilization in this type of investigation.
2) The average normal blood plasma iron concentration of seven groups of five twenty-five gram male albino mice is 249 plus or minus 10.63 micrograms per one hundred milliliters.
3) The blood plasma iron concentration of groups of five young male mice was significantly reduced twenty-four hours after single injections of twenty milligrams of similarly prepared toxohormone-containing fractions extracted from each of the seven human malignant tumors tested. Depressed plasma iron values ranged from 125 to 212 plus or minus 10.63 micrograms per one hundred milliliters.
4) The plasma iron level of groups of young male mice appears to be quite sensitive to the effects of injections of even small amounts

of crude toxohormone-containing tumor fractions. An average depression of 30 per cent was observed.

5) Various individual malignant tumors among the seven tested contained varying amounts of toxohormone or toxohormone of varying degrees of toxicity.

6) It generally appeared that of the seven malignant tumors investigated, the more malignant a tumor was the less toxohormone (or less toxic) it produced, and conversely, the less malignant a tumor was the more toxohormone (or more toxic) it produced.

BIBLIOGRAPHY

Adams, D. H. "The Mechanism of the Liver Catalase Depressing Action of Tumors in Mice," Brit. J. Cancer, 4: 183, 1950.

Adams, D. H. "Further Observations on the Liver Catalase Depressing Action of Tumors," Brit. J. Cancer, 5: 115, 1951.

Barkan, G., and Walker, B. S. "Determination of Blood Plasma Iron," J. Biol. Chem., 135: 37, 1940.

Batson, H. C. An Introduction to Statistics in the Medical Sciences, Minn.: Burgess Publishing Co., 1960.

Begg, R. W. "Tumor-Host Relations," Adv. Cancer Res., 5: 1, 1958.

Blumenthal, F., and Brahn, B. "Die Katalasewrikung in Normaler und in Carcinomatöser Leber," Z. Krebsforsch, 87: 436, 1910.

Boyd, William. A Text-Book of Pathology, Philadelphia: Lea and Febiger, 1953.

Brahn, B. "Weitere Untersuchungen über Fermente in der Leber von Krebskranken," Sitzber. kgl. preuss. Akad. Wiss., 478, 1916.

Buckley, L. Duncan. Cancer and Its Non-Surgical Treatment, New York: William Wood and Co., 1921.

Busch, Harris. Biochemistry of the Cancer Cell, New York: Academic Rress, Inc., 1962.

Day, E. D., Gabrielson, F. C., and Lipkind, J. B. "Depressions in the Activity of Liver Catalase in Mice Injected with Homogenates of Normal Mouse Spleen," J. Natl. Cancer Inst., 15: 365, 1955.

Endo, H., Sugimura, T., Ono, T., and Konno, K. "Catalase Depressing Tissue Factors: Toxohormone and Kochsaft Factor," Gann, 46; 51, 1955.

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, 1960.

Fukuoka, F., and Nakahara, W. "Mode of Action of Toxohormone. A Third Study on Toxohormone: A Characteristic Toxic Substance Produced by Cancer Tissue," Gann, 42: 55, 1951.

Fukuoka, F., and Nakahara, W. "Toxohormone and Thymus Involution in Tumor Bearing Animals. A Fourth Study on Toxohormone: A Characteristic Toxic Substance Produced by Cancer Tissue," Gann, 43: 55, 1952.

Fukuoka, F., and Nakahara, W. "Amino Acids and Toxohormone Synthesis. A Fifth Study on Toxohormone: A Characteristic Toxic Substance Produced by Cancer Tissue," Gann, 44: 1, 1953.

Greenfield, R. E., and Meister, A. "Effect of Injections of Tumor Fractions on Liver Catalase Activity of Mice," J. Natl. Cancer Inst., 5: 997, 1951.

Greenstein, Jesse P. Biochemistry of Cancer, New York: Academic Press, Inc., 1954.

