THE CALCIUM, POTASSIUM, AND SODIUM CONTENT OF MOUSE LIVER MITOCHONDIA DURING THE COURSE OF HEPATOMA INDUCTION WITH CARBON TETRACHLORIDE

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ABSTRACT

THE CALCIUM, POTASSIUM, AND SODIUM CONTENT OF MOUSE LIVER MITOCHONDIA DURING THE COURSE OF HEPATOMA INDUCTION WITH CARBON TETRACHLORIDE

by Wilbur Lesley Dungy

Strain-A mice were force-fed thirty doses of 40% CCl_4 in olive oil, in order to induce hepatoma. It has been demonstrated that a dose of 0.005 ml 40% CCl_4 solution per gram of body weight administered on a tri-weekly basis will induce hepatoma formation. Attempts were made to accelerate hepatoma induction by administering doses of greater concentration (0.010 ml to 0.025 ml/gm. body wt.). Female mice tolerated dose levels as high as 0.015 ml., but males failed to survive the feeding regime at dose levels exceeding 0.005 ml/gm. body weight. Castrate females treated with testosterone propionate were as sensitive to CCl_4 as intact males, while castrate males treated with stilbesterol tolerated CCl_4 as well as intact females. Castrate males and females tolerated CCl_4 equally well. This suggested that the presence of androgens in some manner increased the sensitivity of mice to the toxin.

Samples of whole livers and liver mitochondria were analyzed periodically during the course of hepatoma

induction for concentrations of calcium, sodium, and potassium. Mitochondria were obtained by homogenization of liver tissue in 0.25M sucrose solution, followed by differential centrifugation. The metal ions were extracted from whole liver and mitochondrial samples by wet ashing with 2% trichloroacetic acid. The extracts were analyzed for metal ion content by flame photometry. The calcium content of mitochondria increased to a maximum of five times the control level between 25 and 72 hours. Potassium concomitantly decreased to below 50% of the control level. During the interval, sodium concentrations did not vary markedly from normal values. Both calcium and potassium levels returned toward normal levels within 112 hours following feeding.

In the multiple feeding experiment, the ion changes during the first nine feedings were similar to the pattern of ion fluctuations of mitochondria following a single exposure to CCl_4. The calcium content increased to a maximum of nearly 8 times control levels, while potassium concentration decreased to 1/3 of the control value. Following the ninth feeding, both ion concentrations approached control values, although remaining significantly different from them.

Oxygen utilization of liver slices was monitored during the course of these experiments as a means of estimating mitochondrial disruption. Oxygen utilization

was depressed to 30% of control levels following a single exposure to CCl_4, but increased toward control values at 48 to 72 hours. The oxygen utilization of liver slices of mice during the multiple feeding experiment decreased to a minimum of 42% of control values at the fifth feeding. From this point there was a generally variable but incomplete approach toward recovery of control levels.

Maximum depression of aerobic respiration of liver slices was associated with maximum accumulation of calcium and loss of potassium by mitochondria. Recovery of respiration closely paralleled the decline of calcium concentrations and recovery of potassium with time.

The relations of the ion fluctuations and the alterations in aerobic respiration to the histological changes observed during the course of the investigation were discussed.

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CHAPTER I

INTRODUCTION

Various chemical agents have been utilized to induce tumors in susceptible species in order to facilitate the study of the origin and development of neoplasia. In early investigations the etiology of the neoplasia was determined primarily by histological criteria. In later studies, however, biochemical analyses have been utilized to an increasingly greater extent as techniques for the determination of disruption of normal cellular functions.

The A-jax mouse is one species in which hepatic tumor may be induced upon sufficient exposure to carbon tetrachloride. It was first demonstrated by Edwards (1941) that high incidences of hepatic tumor can be induced in strain-A mice that have been force-fed carbon tetrachloride for eight to sixteen weeks. Many investigators have reported extensive necrotic destruction of liver tissue of various species, as well as general disruption of metabolic functions, following a single dose of carbon tetrachloride. Vallee (Thiers *et al*. 1960) has demonstrated that rat liver mitochondria exposed directly to a single dose of carbon tetrachloride *in vitro*, or indirectly *in vivo* by portal circulation of an absorbed, orally-administered dose,

degenerate within ten hours as evidenced by cloudy swelling, loss of bound DPN, and uncoupling of oxidative phosphorylation. The time course of this degeneration follows the rapid sequestering of calcium and the concomitant loss of potassium by these organelles.

In view of the fact that these striking changes in metal ion content of rat liver cells and mitochondria occur following a single feeding of carbon tetrachloride, and that this particular chemical lesion appears to be relatively specific to carbon tetrachloride-induced liver injury, the question arose as to whether the initial disturbances evidenced following a single dose of carbon tetrachloride persisted until and beyond the definitive tumor development requiring approximately thirty tri-weekly doses. It also seemed of interest to know whether these early and subsequent ion effects, and their consequent metabolic disturbances could be utilized with any degree of accuracy as indications of histological and biochemical changes associated with neoplastic development.

