To the Graduate Council:

I am submitting herewith a thesis written by Patsy Ann Odom entitled "Copper and Cyotchrome Oxidase Distribution in Tissues and Serum of Mice Bearing Harding-Passey Melanoma." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry.

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COPPER AND CYTOCHROME OXIDASE DISTRIBUTION IN TISSUES AND SERUM OF MICE BEARING HARDING-PASSEY MELANOMA

A Thesis

Presented for the

Master of Science

Degree

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Patsy Ann Odom

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ABSTRACT

The objective of this investigation was to determine whether cytochrome oxidase and copper levels were changed in mice bearing Harding-Passey melanoma. Mice were divided into four groups (1) normal, no tumor, mice on regular diet (2) normal, no tumor, mice on copper-free diet (3) mice with Harding-Passey melanoma on regular diet (4) mice with Harding-Passey melanoma on copper-free diet.

It was observed that animals with tumor had increased serum copper levels. Copper-free diet decreased serum copper levels as expected, but presence of tumor increased serum copper levels, both with normal and copper-free diets. Differences in copper content between control and tumor bearing animals were not statistically significant for liver, brain, muscle, and skin. In animals raised on copper-free diet the copper contents of tumor was significantly decreased, while there was no copper depletion in liver, brain, muscle, and skin.

It was found that homogenates of liver and brain had lower cytochrome oxidase activity in animals with tumor (on both types of diet). Liver and brain homogenates from animals on regular diets with no tumor had pooled mean values of 4.41 and 3.54 μmoles O_2/min/gram, respectively. On the other hand, liver and brain homogenate from animals on regular diets with tumor had pooled mean values of 2.64 and 2.57 μ mole O_2/min/gram, respectively. In liver and brain on copper-free diets, with no tumor, cytochrome oxidase was found to be 3.96 and 2.89 (units same as above, respectively). On copper-free diet with tumor, liver and brain tissue had 1.11 and 1.84 μ moles O_2/min/gram activity, respectively. Statisti-

cal analysis supported the belief that presence or absence of tumor had an effect on cytochrome oxidase levels observed on liver and brain from animals on copper-free or regular diets.

A sucrose gradient method proved useful in obtaining tumor mitochondria, which upon examination by electron microscopy appeared to be free of melanin granules.

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CHAPTER I

INTRODUCTION

A. LITERATURE REVIEW

A search of the literature indicates that trace elements appear to undergo a change in concentration from normal levels in cases of cancer. Some of the trace elements may even exert an inhibitory influence on tumor development. Studies indicate that the distribution of copper in tissues and serum can become altered in tumor-bearing hosts. Records also show that in cancerous hosts activities of copper-containing enzymes, such as cytochrome oxidase and tyrosinase may deviate from normal levels.

There has been much interest in the relationship of nutrition to cancer. The new cell type appears to obtain its nutrients for growth from the host. Calorie restriction has been shown to influence the acceptance of virtually all mouse tumors studied. Tannenbaum (27) found that calorie restriction repressed the growth of spontaneous mammary carcinoma, skin tumors, and induced sarcomas, mainly during the developmental stage. Chronic caloric restriction resulted in many changes, such as absolute and relative weights of organs, mitotic activity, hormone production, and metabolism. The incidence of tumors decreased and time of appearance was delayed. He found any dietary change could have effects on the state of the host. This work also indicated that due to restricted diet there may be a loss of body weight of host, which may be responsible for low tumor occurrence.

Early work performed by Greenstein (10) investigated copper content in tumor-bearing mice and rats. He performed studies on tissues of rats with transplanted sarcoma, with transplanted hepatoma, and on tissues of mice bearing transplanted hepatoma and spontaneous mammary tumors. The copper content of livers from tumor-bearing rats was distinctly elevated over the level in normal rats. An increase of copper was also observed in livers of tumor-bearing mice.

The copper content of human carcinoma tissue has been determined. In 1942 Sandberg (20) found that excessive storage of copper occurs in livers in various forms of human cancer. He studied 146 cases of disease accompanied by secondary anemia. In some cases of cancer the increase in copper and iron storage was out of all proportion to the anemia. In some cases such increase in storage occurred in the absence of anemia. Even greater storage of copper and iron occurred in the liver and spleen when extensive metastasis was present.

Studies were also done to see if serum copper became altered in cases of carcinoma. Greenstein (12), using rats with Jensen sarcoma, showed that serum copper levels were slightly lower in tumor-hosts compared to normal hosts. Copper levels on whole blood from animals with tumors were significantly reduced compared to normal animals.

In 1953 Keiderling and Scharpf as cited by Schutte (22) showed that in cases of cancer such as chronic and acute leukemias,

lymphogranulomatoses, bronchial carcinomas, and carcinoma of the stomach and intestines, the blood-serum level of copper is greatly increased above the normal range.

Donath (6) reported elevated serum copper in 77 percent of 357 cases of cancer. In a thorough investigation on 209 cases of mammary, uterus, and rectum carcinoma, he found hypercupremia and accumulation in tumor tissue. Donath believed that the changes which he observed were a result of a defense response of the organism to the tumor.

Kolaric (15) examined serum copper levels in cases of solid tumors. He found that in 82 percent of bronchoid carcinoma cases, serum copper was elevated while in other types of carcinoma copper was not significantly different from normal serum copper levels. Included in his study were 25 patients with metastatic melanoma. In these patients serum copper levels were increased in 12 (48 percent) and normal in 13 (52 percent). With metastatic melanoma no significant increase in serum copper levels was observed compared to patients with no tumor.

Further research involved the changes in cytochrome oxidase levels in animals with tumors. Green and Haven (9) showed an <u>increase</u> in cytochrome oxidase activity in liver homogenates or mitochondria from livers of rats bearing Walker carcinoma 256. Higher cytochrome oxidase was found in animals with large tumors compared to animals with small tumors, tumor size being percent of total body weight. The increase in enzyme in liver homogenates or mitochondria could readily be seen after the tumor had reached 30 percent of the total weight of the rat. They examined several possibilities which

might have been responsible for the increase. Since it was observed in tumor-bearing rats that on dry weights and fat-free dry weights enzyme activity was increased when compared to normal animals, lipid differences between normal and tumor-bearing hosts did not explain the increase. The increase could not be explained by a greater mitochondrial permeability to substrate on the part of the tumor-bearer. In conclusion, they found no explanation for the increase of cytochrome oxidase in the livers of tumor-bearing rats. Finally they suggested that the increase may have been related to metabolic changes in the tumor-bearing rats since earlier experiments had reported that in rats with Walker 256 carcinoma there was an increase in caloric requirement and expenditure.

Later Haven's group (14) observed a decrease in cytochrome oxidase of liver in C3H mice with spontaneous mammary tumor. An average of 16250 natoms O /min/mg. dialysed dry weight for tumor free mice was found which differed from the average value of 13660 $\pm$ 1250 natoms O /min/mg. dialysed dry weight for tumor-bearing mice. Tumor-free mice had significantly higher cytochrome oxidase activity than rats of different strains. They believed that the difference in cytochrome oxidase activity between mice and rats was due to a difference in the size of the two species. Rats had received only transplanted tumors but mice had received transplanted and spontaneous tumors. In order to eliminate type of tumor growth as the reason for the increase, cytochrome oxidase levels were measured in mice with transplanted tumor and mice with spontaneous tumor. They found that cytochrome oxidase activity in mice was the same regardless of the type of tumor.

Further studies investigated copper dependent systems. Smyshlyaw (25) studied the activity of cytochrome oxidase, cytochrome c system, and copper content of mice during experimental leukemia. A reduction in cytochrome oxidase and cytochrome c was observed for all tissues of C 57/B/ mice with experimental leukosis. There was also a reduction of copper levels in spleen and kidney. An increase of copper was observed in muscle.

Greenstein (11) reported on the cytochrome oxidase activity in normal and neoplastic tissues of mice. Normal liver and brain tissue homogenates had values of 714 and 893 natoms of O /mg. tissue/hr. at 38°, respectively. Cytochrome c was added in excess ($1x10^{-4}M$) in the presence of p-phenylenediamine. Neoplastic tissue (melanotic tumor) had a cytochrome oxidase activity of 178 by the above same procedure. He summarized that cytochrome oxidase is among the least active enzymes in tumors. Greenstein also looked at cytochrome oxidase and copper in normal and neoplastic human tissues. Normal human liver was reported as having a cytochrome oxidase value of 250, activity expressed as above. Human melanoma had cytochrome oxidase activity of 26 - 31. Normal liver tissue and human melanoma tissue contained 0.8 and 0.4 - 0.5 milligrams copper per 100 grams wet tissues, respectively.

It has been reported that in cases of melanoma the activity of tyrosinase, a copper containing enzyme, becomes elevated. Melanoma has its start in pigment producing cells, melanocytes. These cells contain specialized organelles called melanosomes which form melanin.

Tyrosinase is bound to these specialized organelles. Tyrosinase is synthesized on ribosomes and transferred to melanosomes by way of the Golgi complex.

Using a density-gradient of sucrose, Baker (1) devised a scheme for separating mitochondria from melanin granules by centrifugation. It was found that the granules contained melanin and enzyme tyrosinase. From a large-granule preparation 94 percent of the total activity of tyrosinase was recovered in fractions 5 (melanosomes in early stages) and 6 (melanin granules) after centrifugation. He found mitochondria lack the enzyme tyrosinase. Until this work, granules were believed to have been "modified mitochondria."

Seiji (24) looked at the chemical composition of melanosomes and mitochondria in B-16 mouse melanoma and in Harding-Passey mouse melanoma. He found tyrosinase localized for the most part in the melanosome. Also the trace element, zinc, was reported in the melanosomal fraction.

Burnett (3) examined a number of patients at various stages of melanoma development. Tyrosinase activity was seen to increase as development of melanoma proceeded. Tyrosinase acts upon tyrosine to convert it into DOPA. An increase in DOPA was also found as the disease progressed. Treatment by surgery or chemotherapy temporarily lowered serum tyrosinase. Enzyme levels went back up before death. Burnett's technique, using radioactive tyrosine to assay tyrosinase, proved useful in detecting all but early stages of the disease.

In summary, past studies have indicated a fluctuation in copper and cytochrome oxidase activity in certain cases of cancer. Generally

there appeared to be an increase of copper storage in the livers of tumor-bearing hosts (rats, mice, and humans). Serum copper levels in tumor-bearing rodents appeared decreased while in most cases of human carcinomas an increase of serum copper was reported. Cytochrome oxidase was seen to be increased in livers of tumor-bearing rats. A decrease of cytochrome oxidase acitivty was observed in mice bearing spontaneous mammary tumors as compared to normal mice. Activity did appear to be species related: normal mice had higher activity than normal rats. Other studies conducted on tumor-bearing mice also indicated decreased cytochrome oxidase activity. Another copper containing enzyme, tyrosinase, has been reported to become elevated in serum in cases of melanoma.

In studies done on tumor-bearing animals, weight, size and overall metabolism have to be evaluated before interpreting the results.