Greenstein, J. P., and Andervont, H. B. "The Liver Catalase Activity of Tumor-Bearing Mice and the Effect of Spontaneous Regression and Removal of Certain Tumors," J. Natl. Cancer Inst., 2: 345, 1942.

Greenstein, J. P., and Andervont, H. B. "Note on the Liver Catalase Activity of Pregnant Mice and of Mice Bearing Growing Embryonic Implants," J. Natl. Cancer Inst. 4: 283, 1943.

Greenstein, J. P., Jenrette, W. V., and White, J. "The Liver Catalase Activity of Tumor-Bearing Rats and the Effect of Extirpation of the Tumors," J. Natl. Cancer Inst. 2: 283, 1941.

Hargreaves, A. B., and Deutsch, H. F. "The in vitro Inhibition of Catalase by a Tumor Factor," Cancer Res., 12; 720, 1952.

Heilmeyer, L., and Plötner, K. "Das Sermeisen und die Eisenmange Krankheit," Fisher. Jena, 1937.

Hoshizima, H. "Studies on the Mechanism of Toxohormone Function," Gann, 48: 239, 1959.

Kampschmidt, R. F., Adams, M. E., and McCoy, T. A. "Some Systemic Effects of Toxohormone," Cancer Res., 19:236, 1959.

Kitzes, G., Elvehjem, C. A., and Schuette, H. A. "The Determination of Blood Plasma Iron," J. Biol. Chem., 155: 653, 1944.

Lucké, B., Berwick, M., and Zeckwer, I. T. "Liver Catalase Activity in Parabiotic Rats with One Partner Tumor Bearing," J. Natl. Cancer Inst. 13: 681, 1952.

Nakagawa, S., Kosuge, T., and Tokunaka, H. "Purification of the Liver Catalase-Reducing Substance Occuring in Cancer Tissues, with Special Reference to Its Activity," Gann, 46: 585, 1955.

Nakahara, W. "A Chemical Basis for Tumor-Host Relations," J. Natl. Cancer Inst., 24: 77, 1960.

Nakahara, W., and Fukuoka, F. "Toxohormone: A Characteristic Toxic Substance Produced by Cancer Tissue," Gann, 40: 45, 1949.

Nakahara, W., and Fukuoka, F. "Purification of Toxohormone. A Second Study on Toxohormone: A Characteristic Toxic Substance Produced by Cancer Tissue," Gann, 41: 47, 1950.

Nakahara, W., and Fukuoka, F. "Dialyzable Form of Toxohormone. A Sixth Study on Toxohormone: A Characteristic Toxic Substance Produced by Cancer Tissue," Gann, 45: 67, 1954.

Nakahara, W., and Fukuoka, F. "Biosynthesis of Toxohormone. A Seventh Study on Toxohormone: A Characteristic Toxic Substance Produced by Cancer Tissue," Gann, 45: 77, 1954.

Nakahara, W., and Fukuoka, F. "The Newer Concept of Cancer Toxin," Advances Cancer Res., 5: 157, 1958.

Nakahara, Waro and Fukuoka, Fumiko. Chemistry of Cancer Toxin Toxohormone, Springfield: Charles C. Thomas, 1961.

Okamura, Y., Fujii, S., Kawachi, T., Kawanami, H., Kuwano, M., and Yamamura, Y. "Effect of Enzymatic Hydrolysis on Toxohormone Activities," Gann, 53: 365, 1962.

Ono, T., Ohashi, M., and Yago, N. "The Effect of Toxohormone on Iron Metabolism," Gann, 51: 213, 1960.

Ono, T., Sugimura, T., and Umeda, M. "Preparation of Potent Concentrates of Toxohormone Free from Nucleic Acid," Gann, 46: 617, 1955.

Ono, T., Sugimura, T., and Umeda, M. "The Purification of Toxohormone," Gann, 48: 91, 1956.

