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Protein Metabolism in Rats with Tumors of the Liver

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RICHMOND — PROTEIN METABOLISM IN RATS

NAME AND ADDRESS	DATE

Protein Metabolism in Rats with Tumors of the Liver

By

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TABLE OF CONTENTS

CHAPTER		PAGE
I	INTRODUCTION	1
II	SURVEY OF LITERATURE	3
III	METHODS AND MATERIALS	12
IV	RESULTS	17
V	DISCUSSION	22
VI	SUMMARY AND CONCLUSIONS	29
	LITERATURE CITED	30

LIST OF TABLES

TABLE		PAGE
1	CARCINOGENIC DIET USING p-DIMETHYLAMINOAZOBENZENE (DAB)	40
2	WATER, SOLIDS, LIPID, AND NITROGEN CONTENT OF LIVERS FROM RATS FED DAB	41
3	CREATININE AND NON-PROTEIN NITROGEN EXCRETION OF RATS FED DAB FOR NINE MONTHS	42

LIST OF FIGURES

FIGURE		PAGE
1	RELATIONSHIP OF WET WEIGHT TO PERCENTAGE WATER IN LIVERS OF ANIMALS FED DAB	43
2	RELATIONSHIP OF WET WEIGHT TO TOTAL WATER CONTENT IN LIVERS OF ANIMALS FED DAB AND BASAL DIETS	44
3	RELATIONSHIP OF WET WEIGHT TO TOTAL LIVER SOLIDS IN LIVERS OF ANIMALS FED DAB AND BASAL DIETS	45
4	RELATIONSHIP OF WET WEIGHT TO TOTAL PROTEIN IN LIVERS OF ANIMALS FED DAB AND BASAL DIETS	46
5	RELATIONSHIP OF WET WEIGHT TO PERCENTAGE PROTEIN IN LIVERS OF ANIMALS FED DAB AND BASAL DIETS	47
6	RELATIONSHIP OF LIVER WEIGHT TO TOTAL PROTEIN IN LIVERS OF RATS FED DAB AND BASAL DIETS; SEMI-LOG PLOTS BASED ON LIVER WEIGHT AND LIVER DRY WEIGHT	48

CHAPTER I

INTRODUCTION

Studies on changes in protein metabolism in tumorous animals are of importance because proteins make up the basic architecture of cells, and are intimately linked with essential metabolic and regulatory activities. Most of the work concerning p-dimethylaminoazobenzene (DAB) induced tumors has been directed toward either the dietary or enzymatic ramifications of carcinogenesis. A large portion of the data related to total protein or nitrogen alterations appeared to be by-products of other studies. In some instances it has been difficult to interpret accurately the data regarding nitrogen alterations in tumorous liver because it was not stated whether the tissue came from tumorous or non-tumorous areas of the liver.

Some workers have postulated that a tumor is a nitrogen trap because tumors are known to contain large amounts of nitrogen. Zamecnik and Stephenson (1947, p.331), reported an increase in total nitrogen in hepatomas as compared to normal liver tissue. Striebich, et. al., (1953, p. 281), found that there was slightly less nitrogen in the livers of azo dye fed rats than in controls on a percentage basis. Tumorous liver tissues have been reported to contain decreased solid contents (Griffin, et. al., 1948, p. 1228). Livers of rats bearing extra-hepatic

CHAPTER I

INTRODUCTION

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Such workers have [illegible] nitrogen than [illegible] amounts of nitrogen. [illegible] and [illegible] ([illegible]) reported an increase in total nitrogen [illegible] compared to normal liver tissue. [illegible] et. al. (1953, p. 281), found that [illegible] gen in the liver [illegible] on a percentage basis. [illegible] reported to contain [illegible] et. al., 1948, p. 1[illegible] tumors also were shown to have increased water contents and decreased solid contents (Green, et. al., 1950, p.773). These findings suggested that on a percentage basis the protein content of entire tumorous livers might not be increased.

The purpose of our study was to determine possible alterations in water, fat, and protein content of the livers from rats fed DAB for long periods. Aliquots of the dry, fat-free liver rather than selected areas were used for the protein determinations.

Studies were also undertaken to compare the amounts of non-protein nitrogen and creatinine in the blood and urine of animals fed DAB with those of rats fed only the basal diet since differences in the level of these compounds in the two groups indicates differences in protein metabolism.

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in protein metabolism.

CHAPTER II

SURVEY OF LITERATURE

The first description of atypical growths due to the injection of scarlet red (an azo dye) was published by Fischer in 1906 (Rusch, et. al., 1945, p. 267). He found that the injection of scarlet red into the ears of rabbits caused the formation of atypical epithelial growths which were difficult to distinguish from cancerous growths. Unlike true cancers, these growths always receded when treatment with the chemical compound was discontinued. In 1924, Schmidt (Rusch, et. al., 1945, p. 267), who was feeding scarlet red to mice, noticed that the dye caused an extensive proliferation of the epithelial cells of the liver, which he considered to be both adenomatous and sarcomatous in nature. According to Sugiura and Rhoads (1941, p. 3), it was Sasaki and Yoshida in 1935 who first conclusively demonstrated the carcinogenicity of an azo dye. These workers obtained hepatomas and cholangiomas in rats by feeding 1 mg. of 2',3-dimethyl-4-aminoazobenzene per gm. of food for about 300 days.

In the following years many related azo dyes were also tested for their carcinogenicity. Sugiura and Rhoads (1941, p. 3), stated that Kinosita in 1937 demonstrated that p-dimethylaminoazobenzene, commonly known as butter yellow and hereafter referred to as DAB, was

CHAPTER II

SURVEY OF LITERATURE

The first [illegible] to the injection of scarlet red [illegible] was published by Fischer in 1906 (Rusch, op. cit., 1944, p. 227). He found that the injection of scarlet red [illegible] rabbits caused the formation of [illegible] which were difficult to distinguish from [illegible]. Unlike true cancers, these growths [illegible] treatment with [illegible]. In 1924, Schmidt (Rusch, op. cit., 1944, p. 227), [illegible] feeding scarlet red to mice, [illegible] an extensive proliferation of the [illegible] of the liver, which he considered [illegible] and sarcomatous in nature. According to [illegible] (1941, p. 3), it was [illegible] conclusively demonstrated [illegible] dye. These workers [illegible] in rats by feeding [illegible] per gm. of food for [illegible].

In the following [illegible] were also tested [illegible]. [illegible] Rhoads (1941, p. 3) [illegible] strated that [illegible], commonly known as butter yellow and [illegible] the most active carcinogen for rats. He was able to produce liver tumors by feeding DAB for 100-170 days to rats. This dye and one of its more potent derivatives, 3'-methyl-4-dimethylaminoazobenzene, have been employed in most of the recent studies on aminoazo dye carcinogenesis.

In Kinosita's experiments the azo dyes were added in olive oil to a basal diet of brown rice and carrots to correspond to the staple food of the rice-eating countries of the orient. The use of this nutritionally poor diet was very important because the addition of wheat bread reduced liver tumor formation. According to Sugiura and Rhoads (1941, p.3), Ando in 1938 first observed that rats fed DAB did not develop liver tumors if yeast were added to the rice diet. In the same year, Nakahara, Mori, and Fujiwara (Sugiura and Rhoads, 1941, p.3), reported that dried beef liver inhibited the carcinogenic action of DAB. These classic findings of the Japanese were confirmed by investigators in this country (Sugiura and Rhoads, 1941, p.15). The specificity of these dyes for the liver in rodents and the relative ease with which the production of tumors occurred made this a valuable tool in the study of the fundamental nature of the carcinogenic process. Moreover, the hepatomas induced by the aminoazo dyes seemed to be the most active carcinogen for rats, [illegible] produce liver tumors by feeding [illegible] to rats. This dye and one of its [illegible], 3'-methyl-4-dimethylaminoazobenzene, have been [illegible] in most of the recent studies of [illegible] azo dye carcinogenesis.

In [illegible] the [illegible] added in olive oil to [illegible] rice [illegible] and carrots to [illegible] the [illegible] rice-eating countries of the world. The [illegible] of this nutritionally poor diet [illegible] produced [illegible] the addition of [illegible] liver [illegible]. According to [illegible] and [illegible] ([illegible]), [illegible] 1938 first observed [illegible] liver tumors if yeast [illegible] diet. In the same year, [illegible] and [illegible], [illegible], p.[illegible]), [illegible] inhibited the carcinogenic action of [illegible]. These findings of the [illegible] in this country ([illegible], [illegible]). The specificity of these [illegible] and the relative ease with which the [illegible] of [illegible] made this [illegible] as [illegible] fundamental nature of the [illegible] process. [illegible]

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of added interest since dietary factors affected the primary carcinogenesis.

The experiments cited above indicated that liver tumor development could be partially controlled and further biological investigations, particularly enzyme studies, were undertaken. Studies of this type offered a new approach to clarification of the factors determining induction and growth of tumors.

In general the liver of the rat was found to be the most susceptible to the carcinogenic action of DAB, the livers of other animals were found to be more resistant. It was shown that mice would develop liver tumors if the length of the dye feeding period was twice that used in rats. Price, et. al. (1951, p.528), showed that mouse liver, which is slightly susceptible to the carcinogenic action of DAB, was altered considerably the the ingestion of this aminoazo dye for 4 months. There were decreased levels of protein and pentosenucleic acid in the large granules (mitochondria) comparable to those levels previously found in rats fed DAB. It was also shown that guinea pigs, rabbits, cotton rats, and chickens were resistant to the carcinogenic action of DAB (Miller, E.C., and Miller, J.A., 1947, p.479).

The effect of the diet on the incidence of DAB induced hepatomas has been extensively studied. Earlier

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primary carcinogenesis.