B. PURPOSE OF STUDY

The present research was undertaken to determine the distribution of copper and cytochrome oxidase, a copper containing enzyme, in tissues and serum of animals bearing Harding-Passey melanoma. This study was intended to furnish the background information in another study on the effects of the administration of radioactive copper on Harding-Passey melanoma and the distribution of the radioactive copper in the sera and tissues of such mice fed normal and copper free diets.

CHAPTER II

MATERIALS AND METHODS

Animals

Female Balb/c mice were used in the experiment. Mice were divided into four groups of ten animals each, (1) normal, no tumor, regular diet, Purina Laboratory Chow and tap water (2) normal, no tumor, commercial copper-free test diet and glass distilled water, (3) mice with Harding-Passey melanoma on regular diet, and (4) mice with Harding-Passey melanoma on copper-free diet. Young mice were given the Harding-Passey melanoma subcutaneously in left axillary region by trocar technique. Transplants were made at three weeks of age for regular diet animals and at six weeks of age for copper-free diet animals, thus allowing at least three weeks for copper deprivation before transplant. Animals were sacrified at approximately 10-13 weeks of age.

Diets

Milk Diet: This diet was prepared daily. It consisted of one drop of formalin solution (prepared 1:10 dilution of stock formaldehyde 40% by volume, 37% by weight), 1 ml. 0.44 percent $FeSO_4 \cdot H_2O$ solution, and 100 ml. whole milk. (2)

Regular Diet (Purina Laboratory Chow): Copper content assayed by the manufacturer was found to be 18 ppm.

Copper-Free Diet: Pelleted copper test diet was obtained from United States Biochemical Corporation. Copper content assayed in this laboratory was found to be less than 0.2 ppm.

Measurement of pH

pH measurement were made at room temperature on a Fisher Accumet, Model 220 pH Meter, with glass and calomel reference electrodes, using standardization and calibration buffers for accuracy.

Centrifugation

Centrifugation of the blood for serum copper determination was carried out at room temperature in Coleman, Model 6-811, serofuge.

Spinning of tissue homogenates and separation of mitochondria were performed in a Sorvall RC2-B (Ivan Sorvall, Inc.) at 0°-4° C using the SS-34 head.

Sucrose gradient tubes were centrifuged in the swinging bucket rotor SW 39L of the Beckmon Model L ultracentrifuge at 0°-4° C.

The mitochondrial pellet for electron microscopy was prepared using the 50 Ti head in the Beckman Model L3- 50 Ultracentrifuge at 0°-4° C.

Spectrophotometric Measurements

Spectrophotometric measurements were made in Beckman DB spectrophotometer.

Precision Balances

Weighing was done on either a Sartorious Balance, Series 2250, or a Mettler Analytical Balance, Type B5.

Glassware

Glassware was made copper-free by soaking first with chromic-sulfuric acid cleaning mixture or sulfuric-nitric mixture (185 ml. sulfuric to 20 ml. nitric) and then rinsed several times with glass distilled water.

Protein Determination

Protein determination was done by modification of the biuret method (5). Crystallized and lyophilized albumin from bovine serum (Sigma Chemical Company) was used in the preparation of the standard curve. Dilutions of standard and of unknowns were made with 0.9 percent NaCl. After addition of proper amount if biuret reagent, standards and unknowns were read in quartz cuvettes on Beckman DB at 330 mu.

Preparation of H Media (8)

The following reagents were weighed out and delivered to a one liter volumetric flask:

Sucrose 23.96 grams

Mannitol 40.08 grams

HEPES (N-2-Hydroxyethylpiperazine-N^1-2-Ethanesulfonic acid) .48 grams

EGTA (Ethyleneglycol-bis-(B-Amino-Ethyl Ether) N,N^1 - Tetra acetic acid) .38 grams

The above reagents were dissolved and brought to 1000ml. with water. The solution was then adjusted to pH7.4. After proper pH adjustment .5 grams of BSA was added to the solution.

Preparation of Liver Mitochondria

1. Five mice which had been fasted overnight were sacrificed (livers were removed). Fat was trimmed and livers were then put into cold H media.

2. Removed tissue was washed in cold H media. Tissue was then weighed and then cut into tiny pieces for homogenization.

3. A 10 percent homogenate was then prepared (example 10g of liver in total volume of 100 ml.) Homogenization was done in a teflon glass homogenizer. For power homogenization, pestle was connected to 1/2" Black and Decker drill, rmp 550. Pestle was passed four to six strokes through homogenate.

4. Prepared homogenate was then centrifuged for 10 min. at 660xg in Sorvall RC2-B.

5. Supernatant was removed carefully and respun for 10 min. at 9000xg in Sorvall RC2-B.

6. The top white fatty layer was aspirated away. With a few ml. H media the fluffy layer was rinsed off the top of the pellet.

7. The pellet was gently resuspended with a cold test tube to half the original volume in Step 3 and then centrifuged at 9750 x g in Sorvall RC2-B for 10 min.

8. Repeat Step 6, then pellet was resuspended to 1/4 the original volume of Step 3. Spin in Sorvall RC2-B at 9750 xg for 10 min.

9. Repeat Step 6. Mitochondria was resuspended with a small volume of H media.

10. A protein determination was performed of mitochondrial pellet.

Preparation of 10% Tissue Homogenate (4)

Steps 1-4 of procedure for preparation of liver mitochondria were followed.

5. Supernatant was removed carefully and saved for cytochrome oxidase assay. Volume of supernatant was recorded. Pellet was discarded.

Separation of Tumor Mitochondria (1)

Large granule preparation

1. Tumor was removed and cut into tiny fragments.

2. 5-9 volumes of H media were added per gram unit weight tissue for homogenate preparation.

3. Homogenization was done in teflon glass homogenizer as in previous homogenizations.

4. Prepared homogenate was then centrifuged for 10 min. at 700xg in Sorvall RC2-B.

5. Pellet was discarded and supernatant was spun at 11,000xg for 10 min. in Sorvall RC2-B.

6. Supernatant was discarded and remaining pellet was resuspended in approximately 13 ml. H media.

7. One ml. of pellet resuspension was layered to sucrose gradient tubes which were spun for one hr. at 103,00xg using SW 39 L head in ultracentrifuge.

8. Six fractions were apparent. Tumor mitochondria appeared in fraction 3, illustrated in Figure 1.

Sucrose gradient tubes (1)

Eight different sucrose solutions were prepared with concentrations of 1.0M, 1.2M, 1.4M, 1.5M, 1.55M, 1.6M, 1.8M, and 2.0M. Measured amounts of sucrose were dissolved in 25 ml. Hepes, 2mM. Prepared sucrose solutions in Hepes were brought to pH 7.4. See Figure 1 for sucrose gradient and appearance of tube before and after ultracentrifugation. Solutions of sucrose were then layered into cellulose nitrate tube and allowed to set at least 16 hrs. before use.

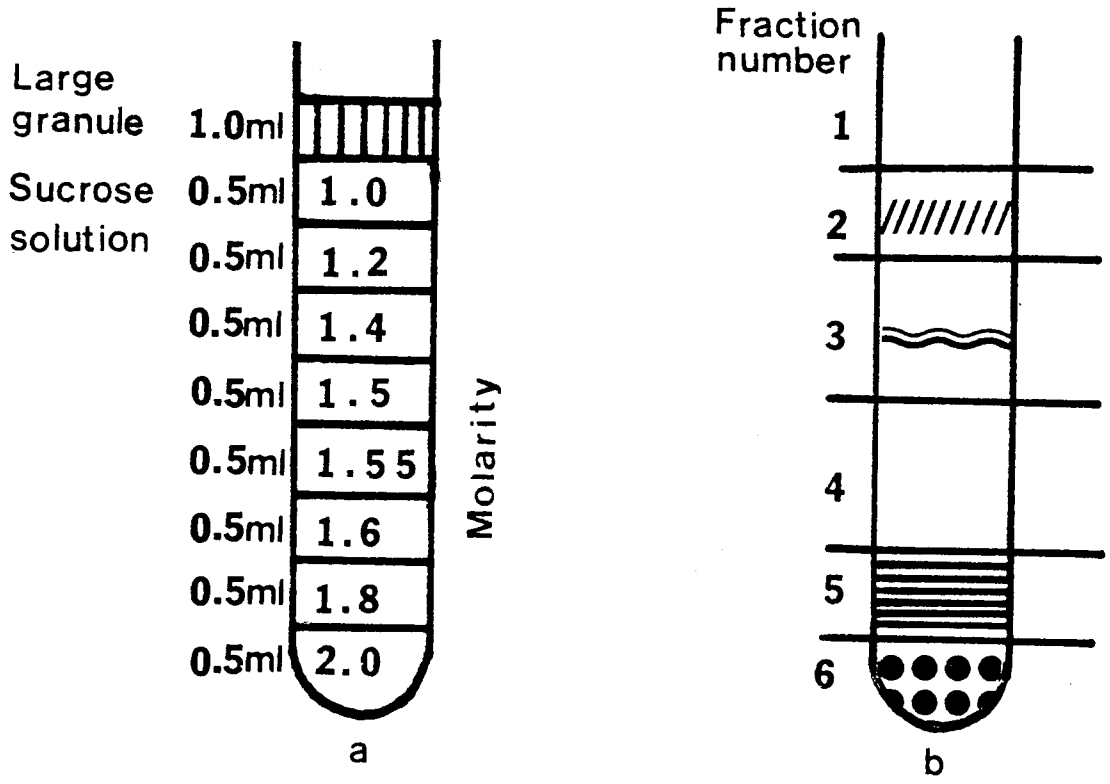


Figure 1. Sucrose Gradient Tube: a, gradient tube before centrifugation; large granule suspension (in H media) was added immediately before centrifugation. The gradient was prepared 16 hr. earlier; b, gradient tube after ultracentrifugation at 103,000 g for 1 hr. Fraction 3 contains mitochondria.

Preparation of Mitochondria for Electron Microscopy

Fraction 3 was removed from gradient and further concentration of mitochondria was achieved according to the flow diagram shown in Figure 2.

Fixation and Embedding Protocol (28)

Solutions needed:

Stocks:

Buffer: A. 0.2M NaH_2PO4

B. 0.2M Na_2HPO4

(23 ml. of A + 77 ml. of B = pH7.2 - 7.3

Note: Used at Final Conc. of 0.1M

Fixatives

Glutaraldehyde: 3.0% in 0.1 M $NaPO_4$ Buffer

Osmium Tetroxide: 2% 0.1 M NaPO4 Buffer

Wash and Storage: 0.2M sucrose in 0.1M $NaPO_4$ buffer

Resin Mixture: For Glass Knives

50 ml EPON 812 (Epoxy resin)
30 ml DDSA (Dodecenyl succinic anhydride)
20 ml MNA (Methyl nadic anhydride)
1 ml BDMA (Benzyledimethylamine)

Solvent-Resin Mixture: 1:1 mix of Resin and 100 percent acetone

Dehydrative: Aqueous acetone

50%, 70%, 95%, 100%.