Ono, T., and Tomaru, T. "The Toxic Tumor Factor Depressing DPN Synthesis in Tumor-Bearing Animals," Gann, 50: 37, 1959.

Ono, T., Umeda, M., and Sugimura, T. "Porphyrin Metabolism in Tumor Bearing Animals. Free Porphyrin in Liver, Harderian Gland and Urine and the Effect Thereon of Toxohormone," Gann, 47: 171, 1956.

Page, Lot B., and Culver, Perry J. Laboratory Examinations in Clinical Diagnosis, Mass.: Harvard University Press, 1961.

Peters, T., Giovanniello, T. J., Apt, L., and Ross, J. F. "A Simple Improved Method for the Determination of Serum Iron. II," J. Lab. Clin. Med., 48: 274. 1956.

Rosenthal, E. "Untersuchungen über Fermente in der Leber und des Blutes bei Krebsmausen," Deut. med. Wochschr., 38: 2276, 1912.

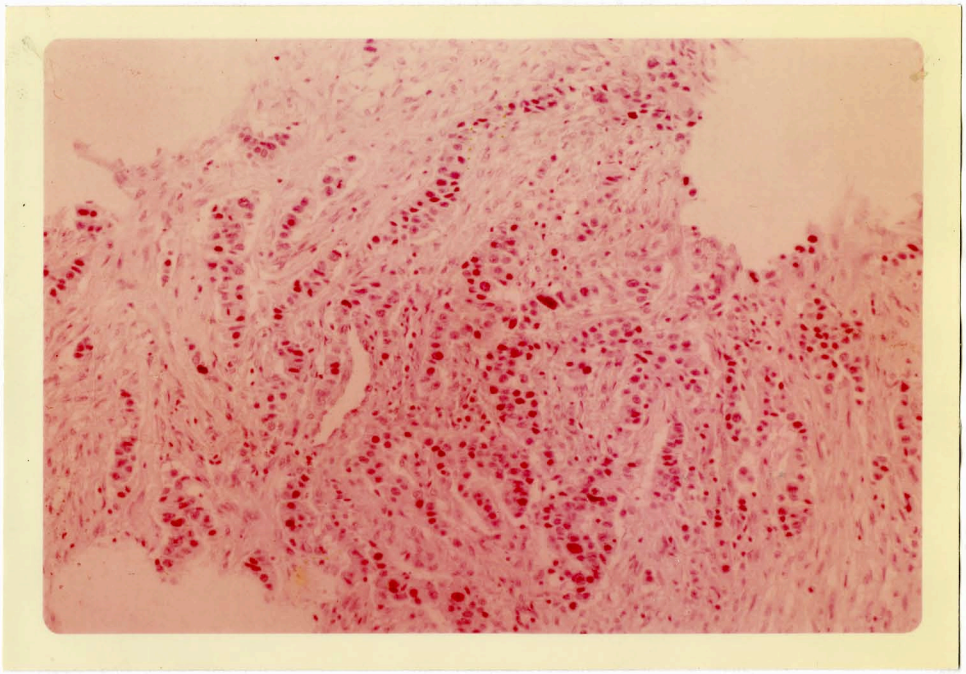
Sandell, E. B. Colorimetric Determination of Traces of Metals, New York: Interscience Publishers, Inc., 1950.

Sugimura, T., Ono, T., and Umeda, M. "Distribution of Radioactive Iron Injected into Tumor-Bearing Animals," Gann, 48: 159, 1957.