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H.C., and Miller, J.A., 19[illegible]

The effect of the diet [illegible]
induced hepatomas [illegible]

workers added an oil solution of 0.06% DAB to a basal diet of unpolished rice. Later workers developed purified diets in which the protein, fat, carbohydrate, and vitamin contents could be varied. Conflicting results were obtained when workers began to vary the amount of dietary protein (casein) in the diets. Harris, et. al. (1947, p.169), reported that the addition of casein caused no delay in tumor development. Griffin, et. al. (1949, p.87), concluded that rats usually developed fewer tumors if the casein content was 24 rather than 12%.

These differences have been explained on the basis of the riboflavin supplement in the diet. The addition of casein and riboflavin together markedly inhibited tumor development according to Kensler, et. al. (1941, p.309). Other workers have shown effects of varying the casein intake on riboflavin content of liver and urine. An increase in the dietary casein was found to produce a decrease in the urinary excretion of riboflavin in the rat (Sarett, et. al., 1942, p.304). Increasing the casein content was also found to produce an increased retention of riboflavin in the liver (Sarett, et. al., 1943, p.181). Apparently high levels of casein were needed for the optimum utilization of riboflavin by the liver; this has an inhibitory effect on tumor development.

The kind and level of fat added to the DAB diet workers added an [illegible] of [illegible] rats. [illegible] workers [illegible] diets in which the protein, fat, [illegible] contents could be varied. [illegible] when workers began to vary the amount of [illegible] (casein) in the diets. [illegible] reported that the addition of casein [illegible] tumor development (Miller, et. al., [illegible]) that rats [illegible] developed [illegible] tumors [illegible] content was 2% [illegible].

These differences [illegible] on the basis of the riboflavin [illegible] addition of casein and riboflavin together [illegible] tumor development according to [illegible] p.309). Other workers have [illegible] of varying the casein intake [illegible] content [illegible]. An increase in the [illegible] of casein [illegible] decrease in the [illegible] rat (Harris, et. al., (1942, p.309). [illegible] content was also [illegible] of riboflavin in the liver (Griffin, et. al., [illegible]). Apparently high levels of casein may cause the optimum utilization of riboflavin [illegible] an inhibitory effect on tumor [illegible].

The [illegible]

seemed to have a marked effect on the rate at which tumors developed. When rats were fed DAB in a diet containing corn oil, the incidence of hepatic tumors at 6 months was 53-64%. However when hydrogenated coconut oil was added to the diet in place of the corn oil the tumor incidence fell below 8% (Miller, J.A., et. al., 1944, p.158). Kline, et. al. (1946a, p.4), also reported protection with hydrogenated coconut oil or the fatty acids of coconut oil and increased incidence with corn oil or its fatty acids. The addition of 5% corn oil caused a ten fold increase in tumor incidence as compared to animals fed the hydrogenated coconut oil. Ingestion of 20% corn oil resulted in a tumor incidence of 100% (Kline, et. al., 1946, p.6).

The histological changes that take place in the livers of animals fed DAB have been extensively studied and variously classified. Opie (1944, p.239), classified the tumors that are produced by DAB as follows: 1. Trabecular hepatomas, 2. Adenohepatomas, 3. Cyst-adenomas, and 4. Cholangiomas. These terms are in part descriptive of the histological characters of the lesions and in part indicate their origin. The term hepatoma has been applied to tumors composed in a large part of cells resembling those of the parenchyma of the liver, whereas cholangiomas maintain the form of bile ducts. Cyst-adenomas are derived seemed to have a marked [illegible] developed. [illegible] corn oil, the incidence of [illegible] 53-54%. However [illegible] to the diet [illegible] fell below [illegible] et. al. (1946a, p.[illegible]) [illegible] genated coconut oil [illegible] increased incidence [illegible]. The addition of [illegible] corn oil [illegible] in tumor incidence [illegible] genated coconut oil. [illegible] in a tumor incidence of 100% [illegible].

The histological [illegible] livers of animals [illegible] and variously classified. [illegible] the tumors that are [illegible] hepatomas, 2. Adenocarcinomas, 3. [illegible] 4. Cholangiomas. These [illegible] the histological characters [illegible] indicate their origin. [illegible] to tumors composed in a large part of [illegible] those of the parenchyma of the liver, [illegible] maintain the form of [illegible] from the cystic ducts.

Harris, et. al. (1947, p.167), described the malignant tumors as "a wide variety of types ranging from well differentiated adenocarcinoma through mixtures of adenocarcinoma and malignant hepatoma (a very common type), to a pure malignant hepatoma, and to a completely undifferentiated type of tumor composed of masses of small basophillic cells."

They found that the tumors varied from microscopic lesions to macroscopic nodules. Occasionally several large tumors, a few centimeters in diameter, of the same or of different types occured in the same liver.

White, et. al. (1942, p.554), described the history and pathology of rat hepatoma 31. The tumor, a malignant hepatoma, arose in the liver of an Osborne-Mendel rat fed DAB and was found to be readily transplantable in the homologous strain of rat. It was very suitable for studies in which liver and a tumor of hepatic parenchymal cell origin were to be compared.

Many researches have been concerned with protein changes of the intracellular components. The Wisconsin group (Price, et. al., 1948, p.348), reported a decrease in liver protein in rats fed DAB which was almost entirely localized in the large granule (mitochondrial) fraction. Increases of protein and desoxypentosenucleic acid in the from the cystic ducts.

Harris, et. al. ([illegible], p. [illegible]) described the malignant tumors in [illegible] a wide variety of types ranging from well differentiated cholangiomas through mixtures of adenocarcinoma or malignant hepatoma ([illegible] type), to a pure malignant [illegible] or complete undifferentiated type of [illegible] containing small basophilic [illegible].

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Walsh, et. al. ([illegible], p. [illegible]) [illegible] history and pathology of [illegible] malignant hepatoma, [illegible] in the liver of [illegible] Kendal rat fed [illegible] plantable in the [illegible] suitable for studies [illegible] parenchymal cell being [illegible] depended.

Many researches [illegible] changes of the [illegible]. [illegible] group (Price, et. al., [illegible], p. [illegible]), [illegible] in liver protein in [illegible] localized in the large granules [illegible]. Increases of protein and [illegible] for the nuclear fractions, and decreases in the amounts of these constituents in the large granule fraction along with an increase of the acid in the supernatant fluid fraction were also reported by the group (Price, et. al., 1950, p.27). Price, et. al., (1949, p.101), also reported a protein increase of 52% in the nuclear fraction of the tumors and a 63% decrease in protein for the large granule fraction as compared to corresponding fractions of the normal liver. This work was also conducted on related azo dyes (Price, et. al., 1949a, p.398).

Succinoxidase activity values were found to correlate most clearly with the values for mitochondrial protein, in accordance with the idea that the enzyme is located in the mitochondria (Potter, et. al., 1950, p.34). Numerous other enzyme studies were undertaken to show possible alterations in tissues with tumors induced by DAB ingestion (Greenstein, 1943, p.423).

Yaekal and Tobias (1951, p.831), suggested that there may be a linear, direct relationship between the amount of nitrogen in the liver and the total mass of the organism in which the liver functions. They stated that the liver size of the normal rat, more particularly its protein content, varied with the body size. If the tissues of the host were augmented by a neoplasm, however, alterations in the liver nitrogen content occured. Rats nuclear fractions, and [illegible] constituents in the large granule fraction, [illegible] increase of [illegible] in the supernatant [illegible] were also reported by [illegible] p.27). Price, et al., ([illegible]) [illegible] protein increase of [illegible] tumors and a [illegible] decrease [illegible] for the large granule fraction as compared to corresponding fractions of the normal liver. [illegible] related [illegible] (Price, et al., [illegible]).

Succinoxidase activity [illegible] correlate most closely with the [illegible] protein, in accordance with the [illegible] located in the [illegible]. Numerous other [illegible] possible [illegible] DAB ingestion ([illegible]).

[illegible] suggests that there may be [illegible] amount of nitrogen in the liver [illegible] the organism [illegible] that the liver [illegible] its protein content, [illegible] tissues of the [illegible] alterations in [illegible].

bearing extra-hepatic tumors (mammary) induced by subcutaneous injections of methylcholanthrene were used in their experiments.

The most recent suggestion concerning the mode of action of the aminoazo dyes in the carcinogenic process was made by Miller, et. al. (1949, p.342), who referred to it as the Protein Depletion Hypotheses. This arose from the observation that the formation of liver tumors in the rats fed DAB, or any of its carcinogenic derivatives, is preceded by the accumulation of protein-bound derivatives of the dye in the liver. These bound-dyes have only been detected in the livers of species susceptible to azo dye carcinogenesis (Miller, E.C., and Miller, J.A., 1947, p.479). They suggested "that the dye-binding phase represented a period during which the liver cells could synthesize the proteins which were bound to the dye faster than they were removed by the dye-binding process and in amounts sufficient for the normal functioning of the tissue." However, once the level of bound-dye starts to fall with continued dye feeding, the dye-binding might have affected specific synthetic mechanisms in the cells to such an extent that they could no longer keep pace with the combined demands of normal function and removal of protein by the carcinogen . Viable cells might finally result which would

have completely lost those systems controlling normal growth and would represent the initial tumor cells. Such a concept is in agreement with the observed absence of protein bound-dye in liver tumors formed during the continuous feeding of the azo dye.

CHAPTER III

METHODS AND MATERIALS

A total of 48 Sprague-Dawley rats of both sexes, ranging in weight from 158 to 290 gms. were used in these experiments. These were housed in groups of four in wire-bottomed screen cages, in an air conditioned room maintained at approximately 75° Fahrenheit.