Equipment

Screw cap or shell vials with #4 corks
Microfuge and tubes
Beem capsules
Welk razor blade
Pasteur pipettes (long), beakers and flasks 4
Stirring apparatus and cylinder
Dessicator, silica gel and vial racks

Preparation of Cell Components for Electron Microscopy

All procedures were carried out in cold environment

(0° to 4°C)

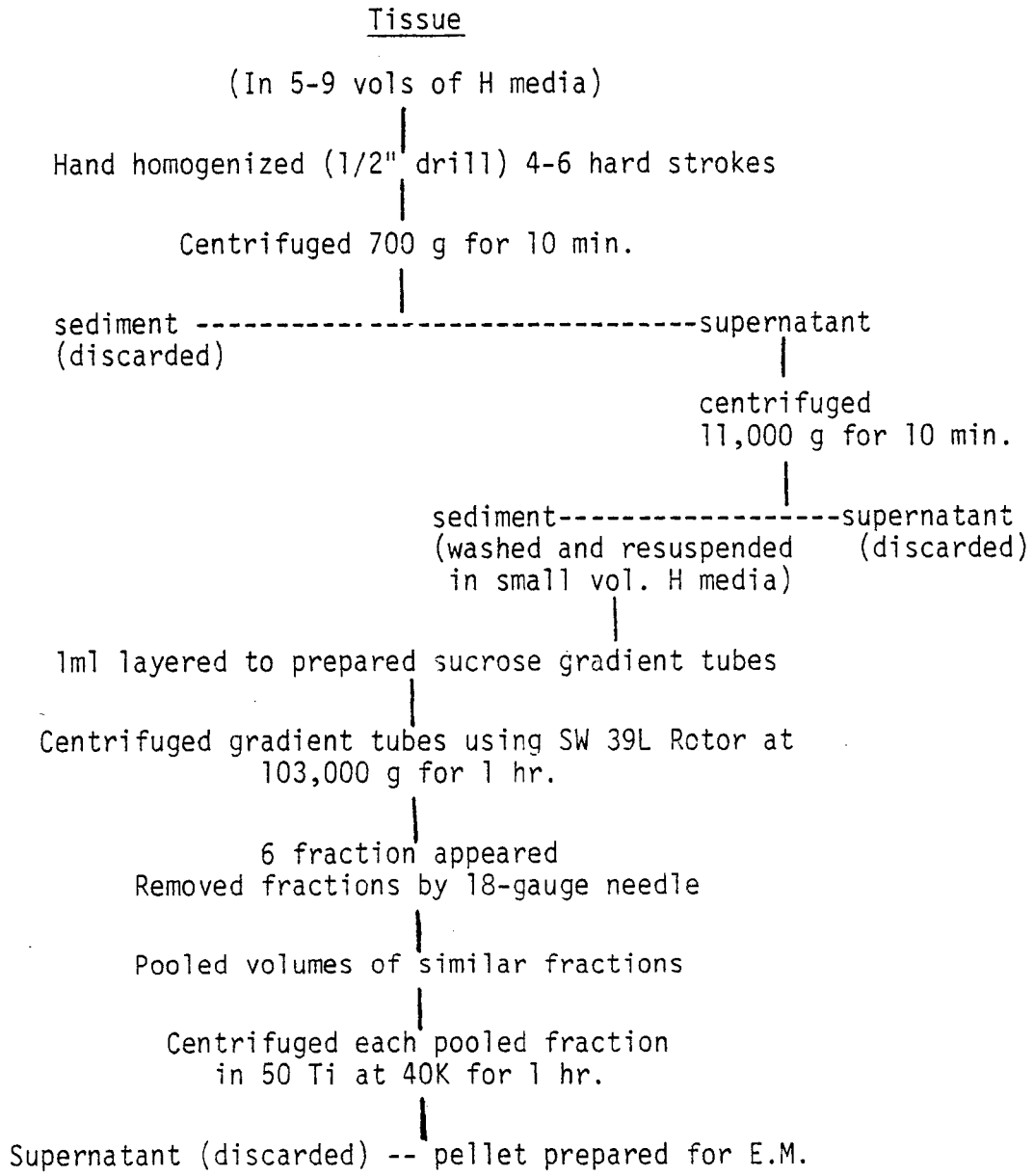


Figure 2. Preparation of cell components for Electron Microscopy. Modification of method of Seiji (23) by Dr. Alfonso Carabez.

Procedure:

Fixation 1. Twenty to 25 µl of a mitochondrial pellet were placed in microfuge tube containing 300 µl of gluturaldehyde (glut.). This was spun 45 sec. in Coleman serofuge - and was allowed to stand 0-4°C for 2 hrs.

2. Gluturaldehyde was removed by shaking (leaving a small amount in tube to keep specimen wet) and was replaced with 3 changes of "wash and storage buffer" and was stored overnight in last change at 0-4°C.

3. In the morning "wash and storage buffer" was replaced with 2.0% OsO_4 (osmium tetroxide) and was allowed to stand for 2 hrs. at 0-4°C.

4. A Pasteur pipette was used to remove OsO_4 and the following reagents were added:

50% acetone cold - 10' x 2. Removed by shaking.
70% acetone cold - 10' x 2. Removed by shaking.
95% acetone cold - 10' x 2. Removed by shaking.

Cold 100% acetone was added for 30' and specimens were removed from ice and cold acetone was removed; 100% acetone at room temperature was added for 30'.

Note: During this time resin and/or solvent resin mix was prepared. Glass vials were properly labeled; vial was filled with 100% acetone at room temperature.

5. To Dislodge Pellets: solid end of plastic from microfuge tube was cut with razor blade leaving a thin wall of plastic

between bottom of tube and specimen. Using blunt object, pellet was dislodged from bottom of tube. A cut was made above the bottom of tube so that the hole was sufficiently large to squeeze out pellet. Tube was held upright to allow the pellet to settle to the opening, so that it could be squeezed out in the first drop into a vial filled with 1:1 mixture of acetone and resin. The pellet was allowed to impregnate overnight in dessicant but without vacuum.

6. When half volume in vials had been reached, the solvent was mostly gone, Beem capsules were prepared with resin and then properly labeled. Specimen was transferred to capsule in a minimum amount of resin mixture. After the pellet had settled to bottom of Beem capsule, the specimen was placed in 60°C oven overnight to polymerize the resin.

7. After allowing the resin to harden, the capsule was cut away and the specimen was trimmed and cut for sections with a fresh prepared glass knife.

Cytochrome Oxidase Assay (Polarographic) (21)

Cytochrome oxidase activity was determined using the Clark oxygen electrode. (7)

Determinations were made on liver mitochondria, tumor mitochondria, and tissue homogenates. Figure 3 illustrates typical cytochrome oxidase activity.

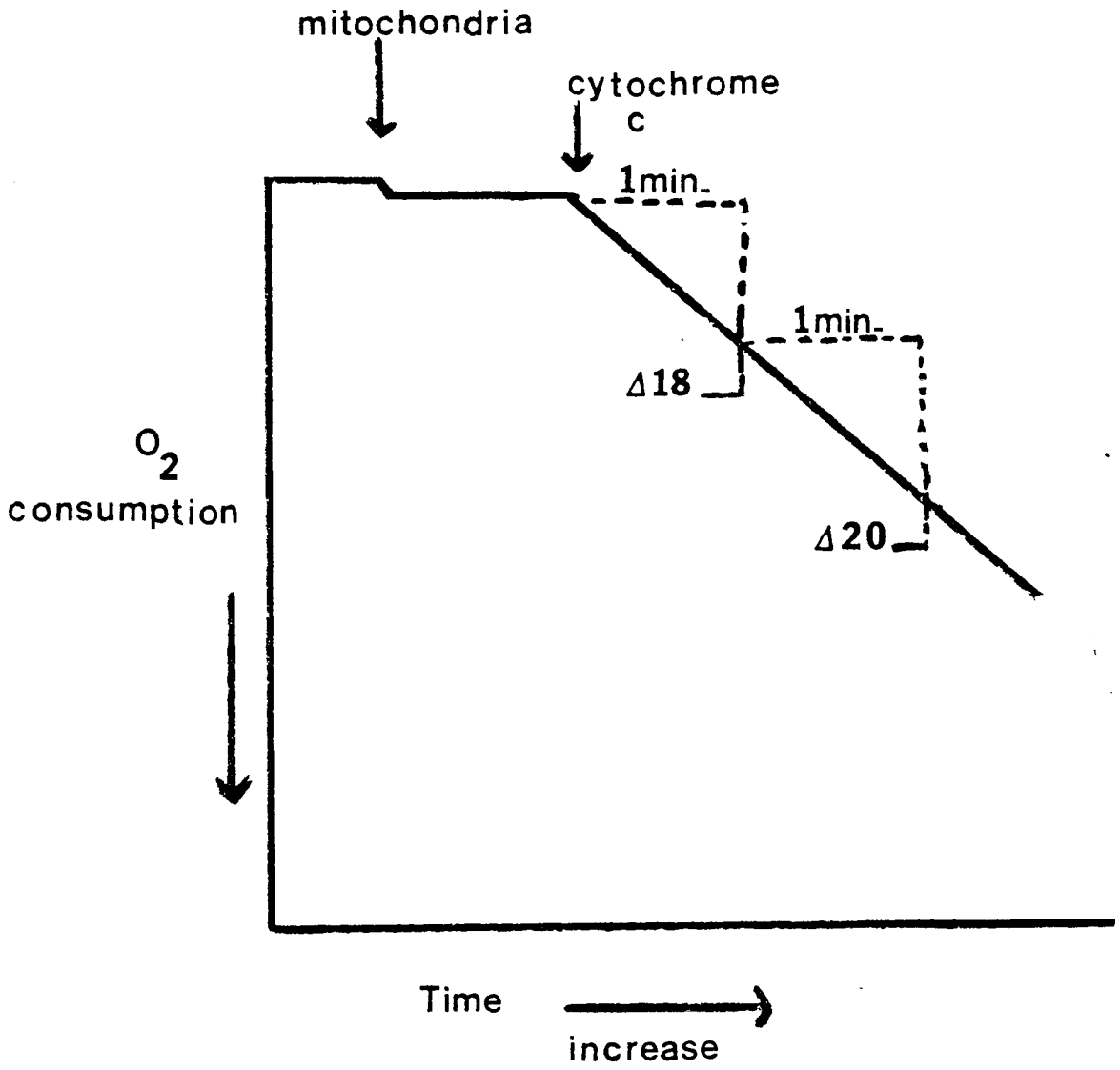


Figure 3. Typical cytochrome oxidase assay. Units change per minute was observed. In this particular assay, liver mitochondria were from tumor-bearing animals on commercial copper-free diet.

Reagents	Volumes
Phosphate buffer, 0.1M, pH 7.4	1.5 ml.
Mammalian cytochrome C, Sigma type III, 1.2mM	0.05 ml.
Na Ascorbate 30 mg/2ml. (freshly made)	0.1 ml.
TMPD (2.8 mg/2ml.) (freshly made)	0.1 ml.
Water + Enzyme	0.25 ml.

Procedure 1. Electrode was standardized at zero O_2 with dithionite and at 96 divisions with air-saturated buffer (100% = 960 natoms O_2 at saturation).

2. Buffer, water, Na ascorbate, TMPD ($N_1N_1N^1N^1$ tetramethyl-p-phenylenediamine) was added to the respiratory chamber; then the enzyme was added. After equilibration, the reaction was started by addition of cytochrome C.