The purpose of this study, then, was to investigate the effects of regular doses of carbon tetrachloride on the oxidative metabolism and the histological appearance of hepatic cells, and to relate these to the calcium, potassium, and sodium content of their mitochondria during the induction process as well as at its termination.

The rationale of the investigation was to determine at weekly intervals, forty-eight hours following every third dose, the changes in mitochondrial ion concentrations which occur in strain-A mouse livers. By taking tissue samples each time mitochondrial suspensions were prepared, it was possible to obtain histological data from each liver as well as total and mitochondrial ion concentrations. The metabolic activity of liver samples was determined by measuring oxygen utilization of liver slices incubated in buffered glucose phosphate medium.

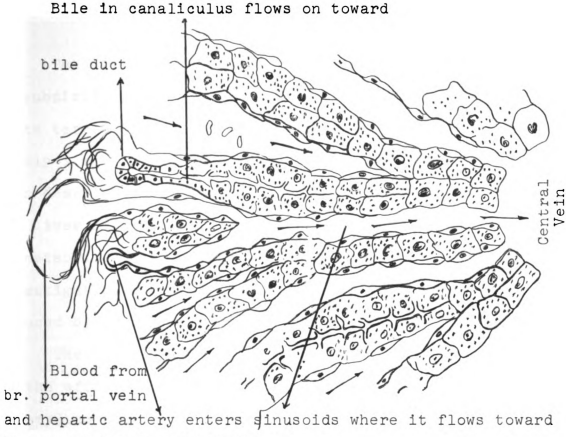

Figure 1.--Drawing at high power magnification showing path of blood flow from portal vein and hepatic artery, into sinusoids, and thence into central vein. (Redrawn from A. W. Ham, Histology, ed. 4 Philadelphia, Lippincott.)

CHAPTER II

LITERATURE REVIEW

The liver of the mouse, as is true of most mammals, is subdivided anatomically into a number of functional units termed lobules. These lobules, which are approximately 1 mm. in diameter and up to 2 mm. in length, have been described by Deane (1954). A brief description of the liver lobule will provide a basis for the correlation of metabolic descriptions observed in this series of investigations to alterations in lobular architecture induced by exposure to carbon tetrachloride.

The liver lobule (Fig. 1) is bounded by branches of the afferent liver vessels (portal vein and hepatic artery) as well as bile ducts. The parenchyma tissue of the lobule is subdivided into radiating plates, two cells in thickness, by the sinusoidal capillaries which transfer blood from the peripheral vessels to the central vein, which connects to the hepatic veins. Studies involving transillumination of intact rat livers, Wakim and Mann (1942) described the intralobular blood flow as proceeding through the sinusoidal spaces from portal branches to central vein. They reported circulation to be intermittent in sinusoids, with 75% being inactive at a given time. A metabolic gradient in terms of the

chemical quality of its blood exists across the lobule, according to Greep (1954). As a consequence of their preemptive position in regard to supplies of exygen and nutrient materials as well as reduced exposure to metabolic waste materials, the peripheral cells exhibit evidence of greater metabolic capacity than cells of the central vein region of lobules.

Additional evidence of a metabolic gradient is cited from the observations of Noel (1923) by Meglitisch et al. (1959) regarding the distribution of mitochondria in the hepatic lobules of the white mouse. He described the following three zones on the basis of mitochondrial morphology: (1) a zone of permanent function at the periphery of each lobule containing granular or rod-like mitochondria (2) a zone of permanent repose surrounding the central vein, containing fine thread-like mitochondria which are not altered by the nutritional state of the organism, and (3) an intermediate zone between central and peripheral zones in which mitochondrial morphology varies with the level of activity of the liver. They suggest that differences in the nature of mitochondria reflect differences in metabolic involvement of cells, and that activity decreases along a gradient from periphery to central zone during the course of digestion.

Pharmacology and Toxicology of Carbon tetrachloride

Carbon tetrachloride is a halogenated hydro-carbon chemically related to chloroform. It is of interest

clinically because its widespread use as an industrial and household solvent makes it a significant source of poisoning. It is of interest to the biologist because it may be used as an effective tool for the induction of experimental injury to various organs.

The effects of CCl_4 on various organs of the body depend upon the amount of CCl_4 circulating in the blood. The blood concentration in turn depends upon the site of absorption. Maximal absorption of CCl_4 occurs when its vapor is inhaled. Absorption is much less rapid from the intestinal tract than from the respiratory tract. As a consequency, according to Goodman and Gillman (1952), a single, large, orally-administered dose, most of which passes unabsorbed, is much less toxic than a series of small doses equalling the same total amount.