The animals were fed a high-fat, low-protein diet following Kline, et. al. (1946, p.5). The composition of this semi-synthetic diet is shown in Table I. The hepatic carcinogen, p-dimethylaminoazobenzene, was added in quantity enough to make the final carcinogen concentration 0.06% of the total diet. The diet was compounded in 10Kg. lots and refrigerated until used. The control diet was the same as the DAB diet except for the addition of the carcinogen. Unless otherwise stipulated the animals were always allowed food and water *ad libitum*.

After a period of 6 to 11 months on the described regimen the animals were sacrificed and the livers were removed and weighed. In cases where the animals died the carcasses were refrigerated until autopsy and liver examination could be completed.

The extirpated, blotted livers were weighed to the nearest tenth of a milligram and dried in an oven at

SECTION III

EXPERIMENTAL PROCEDURE

A total of [illegible] Sprague-Dawley [illegible] ranging in weight from [illegible] were used in these experiments. These were housed in [illegible] bottomed screen cages, [illegible] at approximately 75° [illegible].

For a [illegible] diet following [illegible] of this semi-synthetic diet is shown in Table [illegible] hepatic carcinogen, [illegible] in quantity enough to [illegible] tion 0.06% of the total diet. [illegible] in 10Kg. lots and [illegible] diet was the same [illegible] of the carcinogen. [illegible] were always allowed [illegible] water and diet ad libitum.

After a period of [illegible] regimen the animals were sacrificed and the livers were removed and weighed. [illegible] ination could be completed.

The [illegible] the nearest tenth of a gram [illegible]

105° C. The livers were then ground in a mortar and dried to constant weight, between weighings they were allowed to equilibrate with room temperature in a dessicator containing calcium chloride. The water content was calculated from the wet and dry liver weights.

Total lipid content of the dried liver samples was then determined by a modification of the Saxton fat extraction procedure as given by Kolmer and Boener (1941, p.249). Two ml. of concentrated hydrochloric acid and 20 ml. of distilled water were added to each sample and the mixture was stirred. Twenty ml. of purified ether were added, the mixture was occasionally stirred and then allowed to stand for 5 minutes. Twenty ml. of ethyl alcohol were added, the mixture was stirred and allowed to stand until the ether layered out on the surface. This layer, containing the lipids, was aspirated into a 250 ml. Erlenmeyer flask; three consecutive washings were made of the original mixture by adding 5 ml. of purified ether, agitating, and aspirating the ether layer into the flask.

Twenty ml. of purified ether were added to each sample and the above procedure was repeated in order to remove all the fat. The ether was distilled off over a water bath and 30 ml. of petroleum ether were added to

105° C. The livers were then [illegible] [illegible]
to constant weight, were weighed [illegible]
to equilibrium with room [illegible]
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the mixture was stirred. Twenty ml. of [illegible] ether
were added, the mixture was [illegible] and
allowed to stand for 5 minutes. [illegible] ml. of ethyl
alcohol were added, the mixture [illegible] allowed
to stand until the ether layer [illegible]
layer, [illegible]
ml. [illegible]
made of the [illegible]
ether, [illegible]
flask.

Two ml. of [illegible]
[illegible]
remove all [illegible]
water bath and 30 ml. [illegible]

the residue. The flask was stoppered with a cork and allowed to stand overnight. The petroleum ether solution was filtered, the filter paper washed several times with petroleum ether and the filtrate collected in a tared 100 ml. beaker. The solvent was evaporated over a hot plate and the residue was dried in an oven at 100°C., cooled in a dessicator and weighed to constant weight.

The nitrogen content of the dry, fat-free livers was determined by a semi-micro modification of the Kjeldahl procedure as given by Marcali and Riemann (1946, p.709). Precautions were taken to remove phosphorous in order to increase the accuracy of the procedure as suggested by Marcali and Riemann (1948, p.381). One-half gm. aliquots of the fat-free dried livers were used in all nitrogen determinations. Repeated checks on this procedure using nitrogen containing compounds and normal rat liver extablished it to be both accurate and precise. Protein concentrations were computed as nitrogen content times the factor 6.25.

Another experiment devised to study protein metabolism in tumor bearing animals as compared to non-tumorous controls was conducted. Five male animals fed the DAB regimen and five male controls fed the basal diet for nine months were used. They were paired as to the residue. The flask was [illegible] allowed to stand overnight. The [illegible] was filtered, the filter paper [illegible] petroleum ether and the filtrate [illegible] 100 ml. beaker. The solvent was [illegible] plate and the residue was dried [illegible] 105°C., cooled in a desiccator and weighed [illegible]

The nitrogen content of [illegible] was determined by a semi-micro modification of the Kjeldahl procedure as given by Hawk [illegible] p.709). Preliminary [illegible] in order to increase the accuracy [illegible] suggested by [illegible] gm. aliquots of the rat-[illegible] livers were used for all nitrogen determinations. [illegible] procedure using [illegible] rat liver [illegible] Protein concentrations were calculated as [illegible] times the factor 6.25.

Another [illegible] metabolism in [illegible] tumorous controls was [illegible] the DAB regime, and five male [illegible] diet for nine months were used. [illegible] weight and general external condition and were pair-fed over a period of 20 days. On the twentieth day the rats were placed in metabolism cages equipped with glass funnels which separated the urine from the feces. The urine was collected under tolulene during an 18 hour fasting period. Water was allowed ad libitum during the fasting period and the quantity consumed was measured. At the end of the fast and urine collection period the animals were sacrificed by decapitation and the blood from the trunk was collected in a heparinized syringe, barrel removed, with the aid of a small funnel. The blood proteins were precipitated with sodium tungstate and sulfuric acid. Urinary proteins were precipitated with 15% trichloracetic acid (4% of the diluted urine volume) in order to obtain accurate non-protein nitrogen values. The latter step was taken because of a report that male rats excrete appreciably more urinary protein than do females (Sellers, et. al., 1950, p.667). Linkswiler, et. al. (1952, p.507), had also reported high urinary protein concentrations in rats bearing liver tumors induced by azo dye feeding.

The blood and urine non-protein nitrogen concentrations were determined colorimetrically according to the method of Koch and McMeekin (1924, p.2066). All original urine dilutions were 1:100. A blank and standard weight and general external condition and were measured over a period of [illegible] days. On the [illegible] the rats were placed in metabolism [illegible] with glass funnels which separated the urine from the feces. The urine was collected under toluene daily, [illegible] fasting period. Water was allowed ad libitum during the fasting period and the quantity consumed was recorded. At the end of the last [illegible] urine collection the animals were sacrificed by decapitation and the blood from the trunk was collected in a [illegible] syringe, barrel removed, with the aid of a small funnel. The blood proteins were precipitated with [illegible] and sulfuric acid. Urinary proteins were precipitated with 15% trichloroacetic acid (5% of the [illegible] urine volume) in order to obtain accurate non-protein nitrogen values. The latter step was taken because of a report that male rats excrete appreciably more urinary protein than do females (Sellers, et al., 1950, p.497). Linkswiler, et al. (1952, p.509), had also reported high urinary protein concentrations in rats bearing liver tumors induced by azo dye feeding.

The blood and urine non-protein nitrogen concentrations were determined colorimetrically according to the method of Koch and McMeekin (1924, p.2066). All original urine dilutions were 1:100 [illegible] were used for each determination. Blood creatinine determinations were made as described in the Klett-Summerson Clinical Manual. Urinary creatinine values were determined colorimetrically on 5 ml. of a 1:100 dilution of the collected urine. A Klett-Summerson photoelectric colorimeter was used for all the colorimetric determinations.

were used for each determination. [illegible] determinations were made as outlined in [illegible] Summerson Clinical Manual. Urinary creatinine values were determined colorimetrically on 1 ml. of a [illegible] dilution of the collected urine. A Klett-Summerson photo-electric colorimeter was used for all the colorimetric determinations.

CHAPTER IV

RESULTS

A. STUDIES ON LIVER COMPOSITION (See Table 2).

Results of the analyses of livers from rats maintained on the previously described dietary regimens (Chapter III) are given below. Results were considered to be significant when the p value was 0.05 or less.

Water Content. The livers of animals maintained on the control diet contained an average of 74.7% water as compared to 78.0% for the livers of animals fed the DAB diet (Table 2). This difference was found to be significant ($p = 0.01$). When the percentage water in livers was plotted against the entire liver weight it was apparent that the liver water tended to increase with the liver size but not in a linear fashion (Figure 1). When the liver weight in grams was plotted against the water weight in grams there was a linear increase in water content with an increase in liver size (Figure 2).

Solids. The liver solids content of the DAB maintained animals was 22.1% as compared to 25.3% for the control animals. This difference was found to be significant ($p = 0.01$). The relationship of the weight of liver solids to total liver weight was determined. As can be seen in Figure 3, there was a near linear increase in liver

CHAPTER IV

RESULTS

A. STUDIES ON LIVER COMPOSITION (Series 2). [illegible]

Results of the analyses of livers from rats maintained on the previously described diets in Series [illegible] (Chapter III) are given below. Results were considered to be significant when the p value was .05 or less.

Water Content. The livers of animals maintained on the control diet contained an average of [illegible] water as compared to 71.0% for the livers of animals on the DAB diet (Table 2). This difference was found to be significant (p=0.01). When the percentage water in livers was plotted against the relative liver weight it was shown that the liver water tended to increase with the liver size but not in a linear fashion [illegible] liver weight in grams [illegible] in grams there was a [illegible] increase [illegible] an increase in [illegible] (Figure [illegible]).

Solids. The liver [illegible] maintained animals [illegible] control animals. This difference [illegible] ficant (p=0.01). The [illegible] solids to total [illegible] seen in Figure 3, [illegible] solids with an increase in liver weight. In other words the total solids increased in the livers but the percentage solids decreased with an increase in size.

Lipids. No significant difference was found between the percentage lipids in the livers of the two groups of animals (Table 2). In general, as would be expected, the total fat content increased with an increase in liver size.