Calculations of cytochrome oxidase activity

in tumor and liver mitochondria

units change per min. x 10 = natoms O

$$\frac{\text{natoms O}}{\text{mg/ml x vol. ml.}} = \text{natoms O/min./mg}$$

tissue homogenate

$$\frac{\text{natoms O}}{\text{grams of wet weight x } \dfrac{\text{vol. amt. used in assay}}{\text{total vol. tissue homogenate}}} = \text{natoms O/min./gram}$$

$$\frac{\text{natoms/O/min./gram}}{2} = \text{nmoles } O_2\text{/min./gram}$$

Then convert

$$\frac{\text{nmoles } O_2\text{/min./gram}}{1000} = \mu\text{moles } O_2\text{/min./gram}$$

Serum Copper Determination (19)

A protein-free filtrate was made of the sample. This filtrate was then aspirated into air-acetylene flame, where the metal ions are converted into atomic vapor. Ground state radiation can be absorbed by the atomic vapor (proper wavelength being produced by a hollow cathode consisting of cathode made of copper). The amount of absorption is proportional to the amount of copper present in serum.

Reagents:

1. Copper stock standard; 1000 µg Cu^{++}/ml. (CuO in 0.1N HNO_3)
2. Sodium/Potassium: 140 mEq/Na., 5 MEq K/L
3. 8% Trichloracetic acid

Intermediate Standards:

1. Intermediate Std. #1: 100 µg Cu/ml.
   Ten ml. stock std. (1000 µg/ml.) was diluted to 100 ml. with distilled water.
2. Intermediate Std. #2 1 µg Cu/ml.
   One ml. of 100 µg Cu/ml. (inter. std. #1) was diluted to 100 ml. with sodium/potassium solution.

Working solutions:

1. Blank: Twenty-five ml. of sodium/potassium solution were diluted to 50 ml. with 8% TCA
2. Working Standard: Twenty-five ml. of 1 µg Cu/ml. (Inter. Std. #2) was diluted to 50 ml. with 8% TCA.

Equipment

1. I L (Instrumentation Lab) 353 Atomic Absorption - Emission Spectrophotometer
2. Boling burner
3. Copper hollow cathode lamp

Procedure:

1. One-half ml. serum and 0.5 ml. control (Cation Cal, Dade American Hospital Supply) were mixed with 0.5 ml. of glass distilled H_2O and 1.0 ml. 8% TCA.
2. Mixture was allowed to stand 5 minutes and then centrifuged.
3. The supernatnat was transferred by pipette into a small plastic tube.
4. The blank was aspirated and used to zero the instrument.
5. The standard was aspirated and used to set the instrument at 100 µg% Cu.
6. The supernatant was aspirated from the sample, and the answer was read directly in µg% Cu in serum.

Wavelength setting: 324.7 (nm)

Lamp current: 5mA

Slit width: 320 µ

Determination of Copper in Tissue

Digestion Procedure (29)

The first step in the digestion procedure was to weigh out and record the weight of the sample used. The appropriate amount of

sample was about 1-3 grams. Digestion was carried out in 50 ml. pyrex tubes. The digestion was initiated with the addition of 3 ml. concentrated nitric acid. Additional nitric was added when necessary. Total volume of nitric acid was recorded. Several samples were digested at one time. Tubes were heated by I.T.E. Quartz tube radiant heaters (Ceilheat, Inc.). The digestion was done in the acid hood with fan turned on. An acid blank was also included in each series of digestions. Initial heating was about 60°C for 30 minutes or until brown fuming decreased. Heat was increased to 120°C after fuming. Tubes were at higher temp. for about 2 hrs. (never allowing complete dryness to occur in the tubes). At the end of digestion the tubes were allowed to cool and glass distilled H_2O was used to bring the sample and blank dilution up to a known volume of 10 ml.

Preparation of Standard Curve

Appropriate standards were made by dilution of the Copper Reference Solution, 1,000 ppm copper oxide dissolved in dilute nitric acid (Fisher Scientific Company). Standards included concentrations of 0.5ppm, 1ppm, 2ppm, and 5ppm. Prepared stds. were assayed as samples and deflection in inches as measured on recorder chart paper was plotted vs. known concentrations. See Figure 4 (Cu std. curve).

Assay Procedure

The Beckman Atomic Absorption Spectrophotometer, double beam, Model 485, was used for copper determination of tissues. The instrument

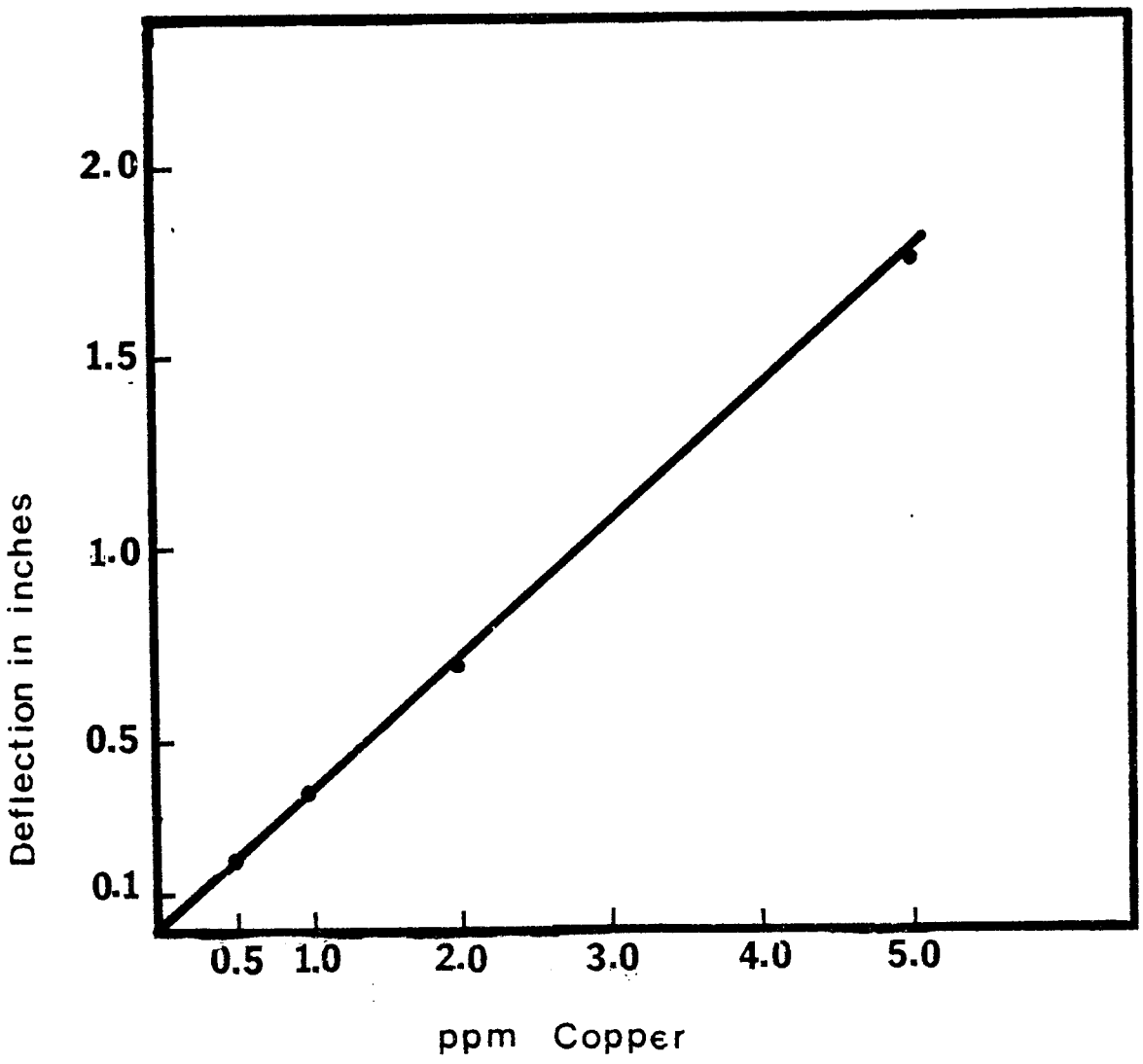


Figure 4. Standard copper curve

was prepared for startup and operation according to Lab Manual for machine operation (18). Prepared standards, blanks, and unknown solutions were aspirated and sprayed into an acetylene flame. A hollow cathode tube emits a high intensity light at specific wavelength (324.8). This beam passes through the sprayed sample and into a monochromator. A photomultiplier amplifies the signal coming from the monochromator. The energy absorbed by the sample is directly proportional to the concentration of element in the sample. Figure 5 shows recorder chart output for copper.

Statistical Design and Analysis

Since disproportionate and unequal subclass numbers were present in these data, analysis of variance was accomplished by least squares methods.

Batch placed within health (reference to presence or absence of tumor) and diet, was the appropriate error term for testing effect of diet x health and health x diet correlation.

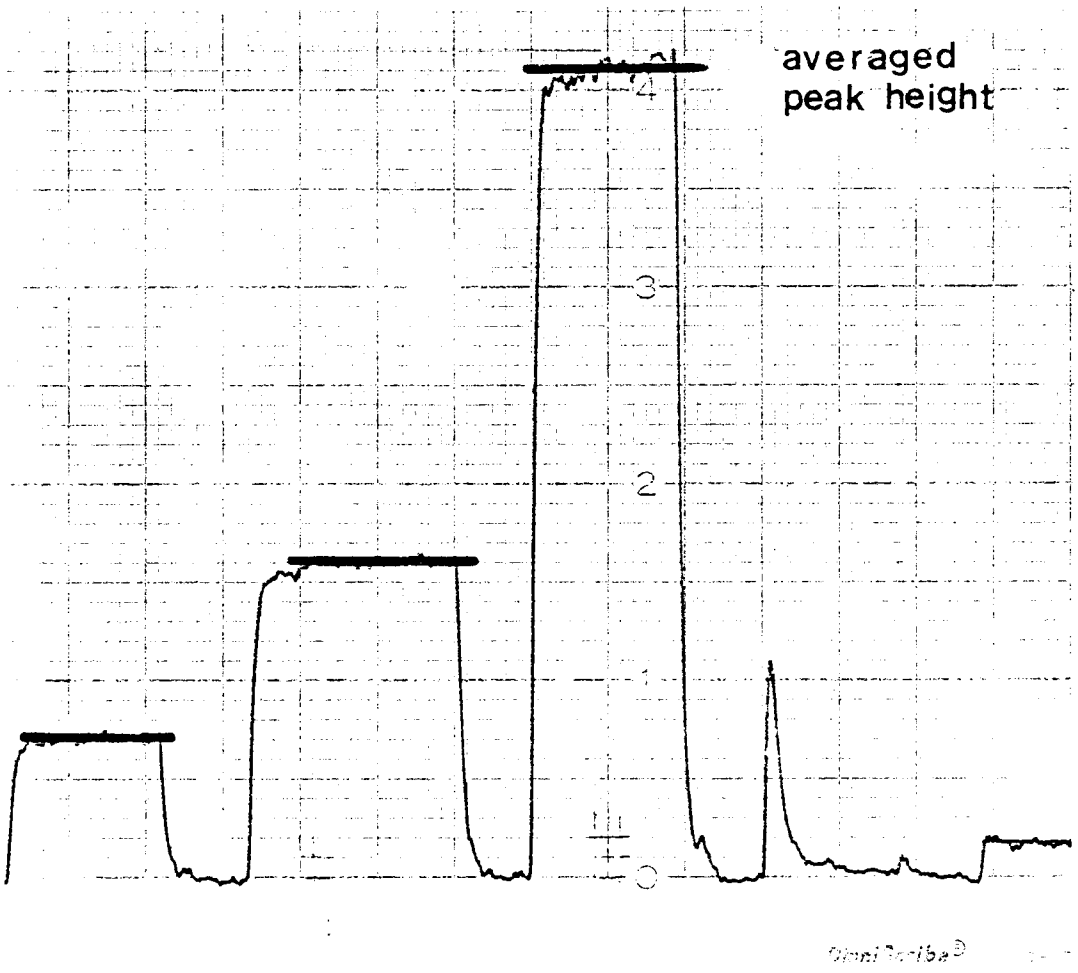


Figure 5. Sample of recorder chart output for Copper. In Atomic Absorption Spectrophotometric assay, the peak height is of interest, not the area of the peak.