Robbins (1929) injected amounts of CCl_4, ranging from 3 cc. (therapeutic antihelminth dose) to 100 cc. directly into the stomachs, small intestines or large intestines of dogs. Ninety-six percent of the therapeutic dose was recovered in the expired air during the subsequent 24-hour period. The highest concentrations of CCl_4 were found in portal blood, while concentrations reaching the arterial circulation (carotid blood) were too slight for detection. The concentrations of CCl_4 recovered in expired air were approximately the same for all doses. If the liver were by-passed by shunting the portal blood directly

into the vena cava, the concentrations of CCl_4 recovered varied with the magnitude of the dose.

Effects of a Single Non-Lethal Dose of CCl_4 on Liver

Numerous investigations have utilized CCl_4 for liver regeneration studies and induction of hepatomas. The majority of these studies have involved a variety of strains of rats and A-jax strain of mice.

Following the administration of a single dose of CCl_4 to rats, Bassi (1960) reported considerable cellular deposition of fat accompanied by extensive swelling of the endoplasmic reticulum and mitochondria of the livers. The liver lobules exhibited a zonal distribution of damaged tissue: the central vein zone was characterized by extensive necrosis accompanied by infiltration of fat, loss of glycogen and nuclear disorganization. The cells of the midzonal region appeared swollen and highly vacuolated (vacuoles contained neither fat nor glycogen), while the cells of the peripheral (lobular vein) zone appeared relatively normal and unaffected. These regional differences are clearly evident within 24 hours. Similar descriptions of liver damage resulting from a single feeding of CCl_4 have been reported by Drill (1958), Christie & Judah (1954). If only a single sublethal dose of CCl_4 is administered, the disruptive changes are transitory; the necrotic tissue of the central zone is cleared by fragmentation and phagocytosis (Edwards 1943)

and autolysis (Rouiller 1964), and is replaced by proliferation of the peripheral cells which are unaffected (Eschenbranner 1946, Morrison 1965).

Vallee (Theirs et al. 1960) reported drastic changes in intracellular cation distribution following a single dose of CCl_4. The mitochondrion was the site of earliest alteration in cation concentration, with the Ca ion increasing 15 fold accompanied by a decrease in mitochondria K^+ to 1/4 its normal value within 48 hours. The concentrations of both ion species were reported to return to normal values within 72 hours. Share and Recknagel (1959) demonstrated similar depletion of K ions in CCl_4-poisoned mitochondria, but reported that these K^+ depleted mitochondria were unable to reaccumulate K^+.

Christie and Judah (1954) have shown that within 10 to 15 hours following ingestion of CCl_4 there was considerable disorganization of the TAC cycle. There was general depression of oxidation of citrate, octonoate, glutamate, and pyruvate; the rate of oxidation of succinate, however, remained unimparied.

Christie and Judah (1954), Basi (1960), Smuckler (1962) presume the hepatic damage to be the result of the direct effects of CCl_4 on the lipid components of cellular membrane systems including those of the endoplasmic reticulum. The possibility that hepatic damage results from anoxia or hypoxia as a consequence of vasoconstriction

arising from increased titers of circulating catecholamines promoted by CCl_4 has been suggested by Calvert and Brody (1960).

Effects of Multiple Doses of CCl_4 on Liver Tissue

Edwards and Dalton (1943), who were the first to report hepatoma induction by CCl_4, determined that a tri-weekly dose of 0.1 cc 40% CCl_4 in olive oil administered orally to mice one to five months of age caused hepatic necrosis. Although definitely toxic to the liver, this dose rate did not cause renal tubular necrosis, and was not lethal when repeatedly administered. There was no evidence of hepatoma in mice receiving less than 23 doses, while most mice exhibiting hepatomas received 35 to 58. The overall incidence of hepatoma in mice of The National Cancer Institute strains C3H, Y, C and A was 88%, while Strain A, with an incidence of 98.4% of 64 mice, showed the greatest susceptibility to the toxin.

Eschenbrenner (1944) in experiments involving variations of quantity of dosage and of interval between doses reported a dependence between these variables. Working with a constant number of doses (30 feedings of a single concentration) ranging from 10^{-4} to 16 x 10^{-4} cc/gm. of body weight administered over intervals of 1 to 5 days, he found the incidence of hepatoma to increase with increase in total time during which the total dose is administered. On the other hand, if the doses

of the concentration range cited above were administered on the same temporal basis, the incidence of hepatoma was found to increase with the magnitude of the dose.

Eschenbrenner (1946) reported that livers of mice developed partial to complete resistance to the necrotizing effects of CCl_4 depending upon the dosage rate. Mice receiving 30 feedings over a period of 120 days developed little resistance. Livers exposed to the same quantity of CCl_4 divided into 120 feedings administered over 120 days, despite some necrosis initially, soon became resistant or insensitive to the agent.

The incidence of hepatoma increased with the degree of necrosis.[1] By utilizing non-necrotizing doses however, it was demonstrated that repeated liver necrosis and concomitant regenerative processes are not essential for hepatoma induction with CCl_4.