Liver nitrogen and protein. The total liver nitrogen and total liver protein values were found to be higher in the DAB fed animals than in the controls (Table 2, and Figure 4).

Percentage liver protein was calculated on the basis of the protein content of the wet liver, the dried liver, and the defatted dry liver. Regardless of how expressed, the percentage protein content was significantly smaller for the livers from the DAB fed animals (Table 2). The greatest difference was obtained on a wet liver weight basis, 17.1 and 13.4% respectively for animals on the basal and DAB regimens. The relationship between the percentage protein and liver wet weight is shown in Figure 5.

The total hepatic protein content of the rats maintained on the DAB and basal regimens was plotted (log scale) against the total liver weight and the dry liver weight (Figure 6). This plot shows that increase of protein in animals fed DAB occurs with increase in liver size solids with an increase in [illegible] the [illegible] increase [illegible] the total solids increased in the livers and the [illegible] age solids decreased with [illegible] increase in [illegible].

Lipids. [illegible] significant difference was [illegible] between the percentages of [illegible] in the livers of the [illegible] groups of animals (Table 2). Therefore, as would be expected, the total [illegible] increase [illegible] in liver size.

Liver nitrogen and protein. [illegible] total [illegible] nitrogen and total liver protein were [illegible] higher in the DES [illegible] animals than [illegible] (Table 2, and Figure 5).

Expressed [illegible] liver protein [illegible] basis of the protein content of [illegible] liver, and the [illegible] expressed, the [illegible] percentage protein [illegible] smaller for the livers from the [illegible] (Table 2). The greatest difference [illegible] basis, 17.1 and 14.[illegible] respectively [illegible] basal and DES [illegible] centage protein [illegible] (Figure 5)

The total [illegible] protein [illegible] maintained [illegible] the DES [illegible] animals [illegible] weight [illegible] and the [illegible] weight (Figure 6). [illegible] tein in animals fed DES [illegible] regardless of the amount of water in the liver. However, as shown previously, the water content does affect the percentage protein values.

Body weight, liver weight, and liver condition. The livers of all the rats maintained on the DAB diet from six to eleven months showed some type of abnormality. In some livers macroscopic nodules were absent, in some they were small and diffusely scattered, and in others only one or several large nodular areas were present. The nodular areas varied in texture as well as in size. Some were firm masses, others appeared somewhat glandular, and still others were fluid filled cysts. In those livers lacking nodules there was either enlargement or discoloration.

The liver weights of these animals ranged from 8 - 71 gm. and averaged 29 gm. None of the livers removed from the control rats appeared abnormal.

Although sex differences may be a factor influencing liver protein content in DAB fed animals no attempts were made to establish such differences in these studies.

B. STUDIES ON NON-PROTEIN NITROGEN AND CREATININE (See Table 3).

Blood creatinine. Although the blood creatinine level tended to be higher in the DAB fed animals (1.6 mg. %)

[illegible] of the amount of [illegible], it were as shown previously, the volume [illegible] from [illegible] [illegible] values.

<u>Gross and [illegible]</u> The livers of all [illegible] were [illegible] from six to eleven [illegible]. In some livers [illegible] they were [illegible] only one or several [illegible]. The nodular areas [illegible]. Some were [illegible], others [illegible] and still others were [illegible]. Incising [illegible] there was [illegible] sition.

The liver weights of [illegible] ranged from 8 - 71 gm. and averaged 28 gm. [illegible] from the control [illegible].

Although sex differences [illegible] Ethionine liver [illegible] attempts were made to [illegible] in [illegible] studies.

E. STUDIES OF [illegible] (See Table 3).

<u>Blood [illegible]</u>. Although the [illegible] level [illegible]

than in the control animals (1.5 mg. %), the difference was not found significant.

Urine creatinine. The concentration of creatinine was significantly higher ($p=0.01$) in the urine of the DAB fed animals (346 mg. %) than in the urine of the control animals (179 mg. %). However the total amount excreted was significantly lower ($p=0.005$) in the urine of the DAB fed animals as compared to the urine of the control rats; 1.7 and 2.6 mg./18 hr./100 gm. body weight respectively.

Blood non-protein nitrogen. The non-protein nitrogen concentration was slightly lower, but not significantly so, in the blood of the DAB maintained animals (55.8 mg. %) as compared to the control animals (56.6 mg. %).

Urine non-protein nitrogen. The concentration of non-protein nitrogen was found to be higher, although not significantly so, in the urine of the control animals (288 mg. %). However the total amount excreted was significantly lower ($p=0.001$) in the urine of the DAB fed animals as compared to the urine of the control animals; 2.1 and 3.7 mg./18 hr./100 gm. body weight respectively.

Water consumption and urine excretion. Both groups of animals consumed equal amounts of water (2.9 ml./18 hr./100 gm. body weight). The DAB fed animals than in the control animals (1.7 mg. %), the difference was not found significant.

Urine creatinine. The concentration of creatinine was significantly higher (p=0.01) in the urine of the DAB fed animals (345 mg. %) than in the urine of the control animals (179 mg. %). However the total amount excreted was significantly lower (p=0.005) in the urine of the DAB fed animals as compared to the urine of the control rats; 1.7 and 2.6 mg./18 hr./100 gm. body weight respectively.

Blood non-protein nitrogen. The non-protein nitrogen concentration was slightly lower, but not significantly so, in the blood of the DAB maintained animals (55.8 mg. %) as compared to the control animals ([illegible] mg. %).

Urine non-protein nitrogen. The concentration of non-protein nitrogen was found to be higher, although not significantly so, in the urine of the control animals (288 mg. %). However the total amount excreted was significantly lower (p=0.001) in the urine of the DAB fed animals as compared to the urine of the control animals; 2.1 and 3.7 mg./18 hr./100 gm. body weight respectively.

Water consumption and urine excretion. Both groups of animals consumed equal amounts of water ([illegible] ml./18 hr./100 gm. body weight). The DAB fed animals excreted a significantly (p=0.01) smaller volume of urine than the control animals; 0.5 and 1.8 ml./18 hr./100 gm. body weight respectively.

Body weight, liver weight, and liver condition. Although the average weight at sacrifice was the same (278 gm.) for the control and experimental animals, the liver weight was significantly greater (p=0.005) for the DAB fed animals (Table 3. The livers of the 5 experimental rats used in the urinary creatinine and non-protein nitrogen excretion studies were examined after the animals were sacrificed. None of these livers contained any large nodular areas but each was enlarged in size and pale in color. The control animals were found to have normal livers with no apparent changes in size, color, or texture.

excreted a significantly (p=0.01) smaller volume of urine than the control animals; 0.6 and 1.2 ml./18 hr./100 gm. body weight respectively.

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CHAPTER V

DISCUSSION

Several workers, some of whom used control animals fed a stock diet in carcinogenic studies, reported that the liver water content of such animals was approximately 70% of the liver weight (Kosterlitz, 1947, p.198; Robertson and Kahler, 1941, p.596; Maver, 1941, p.281). Griffin, et. al. (1948, p.1227), found the liver water to range from 74 - 76% in their control rats fed the basal ration lacking the carcinogen. These figures are in agreement with the values found in the present study (See table 2). These workers also found that after an azo-dye feeding period of 8 weeks the liver water content of rats ranged from 75 - 78% (Griffin, et. al., 1948, p.1228). The average liver water content of rats fed DAB in our prolonged study was found to be 78% (Table 2).

It has been generally accepted that the presence of atypical hepatic growth due to azo-dye ingestion caused increased content of liver water. Greenstein (1943, p.430), suggested that, "the proportion of water in hepatomas irrespective of origin, strain, or species of animal appears to be distinctly greater than in normal liver". It has also been found that the livers of animals bearing extra-hepatic tumors (Walker Carcinoma 256) contained an

CHAPTER V

DISCUSSION

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extra-hepatic [illegible] [illegible] [illegible] increased water content (Green, et. al., 1950, p.773). McEwen and Haven (1941, p.150), also reported that the percentage of water in the livers of rats bearing carcinosarcoma 256 was found to be significantly higher than that in the livers of normal rats. The increase in liver water occured independently of the diets used and was not accounted for by a decrease in the dry residue.

From our data we concluded that as the liver enlarges, due to atypical growth, large quantities of water accumulate and in general a greater water content, both total and percentage-wise, is associated with an increased liver size. Livers with huge tumors contained as much as 55 gm. of water, an amount equivalent to the weight of approximately 6 entire normal livers. Increase in water content with increased liver size was found for the livers of control animals but no percentage change occurred.

In studies of the type reported here it is pertinent to stress the importance of feeding control animals the same dietary ingredients, excepting the carcinogen, as the experimental animals. It was noticed while surveying the literature on liver water in azo dye fed rats that the difference in water content between stock-fed and basal-diet fed animals was not emphasized. Other workers in our laboratory have observed this difference and have increased water content (Green, et. al., 1956, p.773). McEwen and Haven (1941, p.150), also reported that the percentage of water in the livers of rats bearing Walker carcinosarcoma 256 was found to be significantly higher than that in the livers of normal rats. The increase in liver water occurred independently of the diets used and was not accounted for by a decrease in the dry residue.

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The results of this experiment also indicate that a decrease in the percentage of total liver solids is associated with increase in liver size. A significant difference was found between the percentage solids contents of the DAB fed and the control animals. The percentage solids content was significantly lower in the livers of the DAB fed rats (Table 2). Robertson and Kahler (1941, p.596), while measuring hepatic riboflavin levels, reported that the percentage solids content was lower in the livers of DAB fed animals than in the control livers. This has also been found by the workers cited above in the discussion on liver water.