Photomicrograph 1

Adenocarcinoma, breast
6339-63

APPENDIX



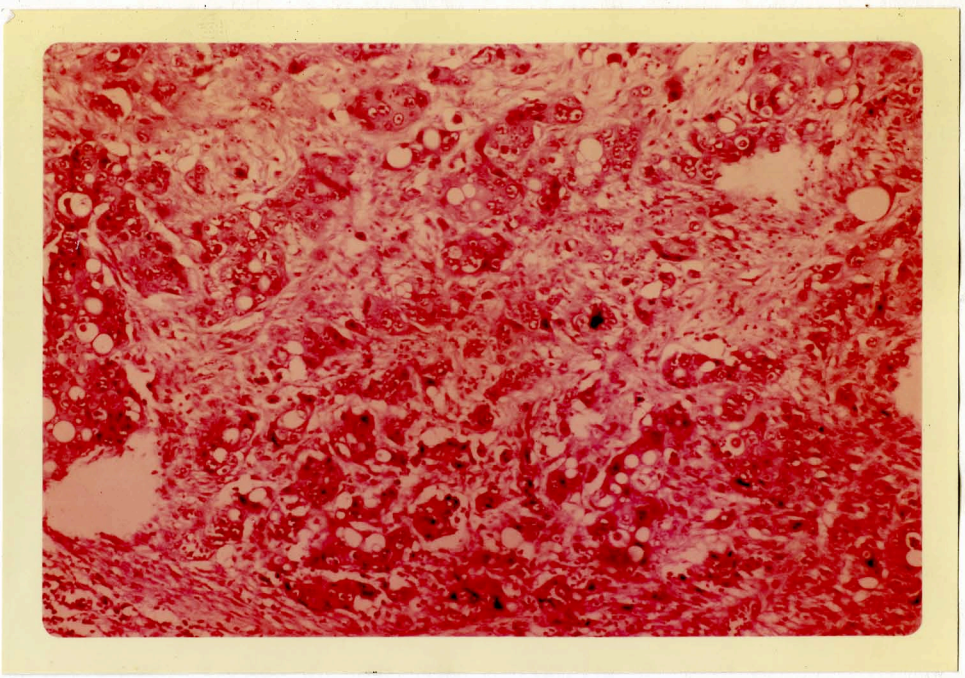
Photomicrograph 1

Photomicrograph 2

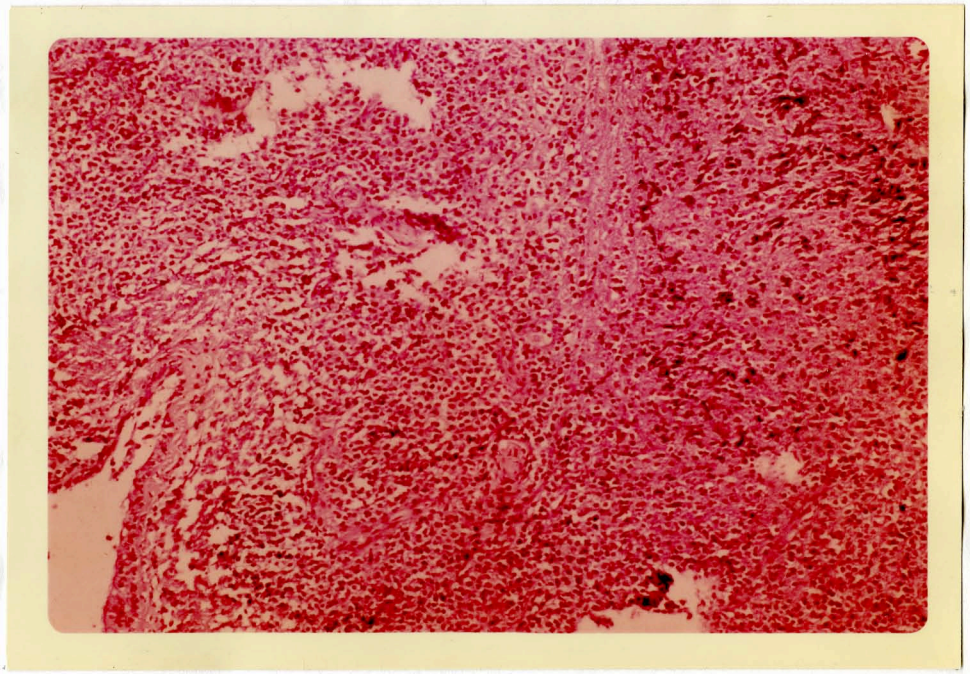
Adenocarcinoma, colon
282-63

Photomicrograph 3

Anaplastic carcinoma, bronchiogenic
279-63



Photomicrograph 2



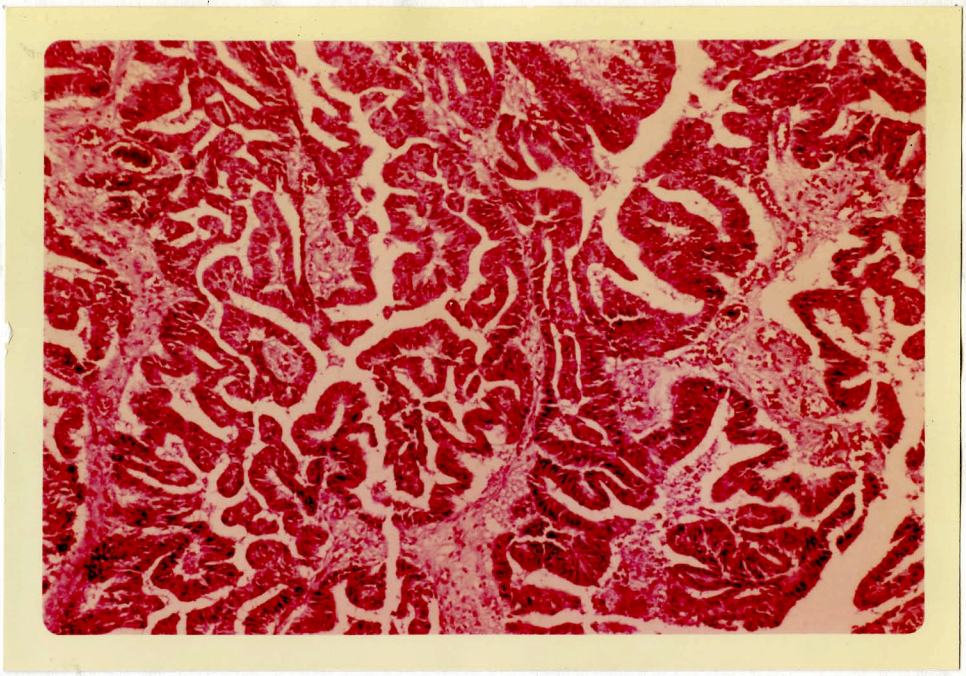
Photomicrograph 3

Photomicrograph 4

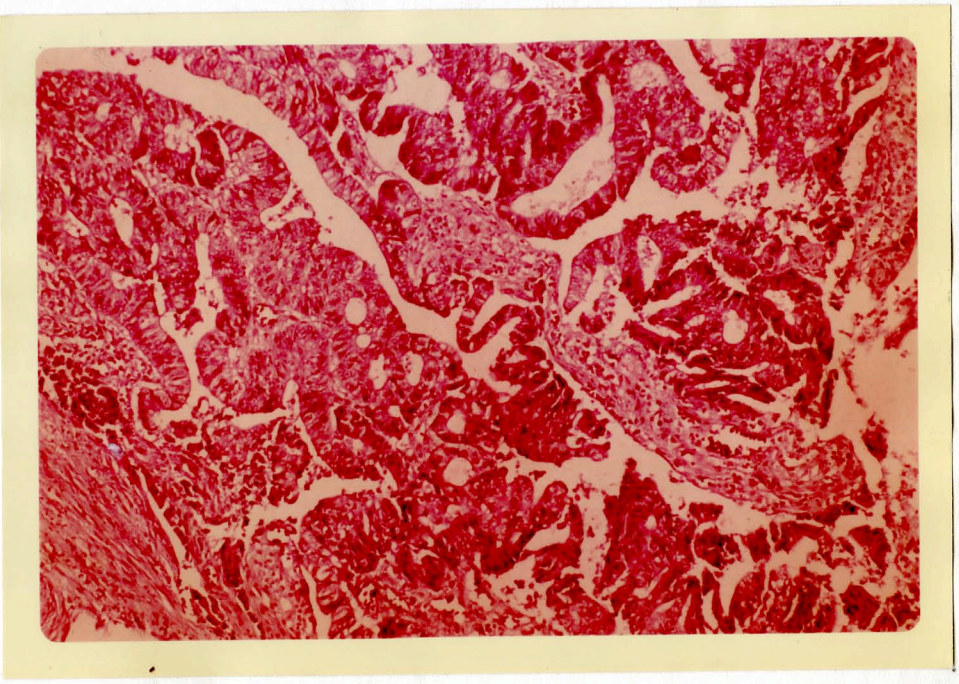
Adenocarcinoma, colon
8719-63

Photomicrograph 5

Adenocarcinoma, rectum
6673-63



Photomicrograph 4



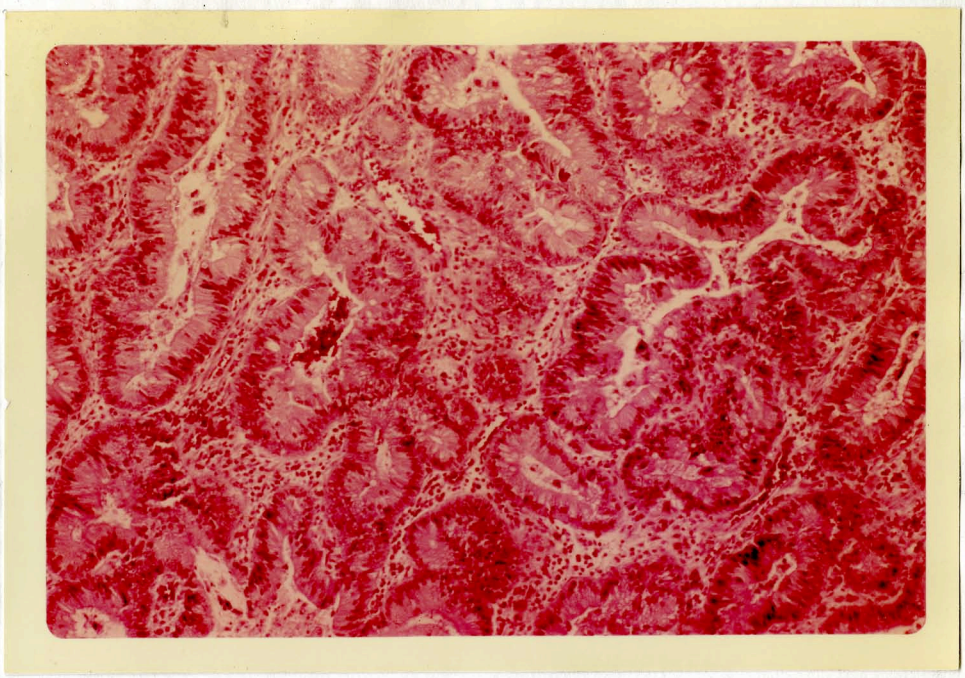