Effects of Carbon Tetrachloride on Kidney

Nephrotoxic effects of CCl_4 are less well understood than hepatic effects. Following ingestion of sufficiently

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[1]The term "necrosis" may be interpreted as either cellular death or the advanced post mortem changes resulting in destruction of the cell following cellular death. Evidence of morphological changes in tissue sections may not occur until some time following irreversible damage to cells. According to Rouiller (1964) the only reliable sign of necrosis is destruction of the nucleus, and this occurs some time subsequent to cellular death. In this report necrosis refers to the visible, morphological changes associated with cellular destruction.

high concentrations, necrosis of lower segments of the renal tubules occurs accompanied by decreased renal blood flow, glomerular filtration and oliguria (Drill, 1958). Eschenbrenner (1946) reported general necrosis of tubular epithelium (with the exception of the capsule and portions of the proximal tubule adjacent to the capsule) in all male mice, but not in females, in response to high doses of chloroform. He reported no evidence of necrosis in the kidneys of female mice at a dose level of chloroform equivalent to the dose level of CCl_4 of our experiments. We have seen, as have Dalton and Edwards (1943) that the dose of CCl_4 administered chronically to produce hepatoma in mice did not produce renal damage.

Regenerative Properties of Liver

In order to understand and to properly interpret the cytological and biochemical changes occurring in liver following mechanical or chemical damage, we attempted to learn the behavior of cells during the process of restoration and regeneration. Since the events associated with recovery from partial hepatectomy and recovery from other forms of experimental liver injury are somewhat comparable, a comparison of regenerative processes following partial hepatectomy and CCl_4 poisoning will be presented.

Yokoyama et al. (1953), in studies of recovery following partial hepatectomy of rats, noted a considerable

increase in mitotic activity which reached a maximum by the third day, then fell off to resting frequency by the seventh day. Liver weight-body weight ratios were observed to fall immediately after surgery, obviously, but returned to normal levels in some cases as early as the fourth day, although total body and liver weights were below normal. Parenchymal cells increased in volume during the period of active mitosis and were noted to increase even more during the period from the fifth to the seventh days, which was suggested to be the time of greatest synthesis of parenchymal cell protoplasm.

Various mechanisms proposed for the regulation of cell division and tissue growth have been reviewed by Swann (1958). He suggests that neither recognized endocrine glands, dietary factors, excretory products nor variations in portal vein blood flow are likely to be critically involved in the regeneration stimulus for the liver. Glinos and Gey (1952) and Glinos (1956 proposed the hypothesis that certain components of normal blood serum inhibit growth at normal circulating levels. The lowering of the serum concentrations of these components by the reduction of the amount of functional hepatic tissue through partial hepatectomy results in initiation of regenerative activity in the liver. They were able to promote cell division in livers of normal rats by decreasing the concentration of plasma constituents by

plasmapheresis, and to inhibit cell division in regenerating livers by increasing the concentration of serum constituents through restriction of fluid intake. Comparable results were reported by Stich (1958) and Zimmerman (1960). Further evidence for promotion of cell division by dilution of serum was obtained from experiments involving parabiotic twin rats and parabiotic triplets (Buchner, 1951). The mean mitotic rate in the liver of the normal rat in each case (associated with partially hepatectomized partners) was reported to increase 6-fold and 50-fold respectively. Thus, in case of the liver there may be a blood-borne inhibitory substance, possibly associated with the albumin fraction. The normal concentrations of this humoral agent could be controlled by some sort of feedback mechanism involving the liver itself.

Tsuboi et al. (1951) studied the events of recovery of mouse liver from CCl_4 and reported extensive regenerative activity within liver lobules superimposed upon the destructive necrotic processes. The bulk of the necrotic tissue developed by the second day and was rapidly removed following the fourth day, with little evidence remaining by the sixth day. Regenerative processes were evident as early as the second day. Mitosis was observed to reach maximal levels by second or third day and to be essentially complete prior to disappearance of necrotic tissue. Comparisons of dry weights of livers of control

and experimental animals following extractions of lipids indicated that CCl_4-treated liver weights exceeded control values by as much as 32% by the fourth day. The nitrogen content of experimental livers exceeded that of controls by 29% by the fourth day, with the bulk of the nitrogen being identified with the protein fraction which exceeded control values by 25% by the sixth day.

Thus, considerable overlapping of destructive and recovery processes were observed during the second, third and fourth days, with regenerative activities dominating the picture from the fourth day. The vigorous restoration processes occurred at rates exceeding the removal of necrotic tissue, and resulted in the eventual overgrowth of the regenerated liver.