The results of our experiments show that there is no significant difference in the percentage fat content of the livers of rats fed DAB as compared to the livers of control animals. Donnan (1950, p.416), found that hepatomas from rats fed DAB gave low percentage fat values, but since the range of values for the remaining tissues of normal appearance was wide, the decrease was not significant. It appeared from her work that different areas of the livers from DAB fed animals have essentially the found it to be a [illegible] of certain enzyme systems [illegible] 1953). Apparently the [illegible] of these [illegible] has not been [illegible] already are [illegible].

The results of this experiment [illegible] that a decrease in the [illegible] is associated with [illegible]. A significant difference was found between the [illegible] of the DAB fed and the control animals. [illegible] solids content was significantly lower [illegible] the DAB fed rats (Table [illegible] [illegible]), while [illegible] that the percentage [illegible] of DAB fed animals than in the control [illegible] also been found [illegible] on liver water.

The results of [illegible] is no significant [illegible] of the livers of rats fed [illegible] of control animals, [illegible] hepatoma [illegible] but since [illegible] of normal [illegible] difference. It [illegible] of the livers from [illegible] same fat content. From these data we concluded that no alteration in the percentage liver fat content results from feeding rats a DAB containing diet.

We found a decreased percentage protein content in the livers of DAB fed animals as compared to control animals. The decrease in percentage protein on a dry as well as wet liver weight basis indicated that the change was not due entirely to the increased water content of the liver (Table 2). It is possible that there was a relative increase in some undetermined liver constituent, possibly carbohydrate. In general, most investigators who have worked with the protein content of livers from azo-dye fed rats have selected tumorous portions for analyses. Mills and Smith (1951, p.691), while working on the B-glucuronidase activity of chemically induced rat hepatomas, noted that there was less protein in the tumor cells than in normal liver cells and that the percentage protein content decreased in the tumorous livers as compared to control livers. Although they used small groups of rats, they found that the control livers averaged 15% protein whereas the tumorous livers averaged 12% protein after a DAB feeding period of 21 weeks.

Price, et. al. (1948, p.348), worked on the intracellular distribution of protein in the livers of rats same fat content. [illegible] these [illegible] compared [illegible] alteration in the [illegible] from feeding [illegible]

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[illegible] cellular [illegible]

fed DAB for 4 weeks. Differential centrifugation methods were used to isolate the intra-cellular components. They reported that there was less protein per gm. of wet liver tissue in the DAB fed animals than in the livers of the control animals. Striebich, et. al. (1953, p.281), worked with liver homogenates and reported that there was only a slightly reduced percentage of nitrogen in the livers of azo-dye fed rats.

Many investigators have been primarily concerned with the nitrogen content of the hepatomas rather than the entire livers from rats fed DAB. Maver, et. al. (1941, p.281), found only a slight total nitrogen increase in the dried hepatoma tissue over the remaining normal tissue of the liver. Apparently there was little difference in nitrogen content between the hepatoma and the remainder of the liver. This suggested that the alteration in protein is not restricted to the malignant areas but is manifest throughout the entire liver. Griffin, et. al. (1948, p.1228), reported that the percentage nitrogen in the hepatoma was lower than in the control liver. These nitrogen data for the hepatoma were found to correlate with our findings for the entire liver, that is, an increased total content but a decreased percentage content.

The results of the experiment concerned with fed DAB for 4 weeks [illegible] [illegible] [illegible] were used to [illegible] [illegible] [illegible] suspected that there [illegible] [illegible] [illegible] tissue in the DAB fed animals than in the [illegible] control animals. [illegible] [illegible] (1951, [illegible]) with liver homogenates [illegible] reports [illegible] a slightly reduced [illegible] [illegible] of azo-dye fed rats.

Many investigators have [illegible] [illegible] with the nitrogen [illegible] [illegible] the entire liver [illegible] rats [illegible] [illegible], et. al. (1947, p.252), found only a slight [illegible] nitrogen [illegible] in the dried [illegible] and [illegible] [illegible] of the liver. [illegible] [illegible] [illegible] difference in nitrogen content between [illegible] [illegible] of the liver. This suggested [illegible] [illegible] tein is not [illegible] [illegible] manifest throughout the whole liver. [illegible], et. al. (1948, p.1228), [illegible] the hepatoma [illegible] the normal liver. [illegible] nitrogen [illegible] with our findings [illegible] liver, [illegible] creased total content [illegible] [illegible].

The results [illegible]

urinary non-protein nitrogen and creatinine excretion must be interpreted with caution. The concentration of both these materials increased (per unit volume) in the DAB fed animals. This apparently represented a faster breakdown of nitrogenous products, metabolically speaking, as compared to the control values. However, when the total amount of creatinine and non-protein nitrogen excreted was calculated (mg./18 hr./100 gm. body weight) it was apparent that the DAB fed animals lost smaller amounts of both these materials than did the controls.

The difference between the values expressed on concentration bases was obviously due to the difference in the volumes of urine excreted by the two groups of animals. The DAB fed animals excreted significantly smaller volumes of urine than did the control animals.

In the chemical determination of non-protein nitrogen and creatinine it was noted that large amounts of protein were precipitated from the urine excreted by the DAB fed animals. This suggested that aside from an important alteration in the metabolism of protein by the liver, a secondary alteration in kidney function resulted from DAB ingestion. Edwards and White (1941, p.177), reported the presence of a brown granular pigment in the tubular cells and macrophages of the renal cortex as a

result of feeding DAB to rats. It was apparently identical, according to them, to the pigment found in the kidneys of rats receiving compounds of lead. This seemed to be added evidence that renal changes occur in DAB feeding.

It was decided that chronic experiments would have to be conducted to determine whether DAB fed animals consistantly excrete such small volumes of urine. This work will be repeated on larger numbers of rats, half of which have been maintained for the past five months on the DAB containing diet. The animals will be forced-fed a liquid diet so as to control the caloric and liquid intake.

CHAPTER VI

SUMMARY AND CONCLUSIONS

1. In comparison with control animals the percentage water in livers of Sprague-Dawley rats fed DAB for 6 - 11 months was significantly increased, whereas the percentage liver solids content was significantly decreased.

2. The total liver nitrogen (protein) content of rats maintained on the DAB regimen, under the conditions of this experiment, increased as the size of the liver increased, but the percentage protein in wet or dry livers was significantly lower than the control values.

3. The percentage fat content in the livers of the DAB fed animals was the same as the control values.

4. Under the stated experimental conditions, the urine of DAB fed animals showed higher concentrations of non-protein nitrogen and creatinine as compared to the urine of control animals. However the total output of non-protein nitrogen and creatinine /18 hr./100 gm. body weight was lower in experimental animals. This was related to a reduced urine volume.

CHAPTER IV

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LITERATURE CITED

Donnan, Sara Kathryn. 1950. The thiobarbituric acid test applied to tissue from rats treated in various ways. Jour. Biol. Chem., vol. 182, no. 1, pp. 415-419.

Edwards, Jesse E., and Julius White. 1941. Pathologic changes with special reference to pigmentation and classification of hepatic tumors in rats fed p-dimethylaminoazobenzene. Jour. Natl. Cancer Inst., vol. 2, no. 2, pp. 157-183, 27 figs., 1 tbl.

Green, John W. Jr., Earl P. Benditt, and Eleanor N. Humphreys. 1950. The effect of protein depletion on the host response to transplantable rat tumor Walker 256. Cancer Res., vol. 10, no. 12, pp. 769-774, 2 tbls.

Greenstein, Jesse P. 1943. Tumor enzymology. Jour. Natl. Cancer Inst., vol. 3, no. 4, pp. 419-447, 6 tbls.

Griffin, A. C., William N. Nye, Lafayette Noda, and J. Murray Luck. 1948. Tissue proteins and carcinogenesis. Jour. Biol. Chem., vol. 176, no. 3, pp. 1225-1235, 1 fig., 3 tbls.

Dennan, [illegible]. 1939. [illegible]
applied to [illegible] trends in [illegible]
[illegible]. [illegible], vol. [illegible]
pp. [illegible].

Edwards, Jesse E., and [illegible]. 194[illegible]. [illegible]
changes with [illegible]
and [illegible] in [illegible]
[illegible]. [illegible]
Cancer Inst., [illegible]
27 figs., 1 [illegible].

Green, John W. Jr., [illegible]
195[illegible]. [illegible]
[illegible]
[illegible] Cancer Res., vol. [illegible], no. [illegible],
p. [illegible].

Greenstein, Jesse P. 194[illegible]. [illegible]
[illegible], vol. 3, [illegible]

Griffin, A. C., [illegible]
[illegible] 195[illegible]. [illegible]
[illegible] Cancer Res., vol. [illegible], [illegible]
no. 3, pp. [illegible].

Griffin, A.C., C.C. Clayton, and C.A. Baumann. 1949. The effects of casein and methionine on the retention of hepatic riboflavin and on the development of liver tumors in rats fed certain azo dyes. Cancer Res., vol. 9, no. 2, pp. 82-87, 5 tbls.

Harris, Paul N., M.E. Krahl, and G.H.A. Clowes. 1947. Paradimethylaminoazobenzene carcinogenesis with purified diets varying in content of cysteine, cystine, liver extract, protein, riboflavin, and other factors. Cancer Res., vol. 7, no. 3, pp. 162-175, 24 figs., 3 tbls.

Kensler, C.J., Kanematsu Sugiura, N. F. Young, C. R. Halter, and C.P. Rhoads. 1941. Partial protection of rats by riboflavin with casein against liver cancer caused by dimethylaminoazobenzene. Sci., vol. 93, no. 2413193, pp. 308-310, 1 fig., 1 tbl.

Kline, B.E., J.A. Miller, H.P. Rusch, and C.A. Baumann. 1946. Certain effects of dietary fats on the production of liver tumors in rats fed p-dimethylaminoazobenzene. Cancer Res., vol. 6, no. 1 pp. 5-7, 1 tbl.