CHAPTER III

RESULTS AND DISCUSSION

Experiments on Effects of Copper Deprivation

A. Effects on Tumor "take" and "growth"

At the very beginning of the research, experiments were set up to observe the effects of copper deprivation on tumor "take" and "growth".

Implants were made in one group (b-1) of mice after copper deprivation had been achieved and in another group (b-2) of mice at the start of copper deprivation. The control group which recieved tumor implants were placed on a regular diet, a-1 and a-2.

Observations were then made on tumor "take" and "growth". Groups consisted of five animals each. Table I shows the results of this study.

This preliminary study seemed to indicate the following (1) "take" and "growth" were more apparent in animals which had been placed on regular diet (designated groups a-1, a-2, 8 takes for 10 animals) as compared to those which had been deprived of copper (groups b-1, b-2, 8 takes for 10 animals). Please note total take did appear the same; however, there was a definite difference in size of tumors. It appeared animals on regular diets had larger tumors and (2) group b-1 which had been deprived of copper a few weeks before implants seemed to have even less "take" and "growth" (3 takes for five animals) as compared to other copper deficient group b-2 which had been deprived of copper at the time of the implant (5 takes for 5 animals).

TABLE I

TUMOR "TAKE" AND "GROWTH"

Group [1]	Date Born	Diet Starting Date	Elapsed Time[2]	Tumor 2 Aug. # of Takes	Tumor 9 Aug. volumes	Tumor 16 Aug. volumes	Tumor 4 Sept. volumes
			days		cm^3	cm^3	cm^3
a-1	6 June	Regular 27 June	17	2	(.25, .5, 1, 0, .25) av = .5	(.25, 1.13, 4, 0, 3) av = 2.1	(5, 12.5, 18, 24.5, 0) av = 15
b-1	6 June	Cu-free 28 June	16	0	(0, 0, 0, 0, 0)	(.25, 0, 0, 0, 0)	(7.5, 7.5, 8, 0, 0) av = 7.7
b-2	20 June	Cu-free 12 July	1	2	(.1, .25, .25, 0, .1) av = .18	(1.5, .25, 1.5, 1.5, 0) av = 1.2	(4, 3, 7.5, 6, 3) av = 4.7
a-2	20 June	Regular 12 July	1	3	(.25, .98, .7, .25, 0) av = .55	(1.5, 3.8, 5, .75, 0) av = 2.76	(9.4, 22.5, 12.5, 12.5, 0) av = 14.2

1 Five animals per group

2 All animals received the transplant on July 13; Time is between start of diet and transplant

B. Effects on State of Host

Included in the preliminary study were growth curves taken on normal mice on regular diets and on milk diets, naturally Cu-free. Weights were taken and recorded every day or two at least once a week for approximately 11 weeks. See Figure 6 for growth curves of animals on these two different diets. Tumor "take" and "growth" studies were performed on these diets. The weight gain for copper deprived group (80 grams) was much less than the weight for regular group (115 gram).

Results observed appeared to be consistent with the literature (27) which reports failure to gain weight by host may be responsible for low tumor occurrence.

Later a commercial copper-free test diet (one used in all the remaining experiments mentioned in this thesis) was compared to the Purina Laboratory Chow. Figure 7 shows the growth curves using these diets. It was observed that commercial copper-free test diet had no apparent effect on weight gain of normal host when compared to weight gain on regular diet. This observation suggested that failure to obtain normal growth rate was not a major factor for results seen in later experiments.

Results of Serum Copper Determinations

Serum copper levels were determined on groups of animals (5 to 10) with or without Harding-Passey melanoma which were also on regular or copper-free diets. Table II tabulates serum copper data. Sera from animals in each group were pooled and duplicate assays were made on each batch.

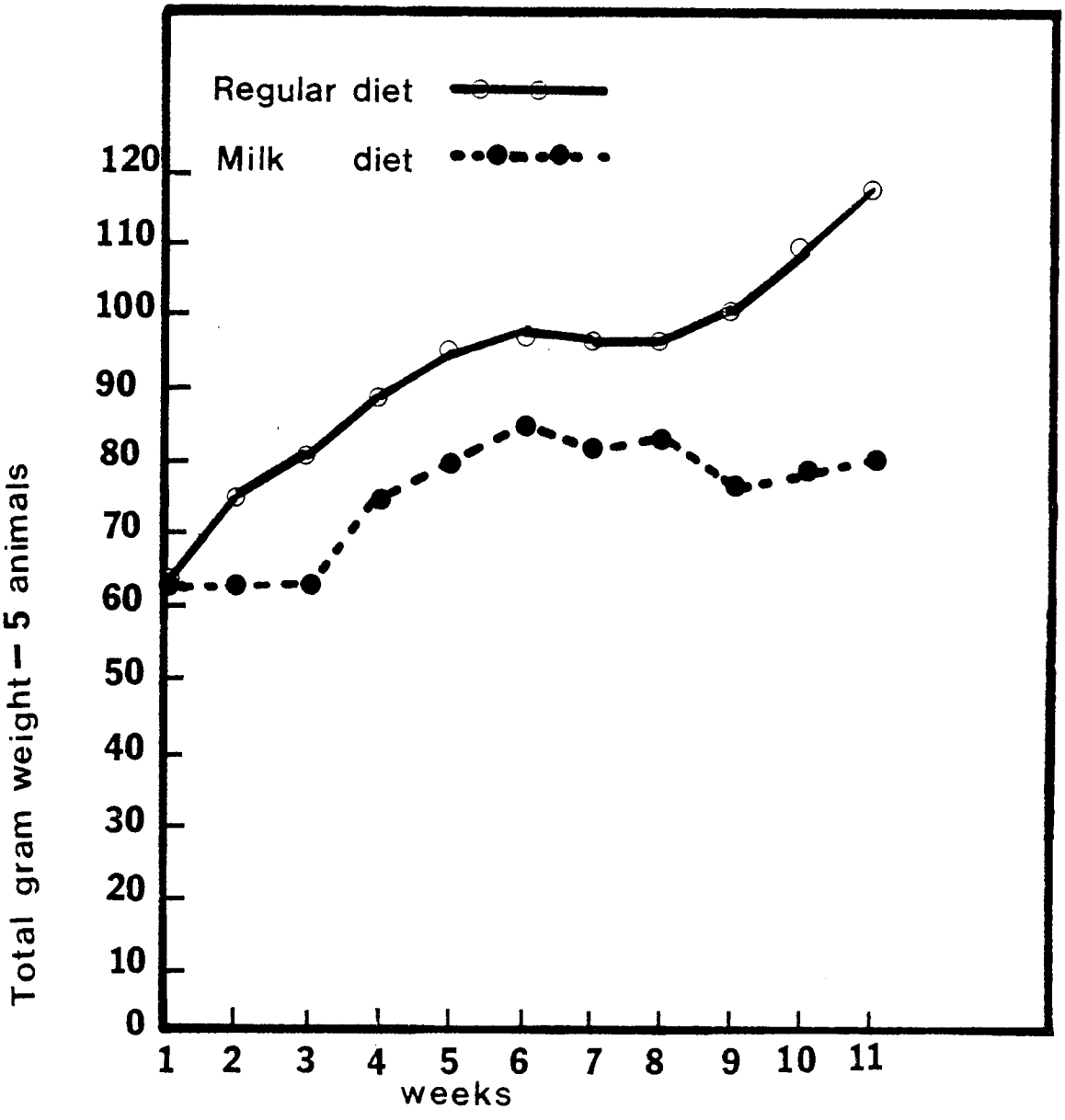


Figure 6: Growth chart for regular diet (Purina Laboratory Chow) and milk diet, naturally copper-free.