Mitochondrial Structure and Function

History of Mitochondrial Study

Cytoplasmic granules, known today as mitochondria, were described as early as 1850 by Kolliker, and isolated from insect flight muscle by him in 1888. Michaelis (1900) introduced Janus green as a supravital stain for mitochondria. In addition, the oxidation-reduction capacity of mitochondria was demonstrated for the first time as they were shown to effect redox changes in the Janus green dye. Extensive investigations since this time have demonstrated the existence of mitochondria

in all cells except those of bacteria and blue-green algae.

Early cytologists suggested that mitochondria possessed considerable plasticity, based upon their observations of variation in size, form and position in fixed preparations. Lewis and Lewis (1914) observed that mitochondria in living cells of chick embryo tissue undergo considerable change in size, form, and position in response to agents such as heat, carbon dioxide, acids, fat solvents and osmotic changes. The conclusion was that there were no definite types of mitochondria, and that any one type could change into another momentarily. Studies of embyonic fibroblasts by Frederick (1956) and Tobioka (1956), based upon utilization of the more recent techniques of phase microscopy and time-lapse photography, support this idea. Furthermore, Lehniger (1964) suggests that the number, size, and location of mitochondria in living cells is an expression of the nutritional or endocrine state of the cell.

Bensley's attempt to isolate mitochondria from broken cell suspensions of liver tissue (1934) laid the ground work for development of the separation of cell organelles by differential centrifugation. Unsatisfactory isolation media hampered early efforts to isolate intact, functional mitochondria. Hogeboom, Schneider, and Palade (1948) reported that when 0.88M sucrose was utilized as

a suspending medium, rat liver mitochondria were readily obtained in the elongate form characteristic of these structures in fixed preparations, and that they were readily stained by Janus green or other specific agents. They demonstrated, on the other hand, that preparations obtained from saline solutions or sucrose solutions isotonic to intact cells yielded spherical, swollen mitochondria which were not readily stained by Janus green.

Following refinement of techniques for isolation of intact, functional cell organelles in the late 1940s biochemists were able to relate specific metabolic functions to these organelles. As a consequence of the coordinated cytological and biochemical investigations of the past twenty years, it has been possible to identify specific biochemical systems with the mitochondrial fractions of cells, and to demonstrate that there is a close correlation between the integration of these biochemical activities and the structural integrity of mitochondria.

Since it has been demonstrated that structural alterations in mitochondria as a result of exposure to chemical or physical agents is reflected in disorganization of metabolic function, a brief account of the presently accepted mitochondrial structural concept, the biochemical systems, and the physiological functions attributed to them will be presented.

Mitochondrial Structure

Lehninger (1964) reported that typical mitochondria of the middle of the spectrum of size and shape (of which rat liver mitochondria are representative) are roughly ellipsoidal rods 3.3u in length by 1.0u in diameter.

The pioneering efforts of Palade (1953) and Sjöestrand (1953), who developed techniques for the study of ultra-thin sections of tissue, have provided considerable information about details of internal structure of mitochondria. The structural model consistent with these studies depicts the mitochondrion as a structure whose lumen is enclosed by a pair of concentrically arranged membrane systems (Fig. 2). The outer membrane is relatively smooth, while the inner membrane is thrown into numerous folds (christae) which exhibit the double membrane structure of the mitochondrial surface. The numbers of christae are related to the metabolic capacity of the tissue involved. (Palade 1956).

The thickness of each of the mitochondrial membranes and the space between have been demonstrated to be relatively constant across the spectrum of cell types studied. With osmium staining, the generalized membrane system can be resolved into a pair of electron dense lines each 40 to 60 A^{o} thick spearated by an electron lucid line 60 to 90 A^{o} wide (Mercer 1960).

Permanganate-stained preparations, on the other hand, show two pairs of electron-dense lines, separated by an