____. 1946a.

Griffin, A.C., [illegible] 19[illegible]. The effect of [illegible] on the [illegible] development [illegible]. [illegible], pp. [illegible].

Harris, [illegible], and [illegible]. 19[illegible]. [illegible] of [illegible], [illegible], liver [illegible], [illegible] vol. [illegible], p. [illegible].

Hensler, C.J., [illegible]. 19[illegible]. [illegible] of rats by [illegible] [illegible]. [illegible].

Kline, [illegible] 19[illegible]. [illegible] production of [illegible] [illegible]. [illegible] p. [illegible].

The carcinogenicity of p-dimethylaminoazobenzene in diets containing the fatty acids of hydrogenated coconut oil or corn oil. Cancer Res., vol. 6, no. 1, pp. 1-4, 2 tbls.

Koch, F.C., and T.L. McMeekin. 1924. A new direct Nesslerization micro-Kjeldahl method and a modification of the Nessler-Folin reagent for ammonia. Jour. Amer. Chem. Soc., vol. 46, no. 9, pp. 2066-2069, 3 tbls.

Kolmer, John A., and Fred Boerner. 1941. Approved laboratory technic. Third Edition. D. Appleton-Century Co., New York City, New York, xxvii & 921 pp., 380 figs., 12 pls., 36 tbls.

Kosterlitz, H.W. 1947. The effect of changes of the dietary protein on the composition and structure of the liver cell. Jour. Physiol., vol. 106, no. 2, pp. 194-210, 1 fig., 3 tbls.

Linkswiler, Helen, May S. Reynolds, and C.A. Baumann. 1952. Factors affecting proteinuria in the rat. Amer. Jour. Physiol., vol. 168, no. 2, pp. 504-508, 3 tbls.

Mc Ewen, H. Douglas, and Frances L. Haven. 1941. The effect of carcinosarcoma 256 on the water content of the liver. Cancer Res., vol. 1, no. 2, pp. 148-150, 5 figs.

Marcali, Kalman, and William Riemann III. 1946. Kjeldahl determination of nitrogen: elimination of the distillation. Indust. and Engineer. Chem., vol. 18, no. 11, pp. 709-710, 1 tbl.

______________________________. 1948. Kjeldahl determination of nitrogen without distillation; application to samples containing phosphorous. Analyt. Chem., vol. 20, no. 4, pp. 381-382, 2 tbls.

Maver, Mary E., G.B. Mider, J.M. Johnson, and J.W. Thompson. 1941. The comparative proteinase and peptidase activities of rat hepatoma and normal and regenerating rat liver. Jour. Natl. Cancer Inst., vol. 2, no. 3, pp. 277-282, 5 tbls.

Miller, Elizabeth C., and James A. Miller. 1947. The presence and significance of bound aminoazo dyes in the livers of rats fed p-dimethylaminoazobenzene. Cancer Res., vol. 7, no. 7, pp. 468-480, 2 figs., 4 tbls.

Mc Ewen, H. Douglas, and Prosser L. Haven. 1941. The effect of carcinogenic 2HS on the water content of the liver. Cancer Res., vol. 1, no. 2, pp. 148-150, 5 figs.

Mazelli, Kalman, and William Siemens III. 1946. Kjeldahl determination of nitrogen: elimination of the distillation. Indust. and Engineer. Chem., vol. 18, no. 11, pp. 709-710, 1 tbl.

________. 1948. Kjeldahl determination of nitrogen without distillation: application to samples containing phosphorus. Analyt. Chem., vol. 20, no. 4, pp. 381-382, 2 tbls.

Maver, Mary E., G.E. Mider, J.M. Johnson, and J.W. Thompson. 1941. The comparative proteinase and peptidase activities of rat hepatoma and normal and regenerating rat liver. Jour. Natl. Cancer Inst., vol. 2, no. 3, pp. 277-282, 5 tbls.

Miller, Elizabeth C., and James A. Miller. 1947. The presence and significance of bound aminoazo dyes in the livers of rats fed p-dimethylaminoazobenzene. Cancer Res., vol. 7, no. 7, pp. 468-480, 2 figs., 4 tbls.

Miller, Elizabeth C., James A. Miller, R.W. Sapp, and G.M. Weber. 1949. Studies on the protein-bound aminoazo dyes formed in vivo from 4-dimethylaminoazobenzene and its C-monomethyl derivatives. Cancer Res., vol. 9, no. 6, pp. 336-343, 5 figs.

Miller, James A., B.E. Kline, H.P. Rusch, and C.A. Baumann. 1944. The carcinogenicity of p-dimethylamino-azobenzene in diets containing hydrogenated coconut oil. Cancer Res., vol. 4, no. 3, pp. 153-158, 2 tbls.

Mills, G.T., and Evelyn E.B. Smith. 1951. The β-glucuronidase activity of chemically induced rat hepatomas. Sci., vol. 114, no. 2974, pp. 690-692, 2 tbls.

Opie, Eugene L. 1944. The pathogenesis of tumors of the liver produced by butter yellow. Jour. Exptl. Med., vol. 80, no. 3, pp. 231-246, 21 figs.

Potter, Van R., J.M. Price, E.C. Miller, and J.A. Miller. 1950. Studies on the intracellular composition of livers from rats fed various aminoazo dyes. III. Effects of succinoxidase and ozalacetic acid oxidase. Cancer Res., vol. 10, no. 1, pp. 28-35, 3 figs., 2 tbls.

Miller, Elizabeth C., James A. Miller, [illegible] 1949. [illegible] bound aminoazo dyes [illegible] Cancer Res., vol. 9, [illegible] pp. 336-343, 5 figs.

Miller, James A., [illegible] 1948. The carcinogenicity of para-dimethylaminoazobenzene in [illegible] Cancer Res., vol. 8, no. 4, pp. 153-158, 2 figs.

Mills, [illegible] [illegible] 592, 2 figs.

Opie, Eugene L. 1944. [illegible] liver produced by butter yellow. Jour. Exptl. Med., vol. 80, no. 3, pp. 231-246, 2 figs.

Potter, Van R., J.M. Price, E.C. Miller, and J.A. Miller. 1950. Studies on the intracellular composition of livers from rats fed various aminoazo dyes. III. Effects of [illegible] oxidase. Cancer Res., vol. 10, no. 1, pp. 28-35, [illegible]

Price, J.M., E. C. Miller, and J.A. Miller. 1948. The intracellular distribution of protein, nucleic acids, riboflavin, and protein-bound aminoazo dye in the livers of rats fed p-dimethylamino-azobenzene. Jour. Biol. Chem., vol. 173, no. 1, pp. 345-353, 4 tbls.

Price, J.M., J.A. Miller, E.C. Miller, and G.M. Weber. 1949. Studies on the intracellular composition of liver and liver tumor from rats fed p-dimethyl-aminoazobenzene. Cancer Res., vol. 9, no. 2, pp. 96-102, 4 figs., 5 tbls.

Price, J. M., E.C. Miller, J.A. Miller, and G.M. Weber. 1949 a. Studies on the intracellular composition of livers from rats fed various aminoazo dyes. I. 4-aminoazobenzene, 4-dimethylamino-azobenzene, 4-methyl-, and 3' methyl- 4-dimethyl-aminoazobenzene. Cancer Res., vol. 9, no. 7, pp. 398-402, 5 tbls.

Price, J.M., R. [illegible] and [illegible] Miller. 19[illegible] [illegible] [illegible] of [illegible] acid, [illegible] and the [illegible] in the liver of [illegible] [illegible]. [illegible] Biol. Chem., vol. [illegible], no. 1, pp. [illegible], [illegible].

Price, J.M., [illegible] Miller, [illegible] and [illegible] 1949. [illegible] of [illegible] of liver and liver [illegible] from rats [illegible] [illegible]. Cancer Res., vol. 9, [illegible] pp. [illegible], [illegible].

Price, J. M., [illegible] Miller, [illegible] Miller, [illegible] 1950. [illegible] in the [illegible] of liver [illegible] [illegible] [illegible]. [illegible] [illegible] [illegible] [illegible]. Cancer Res., vol. [illegible], pp. [illegible], [illegible].

____________________________. 1950. Studies on the intracellular composition of livers from rats fed various aminoazo dyes. II. 3'-methyl-, 2'-methyl-, and 2-methyl-4-dimethylaminoazobenzene, 3-methyl-4-monomethylaminoazobenzene, and 4'-fluoro-4-dimethylaminoazobenzene. Cancer Res., vol. 10, no. 1, pp. 18-27, 8 figs., 6 tbls.

Price, J.M., J.A. Miller, and E.C. Miller. 1951. The intracellular distribution of protein, nucleic acids, and riboflavin in the livers of mice and hamsters fed 4-dimethylaminoazobenzene. Cancer Res., vol. 11, no. 7, pp. 523-528, 2 figs., 5 tbls.

Robertson, William V.B., and Herbert Kahler. 1941. Riboflavin content of liver tumors. Jour. Natl. Cancer Inst., vol. 2, no. 6, pp. 595-600. 4 tbls.

Richmond, D.R., and W.J. Eversole. 1953. Unpublished data.

Rusch, H.P., C.A. Baumann, J.A. Miller, and B.E. Kline. 1945. Experimental liver tumors. <u>In</u> Moulton, F.R. Editor. A.A.A.S. research conference on cancer. A.A.A.S., Washington, D.C., pp. 267-287, I fig., 3 tbls.

______, 1950. Studies on the intracellular composition of livers from rats fed various aminoazo dyes. II. 3'-Methyl-, 2'-methyl-, and 2-methyl-4-dimethylaminoazobenzene, 3-methyl-4-monomethylaminoazobenzene, and 4'-fluoro-4-dimethylaminoazobenzene. Cancer Res., vol. 10, no. 1, pp. 18-27, 8 figs., 6 tbls.