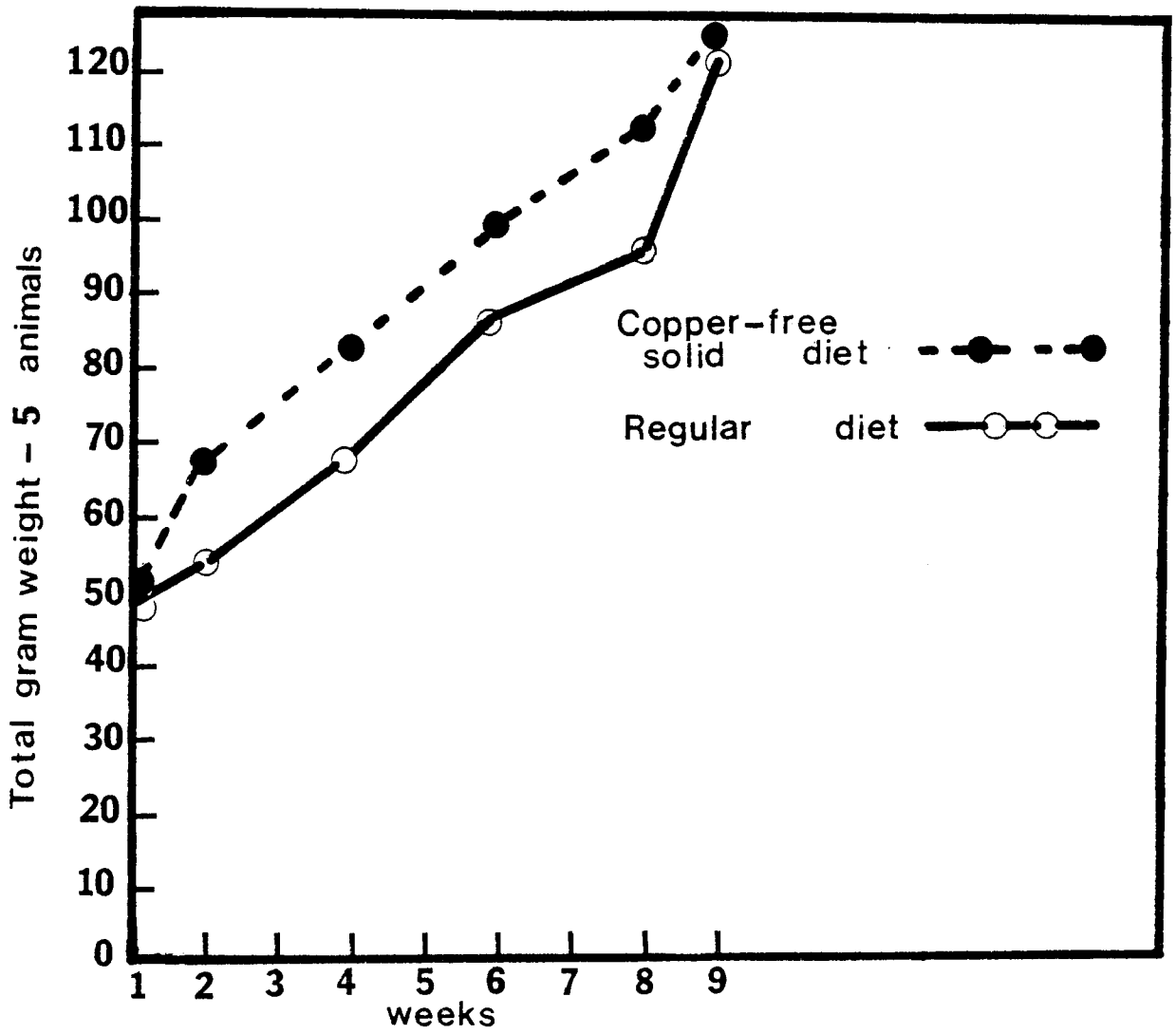


Figure 7. Growth curve for regular diet (Purina Laboratory Chow) and commercial copper-free diet

TABLE II

SERUM COPPER LEVELS OF NORMAL AND MELANOMA BEARING BALB/C FEMALE MICE

Source	Copper Levels, μ gram percent
Experiment 1	
Normal mice on regular diet[1] (9 wks.) (10 animals)	70 72
Mice with Harding-Passey melanoma on regular diet[1] (10 wks.) (7 animals)	105 102
Normal mice on special copper-free diet[2] (18 wks.) (5 animals)	34 34
Mice with Harding-Passey melanoma on special copper-free diet[2] (13 wks.) (5 animals)	52 54
Experiment 2	
Normal mice on regular diet[1] (8 wks.) (8 animals)	74 76
Mice with Harding-Passey melanoma on regular diet[1] (10 1/2 wks.) (7 animals)	116 118
Normal mice on special copper-free diet[2] (19 wks.) (5 animals)	32 34
Mice with Harding-Passey melanoma on special copper-free diet[2] (13 1/2 wks.) (8 animals)	100 102

1 Purina Laboratory Chow (diet relates to one which is illustrated in Figure 7)

2 Copper-free test diet (United States Biochemical Corporation (Diet relates to one which is illustrated in Figure 7)

It was found that regardless of diet, animals bearing Harding-Passey melanoma had increased serum copper levels. Animals with melanoma on Cu-free diet showed unexplained large variability in results (52 & 54 compared to 100 & 102, respectively). Perhaps the latter group was not entirely copper deficient. The finding of increased serum copper agrees with similar earlier studies done on various types of cancer in animals and humans. One possible explanation for the increase is that perhaps due to the sudden rise of tyrosinase levels in serum of these animals, copper which is known to be associated with tyrosinase (17) becomes readily detected too. Statistical analysis indicates that diet and presence or absence of the tumor have a significant effect on the results seen. Results are not affected by diet or the presence or absence of the tumor individually. However, the combination of the two factors did influence data. Table II illustrates these factors.

Distribution of Copper in Tissues

Copper content in various organs of mice, with and without Harding-Passey melanoma was assayed using the atomic absorption technique. Assayed copper values, reported in ppm, are listed in Table III. Individual values listed represent results of different wet weight samples. Tumor tissue did contain some copper. The value found in tumor was comparable to the amount found in skin which seemed reasonable considering tumor implant was made just under the skin of hind leg. No evidence for increased copper storage in liver was observed in animals bearing Harding-Passey malanoma when compared to normal animals. The finding that copper was not increased in liver

TABLE III

THE INFLUENCE OF AGE, COPPER INTAKE, AND PRESENCE OF MELANOMA ON THE COPPER CONTENT[1] IN ORGANS OF BALB/C FEMALE MICE

Treatment	Liver	Brain	Muscle	Skin	Tumor
Normal mice on regular diet[2] (8 wks.) (10 animals)	3.17 2.69	1.76 --	1.33 --	1.33 1.2	
Normal mice on regular diet (9 1/2 wks.) (6 animals)	3.5 3.38	2.0 --	.68 --	1.79 3.0	
Mice with Harding-Passey on regular diet (10 wks.) (4 animals)	3.06 --	1.47 --	.56 --	1.79 1.75	2.2 1.32 1.5 1.56
Mice with Harding-Passey on regular diet (11 wks.) (3 animals)	--	3.0	--	.93	1.67
Mice with Harding-Passey on copper-free diet[3] (13 wks.) (5 animals)	2.76	1.5	1.32	.83	.97 1.2
Mice with Harding-Passey on copper-free test diet (14 wks.) (5 animals)	5.08 3.0	2.31 --	.75 --	.75 --	1.42 .93
Mice with Harding-Passey on regular diet (12 wks.) (3 animals)	10.5	--	--	--	2.7
Normal mice on copper-free diet (18 wks.) (5 animals)	5.8 5.13 5.31	3.23 -- --	1.86 -- --	3.47 1.55	
Normal mice on copper-free diet (13 wks.) (5 animals)	3.83 4.5	1.29 --	.5 --	-- --	

TABLE III (Continued)

Source and Description	Liver	Brain	Muscle	Skin	Tumor
Mice with Harding-Passey on copper-free diet (16 wks.) (2 animals)	3.6	5.0	6.45	3.89	1.68

1 Reported in ppm (wet weight basis)
2 Purina Laboratory Chow
3 Copper-free test diet (United States Biochemical Corporation)

was in contrast to the observation of increased copper storage in previous studies of animals bearing various tumors. Generally, no significant differences were seen between copper content of organ, comparing tumor-bearing to tumor-free mice. Perhaps a better indication of true copper content in tissues could have been made had the assays been determined on dry weight samples. Greenstein (12) reported that tissues of tumor-bearing animals contained a slightly higher water content than similar tissues in normal animals. He suggested that the 1-4 percent increase reflected a disturbance in osmotic equilibrium between intracellular and extracellular fluid. Statistical analysis of the data on the amounts of copper found in tissues demonstrates the following: (1) differences in copper content between control and tumor-bearing animals were not statistically significant for liver, brain, muscle, and skin; (2) in animals raised on copper-free diet the copper content of tumor was significantly decreased, while there was no copper depletion in liver, brain, muscle, and skin.

Cytochrome Oxidase in Tissue Homogenates

Cytochrome oxidase activity was measured on tissue homogenates (liver, brain, and tumor) using the Clark oxygen electrode. Tissue was pooled from a designated group of animals. Values listed indicate replicates on the pooled homogenates. Table IV shows the results. The presence of tumor lowered the cytochrome oxidase activity in animals on both types of diet. (Example: on a regular diet, the presence of tumor decreased the activity in liver and brain 59% and 72% respectively, while on a copper-free diet, corresponding

TABLE IV

CYTOCHROME OXIDASE ACTIVITY ON TISSUE HOMOGENATES

Groups	Tissue (liver, brain and tumor) Activity (μ moles O_2/min/gram)			
	#1	#2	#1	#2
Regular diet, no tumor group #1 (10 animals) (9 wks.) group #2 (5 animals) (7 wks.)	Liver 7.29 4.42 3.92 3.92 $\bar{x}$ = 4.89 s = ± 1.619	Liver 4.17 4.17 3.67 3.92 4.17 $\bar{x}$ = 4.02 s = ± .224	Brain 3.73 3.81 4.15 $\bar{x}$ = 3.90 s = ±.223	Brain 3.28 3.28 3.28 3.28 $\bar{x}$ = 3.28 s = ± 0
Copper-free diet, no tumor group #1 (5 animals) (9 wks.) group #2 (5 animals) (10 wks.) group #3 (5 animals) (12 wks.) group #4 (5 animals) (13 wks.)	#1 Liver 3.62 3.08 3.15 $\bar{x}$ = 3.28 s = ± .294	#2 Liver 2.46 2.46 2.46 $\bar{x}$ = 2.46 s = ±0	#3 Liver 6.02 4.92 5.63 $\bar{x}$ = 5.52 s = ± .56	#4 Liver 4.59 5.1 4.08 $\bar{x}$ = 4.59 s = ± .51
	#1 Brain 2.68 3.3 2.68 3.57 $\bar{x}$ = 3.06 s = ±.4496	#2 Brain 1.47 1.47 1.47 $\bar{x}$ = 1.47 s = ± 0	#3 Brain 3.65 3.35 3.24 3.35 $\bar{x}$ = 3.4 s = ± .18	#4 Brain 4.0 3.2 3.0 $\bar{x}$ = 3.4 s = ± .53
Regular diet, with tumor group #1 (4 animals) (10 wks.) group #2 (3 animals) (10 1/2 wks.)	#1 Liver 1.7 1.7 4.52 $\bar{x}$ = 2.64 s = ± 1.628	#2 Liver 2.63 2.63 $\bar{x}$ = 2.63 s = ± 0	#1 Liver 2.45 2.21 3.75 $\bar{x}$ = 2.8 s = ± .829	#2 Brain 2.29 2.15 $\bar{x}$ = 2.22 s = ± .099
	#1 Tumor 2.38 2.38 4.0 $\bar{x}$ = 2.92 s = ± .935	#2 Tumor 1.29 2.58 $\bar{x}$ = 1.94 s = ± .912		

TABLE IV (Continued)

Groups	Tissue Activity (liver, brain and tumor) μ moles O_2/min./gram)			
Copper-free diet, with tumor	#1 Liver	#2 Liver	#1 Brain	#2 Brain
group #1 (5 animals)	.821	1.41	2.31	1.32
group #2 (5 animals)	.821	1.2	2.48	1.78
	.821	1.23	$\bar{x}$=2.4	1.32
	1.23	$\bar{x}$=1.28	s=± .1204	$\bar{x}$=1.47
	1.36	s=± .11		s=± .27
	$\bar{x}$=1.01			
	s=± .264			
	#1 Tumor	#2 Tumor		
	.83	1.45		
	.83	1.16		
	$\bar{x}$=.83	.98		
	s=± 0	$\bar{x}$=1.2		
		s=± .24		

decreases were 22% and 64% due to tumor. This observation is consistent with earlier studies reported on mice with carcinoma. Enzyme activity in brain in tumor-bearing animals on copper-free diet seems to remain slightly higher than in liver. These animals had been on regular diet three weeks before, therefore the copper storage in brain had started to build up and because of the blood-brain barrier this area of the animal did not become depleted of copper soon. Figure 8 presents a histogram of the above data. This histogram is a diagram of the pooled standard deviations and pooled means observed on the various experimental groups. In this particular study, statistical analysis supported the belief that presence or absence of tumor had an effect on cytochrome oxidase levels observed on liver and brain from animals on copper-free or regular diets.