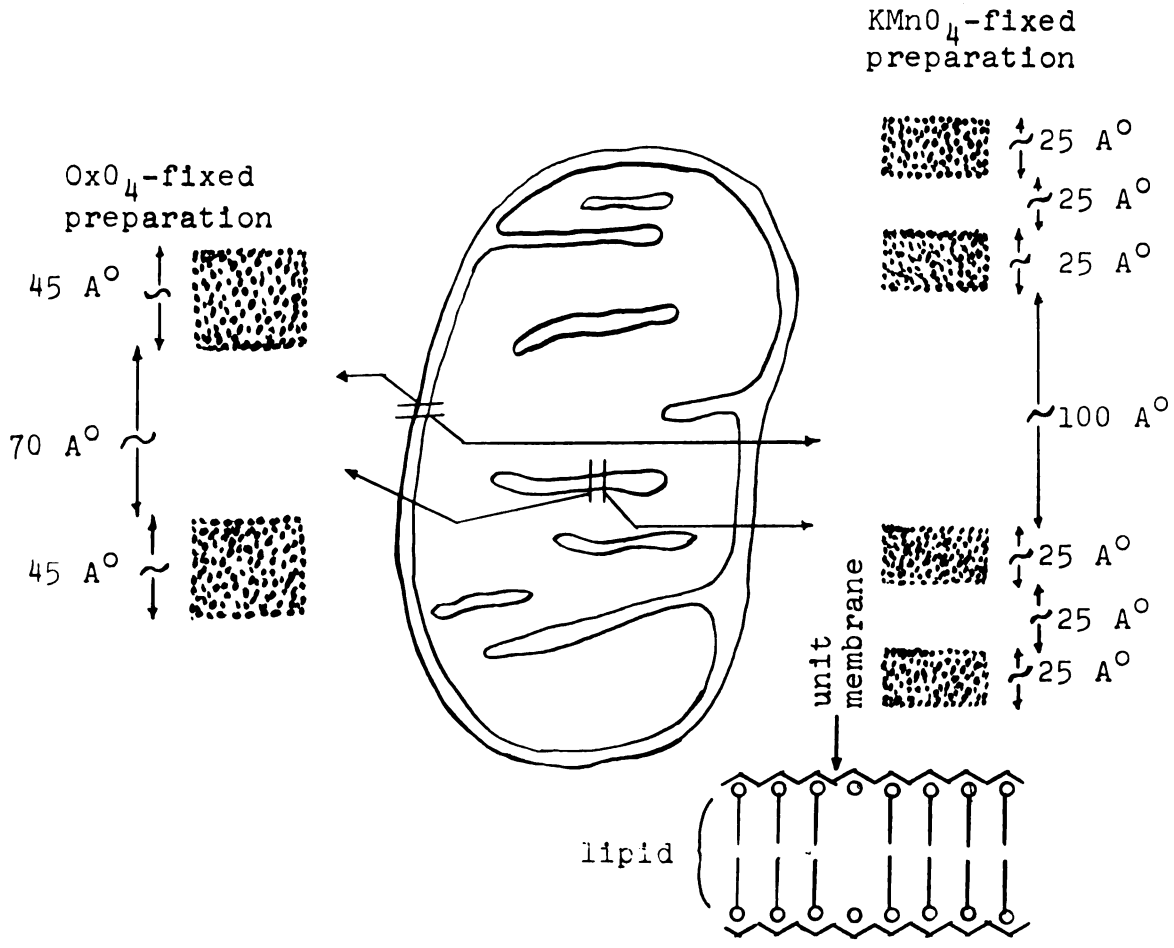

Figure 2.--Comparison of osmium-dense and $KMnO_4$-dense lines of mitochondrial membrane systems. (After J. B. Finean, Chemical Ultrastructure in Living Tissues, Charles C. Thomas, Springfield, Illinois, p. 86.)

electron-lucid line approximately 100 A^{o} in thickness. Each of the pair of dense lines is 25 A^{o} in thickness and separated by a light space of 25 A^{o} in width. Each of the pair of membranes when properly stained exhibit dimensions similar to those of the plasma membrane and apparently conform to the unit membrane concept proposed by Robertson (1959). The patterns arising from the two staining procedures are compared in Fig. 2.

Fernandez-Moran (1963) applied the principle of negative staining to the study of the ultrastructure of the beef heart mitochondria, and his negatively stained images demonstrated the presence of many spherical or ellipsoidal particles approximately 80 A^{o} in diameter which were connected by stalks to the electron lucid area of the inner membrane system primarily. Stoeckenius (1963) has reported the existence of a similar sub-mitochondrial component in mitochondria of Neurospora, while Parsons (1963) has demonstrated the existence of these mitochondrial subunits on the christae of eleven different types of mitochondria, including liver, brain, kidney, pancreas, and hepatoma.

Green (Ziegler 1958) identified particles associated with the christal membranes of rat liver mitochondria which, in contrast to those described by Fernandez-Moran, were larger (120 A^{o} in diameter) and stalkless. It was suggested that these structures called elementary particles (EP by Fernandez-Moran), and electron transport

particles (ETP) by Green, may exhibit modifications in size and form depending upon the tissue in question.

Biochemical Systems

To date, four integrated enzyme systems or functional units have been identified in mitochondria in general (Mercer 1960). Studies beginning with Schneider and Potter (1948), Schneider and Hogeboom (1956), Schneider (1959), and recently by a great number of workers, have provided evidence that some of the enzymes associated with the TCA cycle apparently are confined to the mitochondrial fraction of the cells exclusively. While Kennedy and Lehninger (1949) found isolated rat liver mitochondria capable of carrying out the oxidation of all the TCA cycle intermediates at rates comparable to those determined from studies of intact liver, Schneider (1948) and Kennedy and Lehninger (1949) provided evidence for the presence of a biochemical system in mitochondria capable of exidizing fatty acids to completion, the activities of which are sufficient to account for the activity of the intact cells.