Photomicrograph 5

Photomicrograph 6

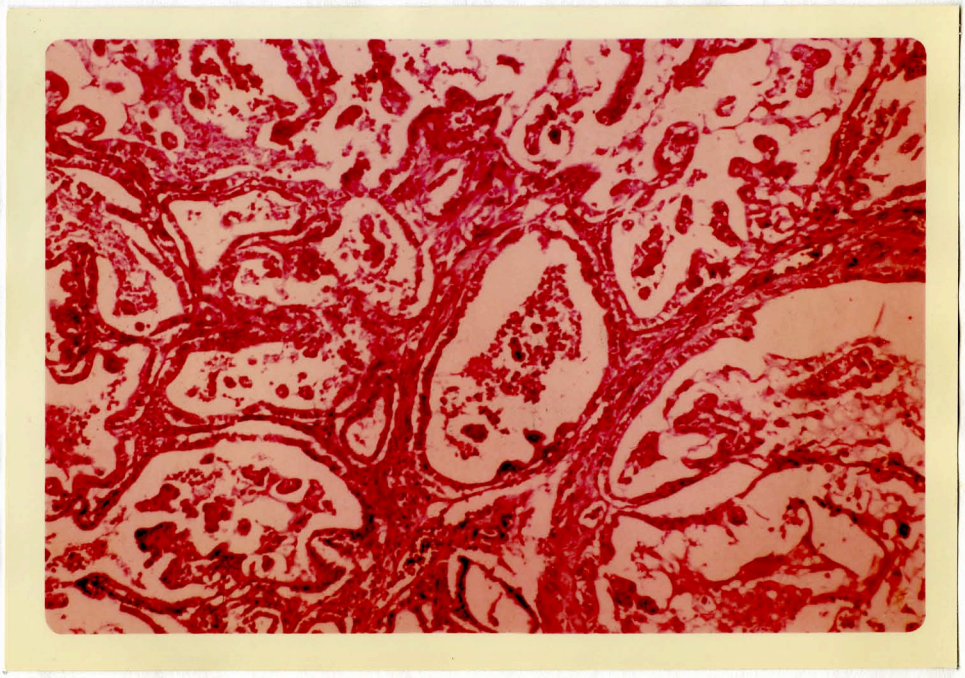
Adenocarcinoma, colon
14187-63

Photomicrograph 7

Adenocarcinoma, colon
9741-63



Photomicrograph 6



Photomicrograph 7

Reagents

1. Acetone

2. Powdered Pure Iron

3. Nitric Acid - 1:3
   (25 ml. concentrated HNO_3 / 75 ml. distilled HOH)

4. Hydrochloric Acid - 1:3
   (25 ml. concentrated HCl / 75 ml. distilled HOH)

5. Standard Iron Solution - concentration of 0.1 mg/ml