Electron Microscopy Preparation

Electron microscopy preparations were made on all fractions taken off the sucrose gradient. Cytochrome oxidase activity was measured on fraction 3, tumor mitochondria; therefore, particular attention was given to the purity of that preparation. Figures 9 and 10 are electron micrographs of tumor mitochondria from mice on regular diet and copper-free diets, respectively. No real difference in size was seen between tumor mitochondria from mice on regular diet and tumor mitochondria from mice on copper-free diet. Most mitochondria from tumors were found to measure approximately 0.8 x 0.6 μ which is comparable to the size of normal mitochondria, measuring 1-2 μ long

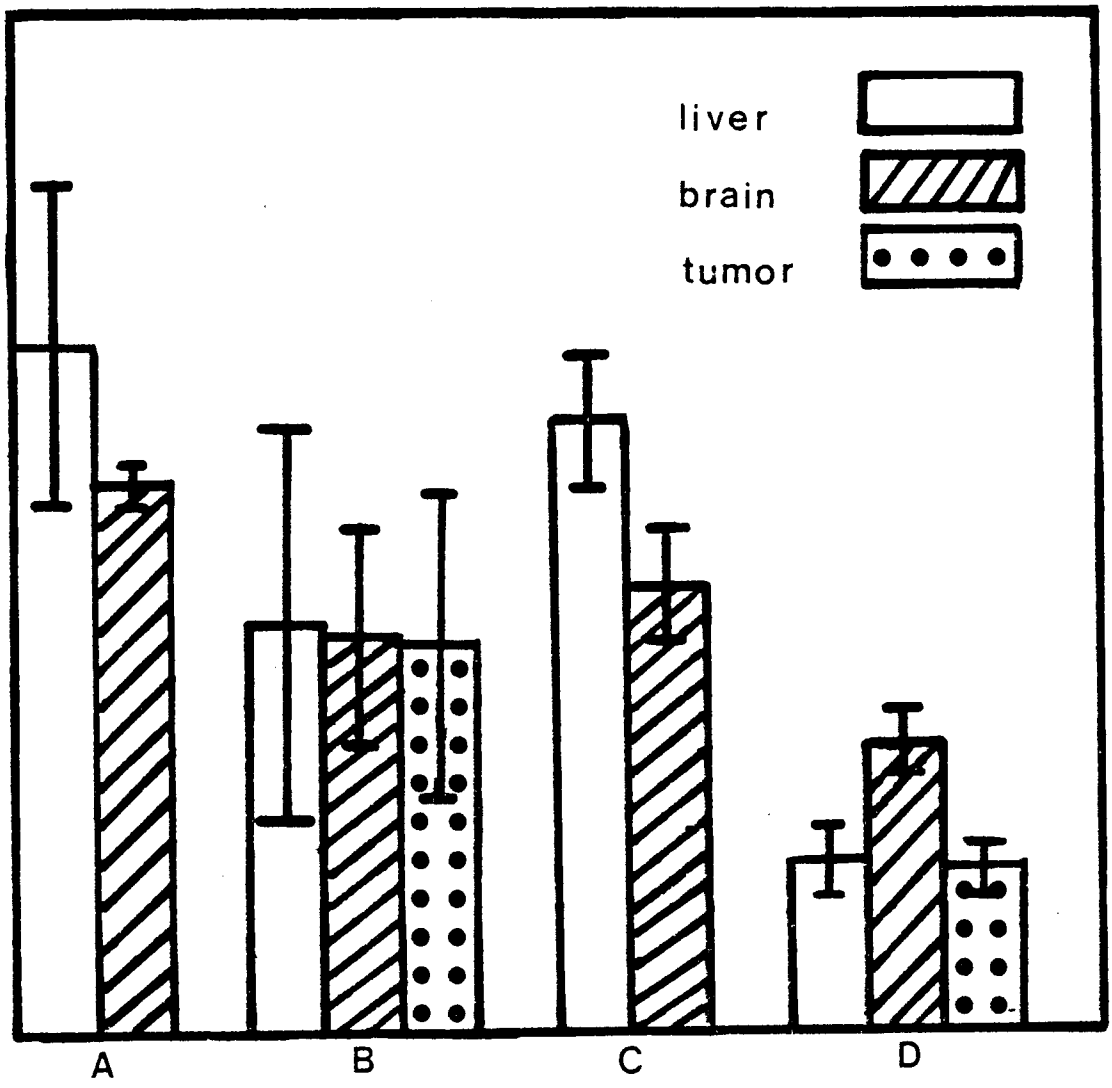


Figure 8. Histogram of pooled standard deviations and pooled means on various groups.

A. The group on regular diet with no tumor
liver: Std. dev. = 1.07 Pooled mean = 4.41
brain: Std. dev. = .14 Pooled mean = 3.54

B. The group on regular diet with tumor
liver: Std. dev. = 1.33 Pooled mean = 2.64
brain: Std. dev. = .68 Pooled mean = 2.57
tumor: Std. dev. = .93 Pooled mean = 2.53

C. The group on copper-free diet with no tumor
liver: Std. dev. = .41 Pooled mean = 3.96
brain: Std. dev. = .35 Pooled mean = 2.89

D. The group on copper-free diet with tumor
Liver: Std. dev. = .23 Pooled mean = 1.11
Brain: Std. dev. = .23 Pooled mean = 1.84
Tumor: Std. dev. = .19 Pooled mean = 1.05

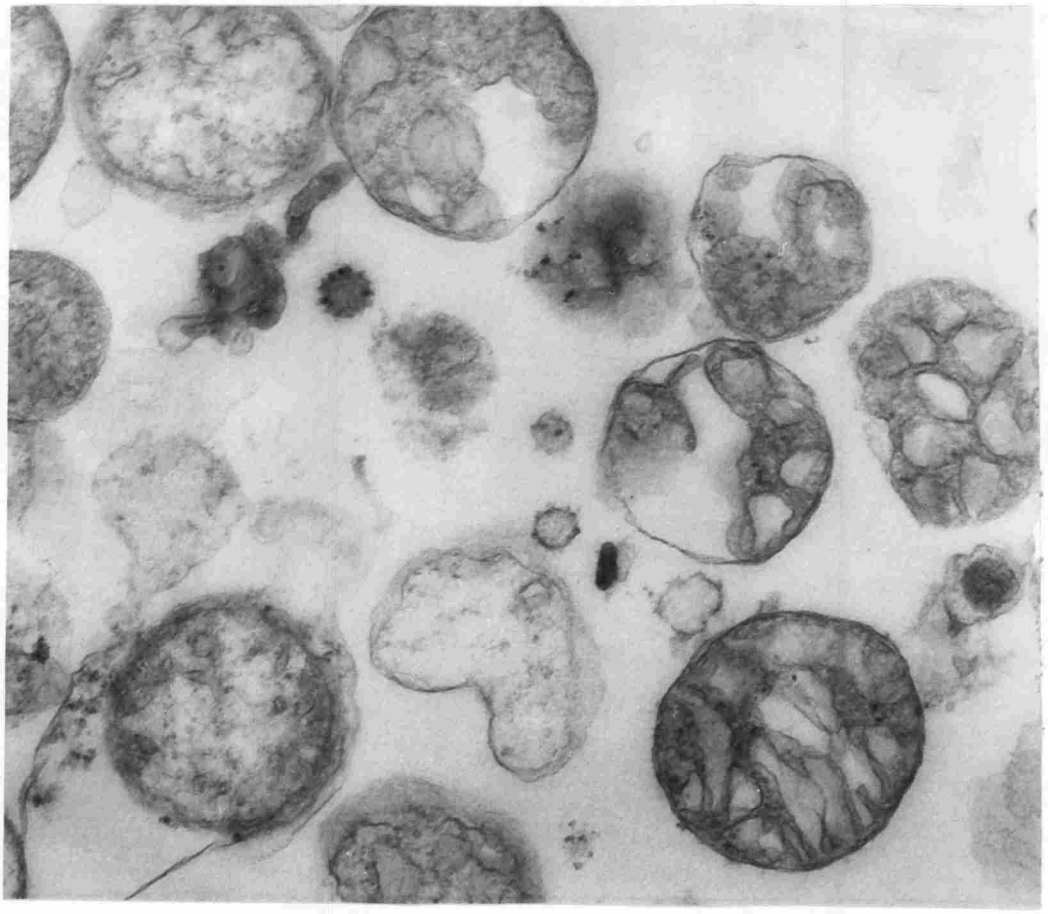


Figure 9. Electron micrograph of fraction 3 (tumor mitochondrial fraction) from mice bearing Harding-Passey melanoma on regular diet.

Scale: 1 3/16 in. = .62 μ

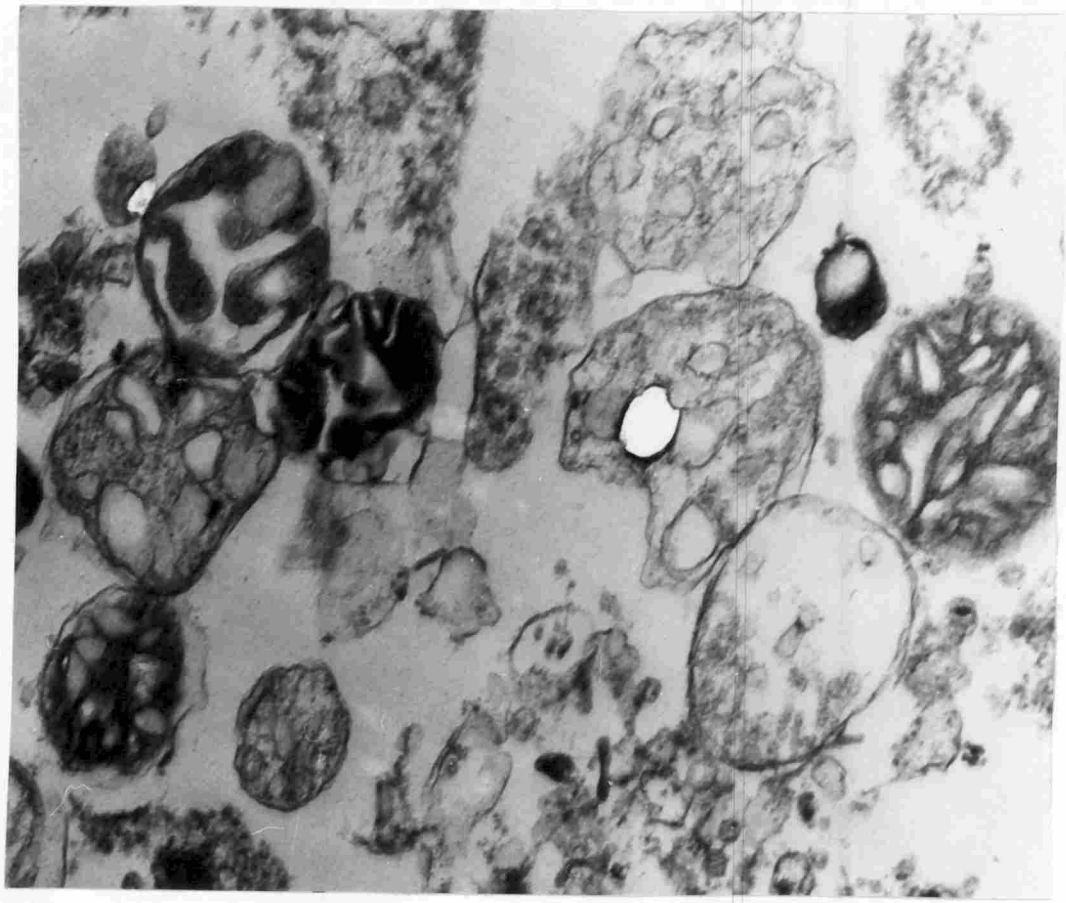


Figure 10. Electron micrograph of fraction 3 (tumor mitochondrial fraction) from mice bearing Harding-Passey melanoma on copper-free diet.