Evidence has been provided by many sources that the enzyme systems associated with formation of high energy phosphates during aerobic respiration reside in the mitochondria, and that the activity of this system in tissue balance studies seems to follow the mitochondrial fraction. Linanne (1958), Pullman (1958) cite evidence for existence of enzyme systems in mitochondria capable

of coupling the various oxidative processes to phosphorylation, while Green (1962) has isolated the soluble proteins from mitochondrial fractions which possess this capacity for phosphorylation.

Osmoregulatory Processes of Mitochondria

Two osmoregulatory processes have been shown to be generally associated with mitochondria: (1) ability to accumulate salts against a gradient, and (2) the ability to undergo controlled volume changes. Considerable evidence has been provided by the work of Bartley and Davies (1954), MacFarlane and Spencer (1953) Spector (1953), and Lehninger and Rossi (1963), and others that *in vitro* preparations of mitochondria maintained under circumstances of adequate oxidative phosphorylation are not only capable of maintaining pre-existing gradients of the cations K^+, Na^+, Ca^{++}, and Mg^{++} but also exhibit the capacity of accumulating these ions from the incubation media. Green (1962) and others have demonstrated the ability of respiring mitochondrial preparations to accumulate inorganic phosphate. Lehninger *et al.* (1963) reported that inorganic phosphate of the medium accompanies Ca^{++} during active accumulation of the latter. Presumably Ca^{++} uptake may passively accompany the active accumulation of phosphate and result in retention of calcium as the insoluble phosphate salt of calcium. Lehninger (1964) reported that rat liver mitochondria accumulate Ca^{++} in the absence of respiration if

ATP or an ATP regenerating system (which is oligomycin, or DNP sensitive but cyanide insensitive) is present.

Changes in volume of mitochondria have been observed in living cell cultures as well as in isolated mitochondrial suspensions. These volume changes have been related both to passive, osmotic processes associated with semipermeability of the membranes as well as to active processes which are respiration dependent.

Tedeschi (1959) accurately predicted the degree of mitochondrial swelling as a function of osmotic activity over a wide range of concentrations of various solutes. Neither permeability nor available surface area varied significantly for lipid-soluble substances, but an increase in available surface area for absorption of polar compounds was noted as swelling progressed. These results suggested a highly convoluted membrane, and that the surface area of the convolutions (presumed to be christae) became available only after being unfolded by swelling. Volume increases in extremely hypotonic solutions were less than those expected by osmotic prediction. This was taken as evidence of membrane damage which permitted loss of osmotically active solute. Mitochondria have been observed to lose their oxidative capacity as a result of loss of low molecular weight soluble components such as NAD, NADP, and adenine nucleotides (Lowey & Siekevitz 1963).

Lehninger (1962) described an "active" type of swelling which was independent of variations in osmotic concentration, dependent upon respiration, and stimulated by such swelling agents as thyroxine, inorganic phosphate, Ca^{++},etc. Swelling stimulated by these agents was reversed by addition of ATP and Mg^{++},and this ATP-driven enzyme mechanism (possibly contractile in nature) was found to be independent of coupled oxidation-phosphorylation processes. (Swelling induced by CCl_4, digitonin, and glutathione was not readily reversed by this mechanism.)

Lehninger suggests that the swelling-contraction phenomena may represent two entirely separate enzymatic processes restricted to different membranes or morphological compartments of the mitochondrion, rather than being a single reversible mechanoenzyme process.

Neifakh and Kazakova (1963) demonstrated the existence of a contractile protein in mitochondrial membranes possessing properties similar to actomyosin. Since relaxed mitochondria permit ready release of endogenuous adenine nucleotides and soluble factors which have a stimulatory effect upon glycolysis, while contraction of membranes renders them relatively impermeable to these substances, it has been postulated that he control of cytoplasmic processes may be regulated by the variable permeability of mitochondrial membranes resulting from the variable degree of contraction of contractile mechanisms.

Relationship of Mitochondrial Ultrastructure to Function

Considerable effort has been directed toward developing techniques that will provide structurally intact mitochondria for studies designed to identify the biochemical and physiological functions of mitochondria and to elucidate the integrated nature of these activities in the functionally intact unit. Concurrently, in a number of laboratories, a series of investigations have been conducted which involve degradation of mitochondria into increasingly smaller units of decreasing complexity. These studies permit the identification of individual steps in the biochemical and physiological phenomena of mitochondria, as well as the identification of the structural subunits to which these various activities are linked.

Studies such as these are of significance because they demonstrate that certain mitochondrial functions follow specific submitochondrial units, and that integration of these activities is lost as the subunits are separated, or severely restricted if the intact mitochondrion is physically distorted. Thus mechanical damage due to isolation techniques may account for disorganization of integrated mitochondrial function under experimental circumstances, and distortion of mitochondrial structure undoubtedly is responsible, to some extent, for metabolic disruptions under certain pathological circumstances.