Price, J.M., J.A. Miller, and E.C. Miller. 1951. The intracellular distribution of protein, nucleic acids, riboflavin, and protein-bound aminoazo dye in the livers of mice and hamsters fed 4-dimethylaminoazobenzene. Cancer Res., vol. 11, no. 7, pp. 523-528, 3 figs., 5 tbls.

Robertson, William v.B., and Norbert Kahler. 1942. Riboflavin content of liver tumors. Jour. Natl. Cancer Inst., vol. 2, no. 6, pp. 595-600. 4 tbls.

Richmond, D.H., and J.E. Edwards. 1953. Unpublished data.

Rusch, H.P., C.A. Baumann, J.A. Miller, and B.E. Kline. 1945. Experimental liver tumors. In Moulton, F.R. Editor. A.A.A.S. research conference on cancer. A.A.A.S., Washington, D.C., pp. 267-287, 1 fig., 3 tbls.

Sarett, Herbert P., Raymond Klein, and William A. Perlzweig. 1942. The effect of the level of protein intake upon the urinary excretion of riboflavin and nicotinic acid in dogs and rats. Jour. Nutrition, vol. 24, no. 3, pp. 295-306, 2 figs., 4 tbls.

Sarett, Herbert P., and William A. Perlzweig. 1943. The effect of protein and B-vitamin levels of the diet upon the tissue content and balance of riboflavin and nicotinic acid in rats. Jour. Nutrition, vol. 25, no. 2, pp. 173-183, 4 tbls.

Sellers, A.L., H.C. Goodman, J. Marmorston, and M. Smith. 1950. Amer. Jour. Physiol., vol. 163, no. 3, pp. 662-667, 2 figs., 1 tbl.

Striebich, Mary Jo, Emma Shelton, and Walter C. Schneider. 1953. Quantitative morphological studies on the livers and liver homogenates of rats fed 2-methyl- or 3'methyl-4-dimethylaminoazobenzene. Cancer Res., vol. 13, no. 3, pp. 279-284, 6 figs.

Sugiura, Kanematsu, and C.P. Rhoads. 1941. Experimental liver cancer in rats and its inhibition by rice bran extract, yeast, and yeast extract. Cancer Res., vol. 1, no. 1, pp. 3-16, 10 tbls.

Wesson, Laurence G. 1932. A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Sci., vol. 75, no. 1943, pp. 339-340, 2 tbls.

White, Julius, Albert J. Dalton, and Jesse E. Edwards. 1942. Pathology of rat hepatoma 31. Jour. Natl. Cancer Inst., vol. 2, no. 6, pp. 539-554, 12 figs.

Yeakel, Eleanor H., and Gordon L. Tobias. 1951. Liver nitrogen in tumor-bearing rats. Cancer Res., vol. 11, no. 2, pp. 830-833, 1 tbl., 1 chart.

Zamecnik, Paul C., and Mary L. Stephenson. 1947. Activity of catheptic enzymes in p-dimethylaminoazobenzene hepatomas. Cancer Res., vol. 7, no. 6, pp. 326-332, 7 tbls.

Wesson, Laurence G. 1932. A modification of the Osborne-Mendel salt mixture containing only inorganic constituents. Sci., vol. 75, no. 1943, pp. 339-340, 2 tbls.

White, Julius, Albert J. Dalton, and Jesse E. Edwards. 1942. Pathology of rat hepatoma 31. Jour. Natl. Cancer Inst., vol. 2, no. 6, pp. 539-554, 12 figs.

Yeakel, Eleanor H., and Gordon L. Tobias. 1951. Liver nitrogen in tumor-bearing rats. Cancer Res., vol. 11, no. 2, pp. 830-833, 2 [illegible], 1 chart.

Zamecnik, Paul C., and Mary L. Stephenson. 1947. Activity of catabolic enzymes in p-dimethylaminoazobenzene hepatomas. Cancer Res., vol. 7, no. [illegible], pp. 326-332, 7 tbls.

APPENDIX

TABLE 1

CARCINOGENIC DIET USING p-DIMETHYLAMINOAZOBENZENE ACCORDING TO KLINE

	gm/Kg
CERELOSE - GLUCOSE MONOHYDRATE (GENERAL BIOCHEMICALS, INC.)	640.0
CASEIN - VITAMIN FREE (G.B.I.)	120.0
+ SALTS MIXTURE	40.0
CORN OIL (MAZOLA)	200.0
++ p-DIMETHYLAMINOAZOBENZENE (EASTMAN ORGANIC CHEMICALS)	0.6

		mg/Kg
THIAMINE HYDROCHLORIDE	(G.B.I.)	3.0
RIBOFLAVIN	"	2.0
PYRIDOXINE HYDROCHLORIDE	"	2.5
CALCIUM PANTOTHENATE	"	7.0
CHOLINE CHLORIDE	"	30.0
# HALIVER OIL	(PARKE-DAVIS)	

+ MODIFICATION OF THE ORIGINAL OSBORNE-MENDEL SALT MIXTURE ACCORDING TO WESSON (1932, p. 339)

++ NOT INCLUDED IN THE BASAL CONTROL DIET

ADMINISTERED ORALLY, ONE DROP PER RAT PER MONTH

TABLE 1.

CARCINOGENIC DIET USING

p-DIMETHYLAMINOAZOBENZENE (DAB)

TABLE 2

LIVER WATER, SOLIDS, LIPID, & NITROGEN CONTENT OF RATS FED p-DIMETHYLAMINOAZOBENZENE

NO OF CASES		SOLIDS %	WATER %	LIPIDS %	TOTAL NITROGEN mg	TOTAL PROTEIN gm	% PROTEIN WET WEIGHT	% PROTEIN DRY WEIGHT	% PROTEIN DRY FAT-FREE WEIGHT
CONTROLS									
15	MEAN	25.3	74.7	3.21	228.4	1.42	17.11	67.2	78.2
	±SE	0.7	0.7	0.55	15.9	0.10	0.45	1.9	2.4
DAB									
23	MEAN	22.1	78.0	3.00	546.6	3.41	13.44	61.7	71.2
	±SE	0.9	0.9	0.25	64.5	0.40	0.43	1.3	1.5
	t	2.73	2.82	NS	4.80	4.85	5.83	2.48	2.48
	P	0.01	0.01		0.001	0.001	0.001	0.02	0.02

TABLE 2.

WATER, SOLIDS, LIPID, AND NITROGEN CONTENT OF LIVERS FROM RATS FED DAB

TABLE 3

CREATININE & NON-PROTEIN NITROGEN EXCRETION OF RATS FED DAB FOR NINE (9) MONTHS

NO. OF CASES		LIVER WEIGHT (GM)	WEIGHT AT SACRIFICE (GM)	WATER * CONSUMED	URINE * EXCRETED	CREATININE BLOOD mg %	CREATININE URINE mg%	CREATININE URINE ★	NON-PROTEIN N. BLOOD mg%	NON-PROTEIN N. URINE mg%	NON-PROTEIN N. URINE ★
CONTROLS											
5	MEAN	7.5	278	2.9	1.8	1.5	179	2.6	56.6	288	3.7
	± SE	0.6	21.2	0.7	0.4	0.11	39.9	0.2	7.68	101	0.3
DAB											
5	MEAN	12.1	278	2.9	0.5	1.6	346	1.7	55.8	425	2.1
	± SE	0.9	53	0.3	0.06	0.14	28.8	0.09	2.3	47.8	0.1
	t	4.38	NS	NS	3.25	NS	3.39	4.0	NS	NS	4.85
	p	< 0.005			< 0.01		< 0.01	< 0.005			< 0.001

* ml/18 HRS/100 gm BODY WEIGHT

★ mg/18 HRS/100 gm BODY WEIGHT

TABLE 3.

CREATININE AND NON-PROTEIN NITROGEN EXCRETION OF RATS FED DAB FOR NINE MONTHS

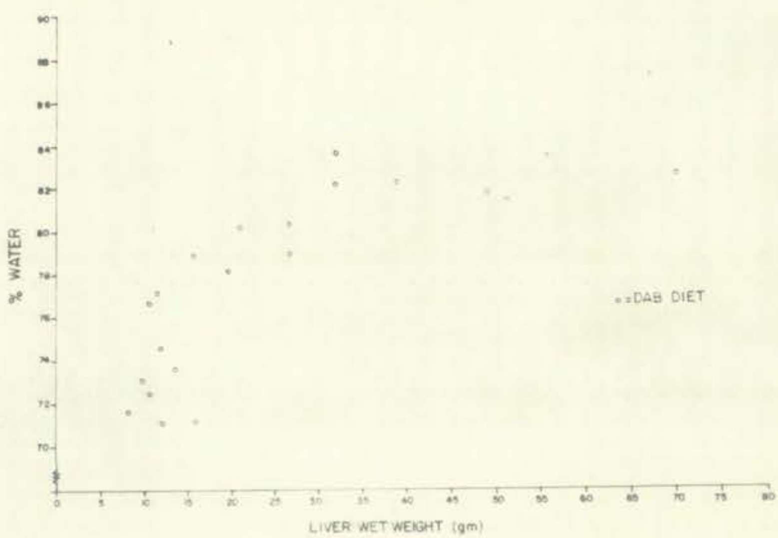

FIGURE 1.