Scale: 1 3/16 in. = .64 μ

and 0.3-0.7 μ wide. Suzuki (26) fed mice cuprizone (a copper chelating agent) which produced giant mitochondria. Depriving the mitochondria of copper produced the structural change. The copper-free diet used in the author's research seems unable, over the 11-week time period used, to reduce copper down to levels where structural change in mitochondria can be seen.

Cytochrome Oxidase Activity of Mitochondria

Liver and tumor mitochondria were isolated from the various experimental groups. Tumor mitochondria were isolated using the sucrose gradient method. Table V lists observed activities.

Liver mitochondria from copper deficient mice had less cytochrome oxidase activity than liver mitochondria from mice on regular diet. This observation parallels the pattern seen in liver homogenates. In the animal's bearing the tumor, cytochrome oxidase appeared to become increased in liver of animals on both types of diets. Generally, tumor mitochondria had the same activity as liver mitochondria isolated from animals with Harding-Passey malanoma.

Analysis of the data presented on cytochrome oxidase activity in liver and tumor mitochondria shows that (1) presence or absence of tumor slightly affects results on liver mitochondria, and (2) there is no effect of diet on results seen on tumor mitochondria.

TABLE V

CYTOCHROME OXIDASE ACTIVITIES OF LIVER AND TUMOR MITOCHONDRIA

Treatment	Group	Activity natoms O /min/mg. protein	Average Activity
Regular diet no tumor, liver	#1 (10 1/2 wks., 5 animals)	114,159,152,155	145
	#2 (13 wks., 5 animals)	142,142,151	145
Copper-free diet, no tumor, liver	#1 (13 wks., 5 animals)	118,103,165,112	125
Regular diet with tumor, liver	#1 (11 wks., 4 animals)	162,169,235	189
	#2 (11 wks., 6 animals)	142,136,139	139
	#3 (13 wks., 5 animals)	595	(595)[1]
	#4 (12 1/2 wks. # animals) not recorded	253	(253)[1]
Regular diet with tumor, tumor	#1 (9 1/2 wks., 5 animals)	1105	(1105)[1]
	#2 (11 wks, 6 animals)	200,179,238	206
Copper-free diet with tumor, liver	#1 (12 wks , # animals) not recorded	414	(414)[1]
	#2 (3 wks., 3 animals)	227,239	233
Copper-free diet with tumor	#1 (18 wks., # animals) not recorded	71	(71)[1]
	#2 (18 wks., 2 animals)	329	(329)[1]
	#3 (13 wks., 3 animals)	229,250,208	229

1 single results

CHAPTER IV

SUMMARY

One of the difficulties encountered in this research was achieving "completely copper-free" animals. Several animals -10- were housed in the same cage which was covered with a stainless steel top; therefore, mice were free to come in contact with the stainless steel top (which contained copper). Attempts were made to determine tissue copper; however, copper values were compared on the basis of wet weight which the author feels is not optimal. One way of judging for copper deficiency was by subjective observation of toughness of skin at time of tumor implant. By this type of evaluation animals on milk diets, naturally copper-free, and on commercial pelleted copper-deficient diets,did seem to achieve copper deprivation (skin was easily penetrated at time of transplant). This is to be expected since copper deprivation will interfere with cross-linking of collagen in the skin.

It has been demonstrated that copper is essential for iron utilization in hemoglobin synthesis (16). In cases of low serum copper it has been seen that anemia often accompanies the condition. Perhaps it would have been advisable to have checked on hemoglobin content in animals with Harding-Passey melanoma. Since high serum copper levels were demonstrated in these animals, the author believes that no profound accompanying anemia would be demonstrated.

From the information observed on tissue copper in animals with Harding-Passey, on regular diet, it appeared that tumor tissue did

not accumulate excess amounts of copper. For instance, tumor tissue contained approximately 1.5 ppm Cu, compared to liver tissue which contained approximately 3 ppm Cu. One would suppose that transport and distribution of copper to tissues would remain fairly normal and that no secondary anemia would be found. It is suggested that future studies on the copper transport protein, ceruloplasmin, may prove helpful.

In the cytochrome oxidase studies done on tissue homogenates, presence of tumor in animals did appear to decrease activity which was measured in liver and brain homogenates. It is known that lipids are associated with cytochromes; therefore, further studies on amounts of lipids in the tumor-bearing host might aid in interpreting these results.

There appeared to be a slight increase in cytochrome oxidase specific activity in mitochondria from livers of tumor-bearing animals. The respiratory control ratio of these mitochondria was checked by the author and found to be normal (approx. 4.0); therefore, uncoupling did not appear to account for the increased enzyme activity. Respiratory control of tumor mitochondria was also checked. It was found that tumor mitochondria had low acceptor control ratio (1.2). Tumor mitochondria had higher cytochrome oxidase specific activity than normal liver mitochondria which may be related to the low acceptor control ratio of the former.

LIST OF REFERENCES

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1. Baker, R. V., M. S. C. Birbeck, H. Blaschko, T. B. Fitzpatrick, and M. Seiji. 1960. Melanin granules and mitochondria. Nature 187: 392-394.

2. Bell, Marvin C. (University of Tennessee, Animal Science) personal communication.

3. Burnett, Jean B. 1975. Melanoma, Methods for Detection and Evaluation. Fractions 1: 1-9.

4. Colowick, Sidney P. and N. O. Kaplan (Eds.) 1955. Tissue Homogenates. Methods in Enzymology, vol I. Academic Press, Inc., New York p. 12

5. Colowick, Sidney P. and N. O. Kaplan (Eds.) Protein Content. Methods in Enzymology, vol 3. Academic Press, Inc., New York p. 702.

6. Donath, I. 1971. Copper and Cancer. Prot. Vitae 16(1): 11-13.

7. Glick, David (Ed.) 1969. Oxygen Electrode Measurements in Biochemical Analysis. Methods in Biochemical Analysis. 17: 5-6.

8. Greenawalt, J. W. 1974. Isolation of Outer and Inner Mitochondrial Membranes. S. P. Colowick and N. O. Kaplan (Eds.) Academic Press, Inc. New York. Methods in Enzymology XXXI. 310-323.

9. Greene, Alice A. and F. L. Haven. 1957. Cytochrome Oxidase in the Liver of Rat Bearing Walker Carcinoma 256. Cancer Research 17: 613-617.

10. Greenstein, Jesse P. 1943. Note on the Copper Content of Tissues of Tumor-Bearing Animals. Journal of the National Cancer Institute 3: 405-408.

11. Greenstein, J. P., J. Werne, A. B. Eschenbrenner, and F. M. Leuthardt. 1944. Chemical Studies of Human Cancer I. Cytochrome Oxidase, cytochrome c, and copper in animal and neoplastic tissues. Journal of the National Cancer Institute 5: 55-76.

12. Greenstein, Jesse P. 1954. Chemistry of Tumors, Chapter VIII. Biochemistry of Cancer. Academic Press, Inc., New York. pp. 545-547.

13. Greenstein, Jesse P. 1954. Chemistry of Tumor-Bearing Host IX. Biochemistry of Cancer. Academic Press, Inc., New York pp. 545-547.

14. Haven, F. L., B. A. Boyd, J. B. Baron, and W. R. Bloor, 1965. Cytochrome Oxidase in the Liver of the C3H Mouse with Carcinoma. Proceedings of the Society for Experimental Biology and Medicine vol. 118: 134-136.

15. Kolaric, Krsto, Ante Roguljic, and Vera Fuss. 1975. Serum Copper Levels in Patients with Solid Tumors. Tumori 61: 173-177.

16. Lardy, Henry. 1978. Fifty Years Ago: Copper as an Essential Element for Iron Utilization. Trends in Biochemical Sciences 3: 93-94.

17. Malmstrom, B. G. 1964. Two forms of copper in copper containing oxidases. Tsoo E. King, H. S. Mason and M. Morrison (Eds.) Oxidases and Related Redox Systems, vol. I: 207-216.

18. McCord, Ray, (University of Tennessee, Ecology). Lab Tricks and Pitfalls Unlimited, manual. personal communication.

19. Parker, M. M., Humoller, and D. J. Mahler. 1967. Determination of Copper and Zinc in Biological Materials. Clinical Chemistry 13: 40-47.

20. Sandberg, M. H., Gross, and Oliver Holly. 1942. Changes in Retention of Copper and Iron in Liver and Spleen in Chronic Disease Accompanied by Secondary Anemia. Archives of Pathology 33: 834-844.

21. Schnaitman, Carl, V. G. Erwin, and J. Greenawalt. 1967. The Submitochondrial Localization of Monoamine Oxidase. The Journal of Cell Biology 32: p. 120.

22. Schutte, Karl H. 1964. Health and Disease as a Function of Trace Elements. The Biology of the Trace Elements. J. B. Lippincott Company, Philadelphia, p. 120.

23. Seiji, M., M. S. C. Birbeck, and T. B. Fitzpatrick. 1961. Melanin Biosynthesis. Annals New York Academy of Science 100 (Part II): 497-533.

24. Seiji, M., T. B. Fitzpatrick, R. T. Simpson, and M. S. C. Birbeck. 1963. Chemical Composition and Terminology of Specialized Organelles (melanosomes and melanin granules) in mammalian melanocytes. Nature 197: 1082-1084.

25. Smyshlyaw, O. K., 1970. Activity of Cytochrome Oxidase, Cytochrome System, and Cu - Content of Animals During Experimental Leukemia. Patol Fiziol EKSP Ter 14 (1): 82-83.

26. Suzuki, Kinuko. 1969. Giant Hepatic Mitochondria: Production in Mice Fed with Cuprizone. Science 163: 81-82.

27. Tannenbaum, Albert, and H. Silverstone. 1953. Nutrition Relation to Cancer. Jesse P. Greenstein and Alexander Haddow (Eds.) Advances in Cancer Research, vol I. Academic Press, Inc. New York, pp. 451-501.

28. Trejo, Alfonso Carabez (University of Tennessee, visiting professor). Application of Electron Microscopy to Problems in Biochemistry and Biology. Biochemistry 5450. personal communication.

29. Weiss, Joseph. (Comparative Animals Research Laboratory, Oak Ridge, Tennessee). personal communication.

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