   a. 0.1 gm. of powdered pure iron dissolved by boiling in 50 ml. 1:3 HNO_3 and 50 ml. 1:3 HCl. Some material remained, probably ferric chloride, which dissloved in distilled HOH.
   b. Dissolved iron diluted to one liter with distilled HOH.

6. 25% Trichloroacetic Acid - CCl_3COOH
   (25 gm. TCA / 100 ml. distilled HOH)

7. 0.5% a,a'-Dipyridyl - (2,2'-Bipyridine)
   (0.25 gm. / 50 ml. distilled HOH)
   a. Approximately 3 ml. of 95% ethyl alcohol should be used to dissolve the material before distilled HOH is added.

8. Undiluted Thioglycolic Acid - (Mercaptoacetic Acid) - $HSCH_2COOH$

9. 25% Sodium Citrate - $Na_3C_6H_5O_7.2H_2O$
   (28.5 gm. / 100 ml. distilled HOH)

$$\frac{25 \text{ gm.}}{258.08 \text{ mol. wt.}} = \frac{x}{294.11 \text{ form. wt.}}$$

$$x = 28.5 \text{ gm.}$$

10. 0.1 molar Sodium Oxalate
    (1.339 gm. / 100 ml. distilled HOH)

APPROVAL SHEET

The thesis submitted by Laurence Lowell McCarthy has been read and approved by three members of the faculty of the Graduate School of Loyola University.

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.

Date May 11, 1964

Gustav W. Rapp, Ph.D.

Signature of Advisor
Gustav W. Rapp, Ph.D.