RELATIONSHIP OF WET WEIGHT TO PERCENTAGE WATER IN LIVERS OF ANIMALS FED DAB

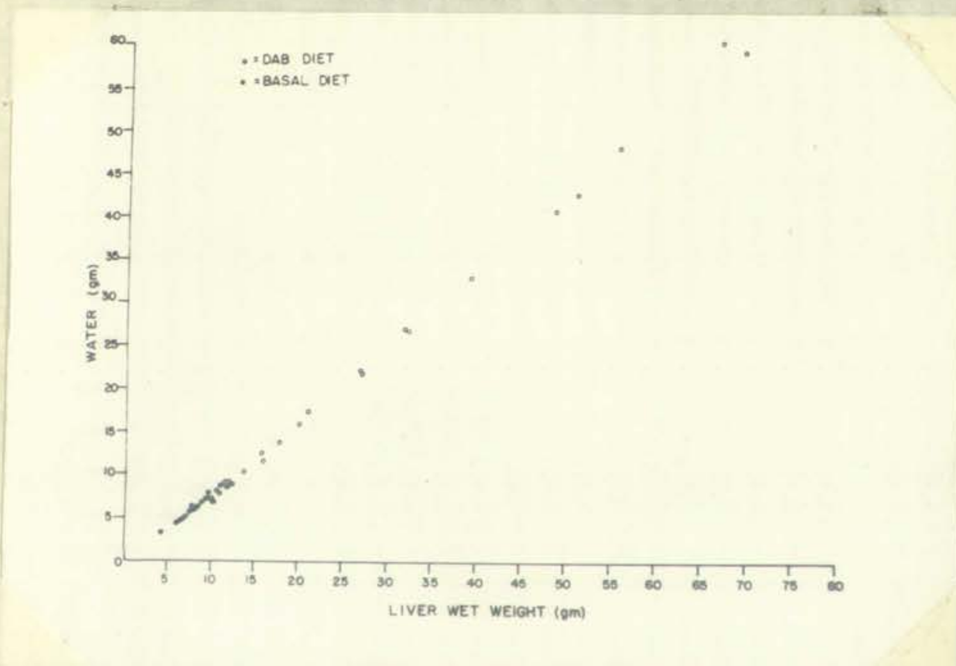

FIGURE 2.

RELATIONSHIP OF WET WEIGHT TO TOTAL WATER CONTENT IN LIVERS OF ANIMALS FED DAB AND BASAL DIETS

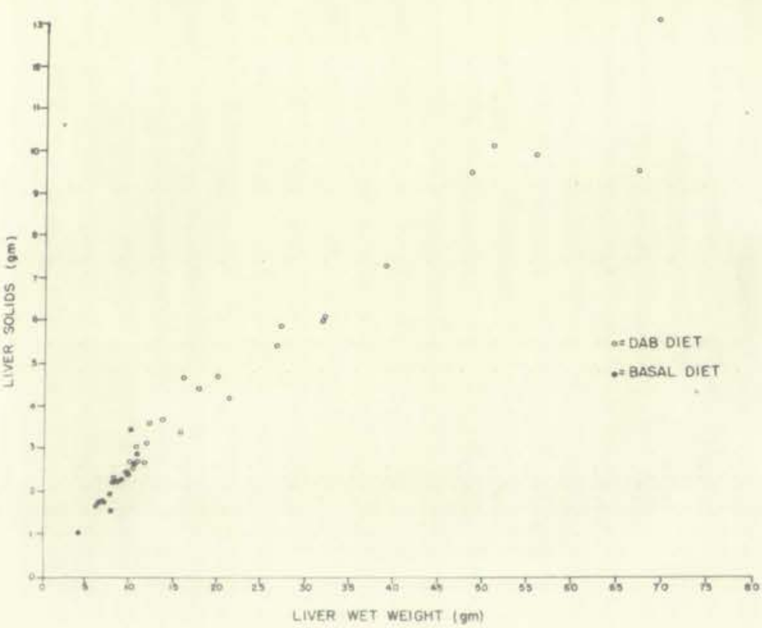

FIGURE 3.

RELATIONSHIP OF WET WEIGHT TO TOTAL LIVER SOLIDS
IN LIVERS OF ANIMALS FED DAB AND BASAL DIETS

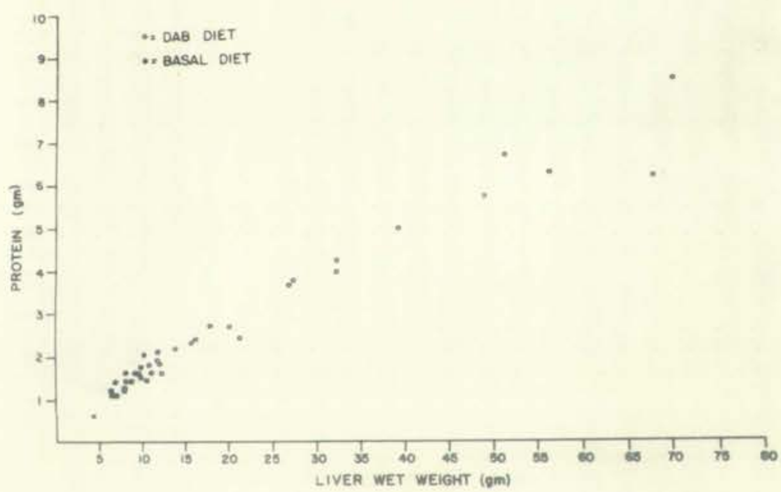

FIGURE 4.

RELATIONSHIP OF WET WEIGHT TO TOTAL PROTEIN IN LIVERS OF ANIMALS FED DAB AND BASAL DIETS

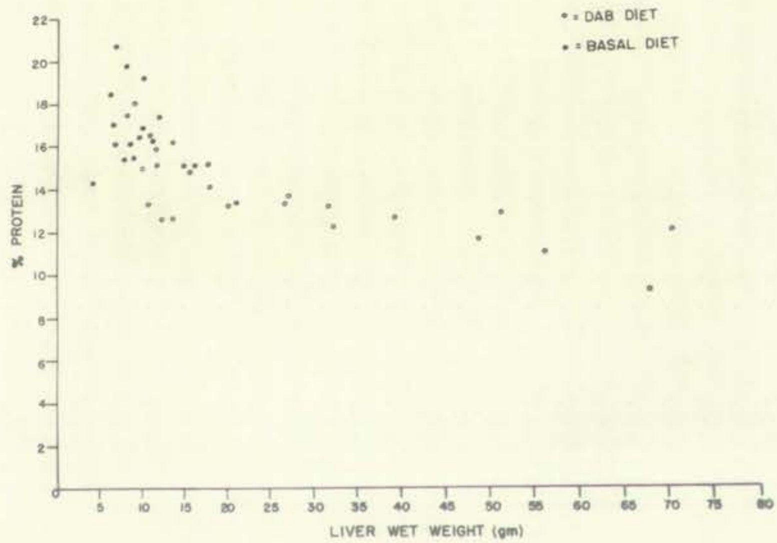

FIGURE 5.

RELATIONSHIP OF WET WEIGHT TO PERCENTAGE PROTEIN IN LIVERS OF ANIMALS FED DAB AND BASAL DIETS

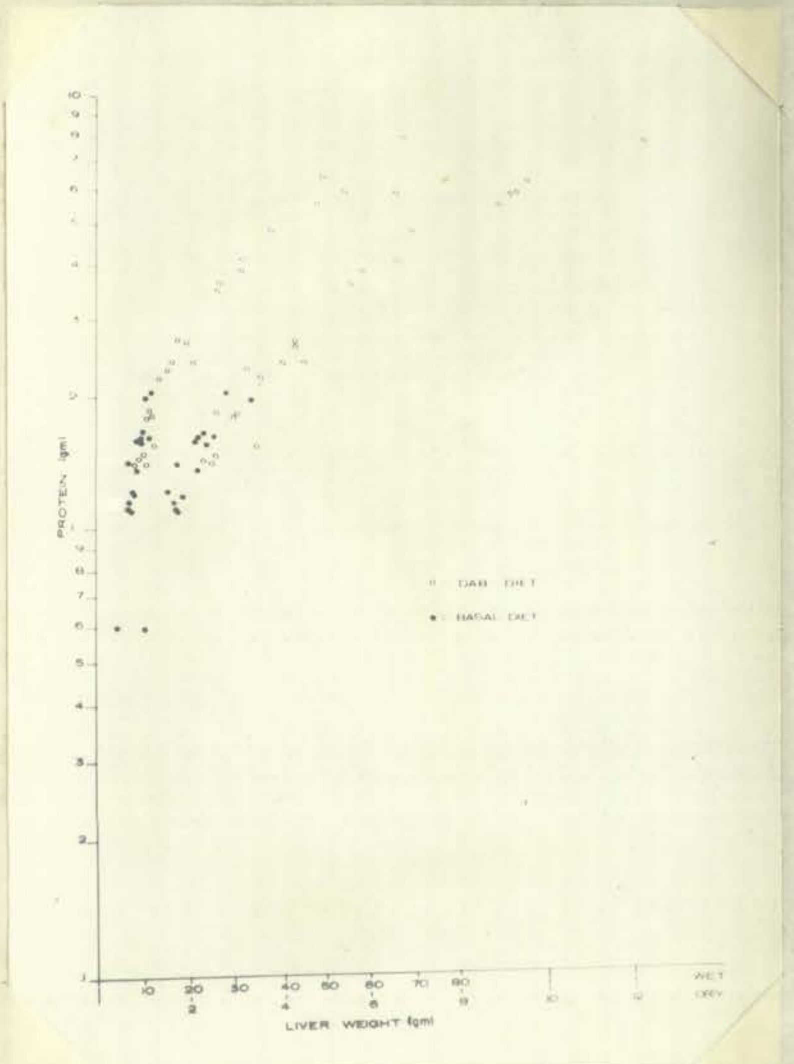

FIGURE 6.

RELATIONSHIP OF LIVER WEIGHT TO TOTAL PROTEIN IN LIVERS OF RATS FED DAB AND BASAL DIETS; SEMI-LOG PLOTS BASED ON LIVER WET WEIGHT AND LIVER DRY WEIGHT