Mitochondria become swollen in hypotonic media, phosphorylation becomes uncoupled from oxidation, and a latent ATPase is activated. Green (1964) considers the enzymes associated with oxidation, as well as those associated with synthetic reactions powered by ATP, to be located in the outer mitochondrial membrane, while those concerned with electron transport are located on the inner membrane. Thus, swelling may alter metabolic function by causing very critical spatial requirements to be exceeded. Novikoff (1956) found that mitochondria isolated in 0.88M sucrose, although retaining their typical red shape, exhibited limited oxidative phosphorylation and enhanced ATPase activity. It was suggested that these mitochondria may have been overly constricted.

Submitochondrial particles produced by the method of Keilin and Hartee (1947), or by more recent modifications, retain considerable succinic and DPNH oxidase activity but do not retain significant capacity for coupled oxidative phosphorylation.

Keilley and Bronk (1957) produced a broad spectrum of submitochondrial fragments by exposure of rat liver mitochondria to sonic vibration. These particles were capable of coupling oxidation and phosphorylation with succinate, DPNH, etc. as substrates, but not with substrates dependent upon presence of DPN^+ which seemed to be lost by these particles.

Devlin & Lehninger (1958) obtained submitochondrial fragments by sedimentation of a digitonin-treated suspendion of rat liver mitochondria at 100,000 x g. These particles retained considerable organization of both electron transport system and phosphorylating enzymes, but were incapable or organized TCA cycle or fatty acid cycle activities.

Green (1963) has reported the isolation and reconstitution of a submitochondrial particle (presumably the EP particles of Fernandez-Moran) that uniquely contains all of the fixed components of the electron transfer system. These isolated particles appear to be physical and functional aggregates of the four complexes known to constitue the electron transport system.

CHAPTER III

METHODS AND MATERIALS

Experimental Animals

The animals used in this series of experiments were strain-A mice obtained from the Roscoe B. Jackson Memorial Laboratory and maintained in our laboratory. Mice of this strain were selected because: (1) much of the related work regarding carbon tetrachloride-induced hepatoma studies has involved this strain, and (2) the incidence of spontaneous hepatoma in strain-A mice has been reported to be less than 1% through 16 months of age (Edwards and Dalton 1942), which is lower than that reported for other strains that have been investigated. The mice utilized were approximately eight weeks of age at the beginning of each experiment, and had attained a body weight of approximately 20 grams. Males and females were kept in separate metal cages with four mice per cage. All mice were fed Purina dog chow pellets and tap water ad libitum. Environmental temperatures were maintained at $75^{\circ}F \pm 1^{\circ}$.

Carbon tetrachloride solutions were prepared by dilution with olive oil to a 40% solution by volume. Measured doses of control and experimental solutions were

administered by stomach tube on a body weight basis. An 18 gauge metal needle, with tip blunted by a mass of solder rounded to the diameter of the mouse esophagus, served as a stomach tube. Measured doses of solutions were administered through this tube from a 1 cc. syringe, graduated in 1/100 cc. units. The mice were force-fed under light either anesthesia in order to prevent puncture of the esophagus or undue damage to teeth as a result of chewing on the metal feeding tube.

Prior to obtaining tissue samples for various experimental observations, the animals were killed by cervical dislocation and exsanguinated. The livers were quickly excised and placed in ice-cold 0.25M sucrose buffered at pH 7.2 with tris buffer. All subsequent preparative procedures were carried out in a cold room at a temperature of 0^{o}- 4^{0}C.

Histological Preparations

Necropsies were performed on mice at predetermined intervals, and whenever possible on mice dying of CCl_4 poisoning during the course of each experiment. The extent of macroscopic hepatic reaction was noted (color, texture, appearance of nodules), and samples of hepatic tissues were prepared for histological studies. All visceral organs were examined grossly, and the kidneys in addition were subjected to microscopic examination periodically for evidence of reaction to carbon tetrachloride.

Analytical Procedures

Preparation of Mitochondria

Liver mitochondrial suspensions were prepared by homogenization of the tissue and differential centrifugation in a 0.25M sucrose medium in essentially the same manner suggested by Hogeboom et al. (1948). Centrifugations were carried out in a Servall centrifuge, Rotor Number SS-34. The homogenates were spun initially at 300 x g for 10 minutes in order to sediment nuclei, incompletely ruptured cells and connective tissue. The resulting supernatants were centrifuged at 10,000 x g for ten minutes in order to sediment the mitochondria. The mitochondria were washed with a fresh volume of sucrose medium equal to that of the supernatant, and centrifuged. This process was repeated. The twice washed mitochondria were then resuspended in a volume of sucrose medium equal to 2 1/2 times the wet weight of the original liver sample if metabolic determinations were to be made, or in a volume equal to 9 x the weight of the liver sample if ion determinations were to be made.

Phosphate fixation-oxygen reduction ratios were determined from randomly selected preparations approximately every two to three weeks in order to demonstrate that the mitochondria were not damaged by the extraction procedures. Swollen or otherwise damaged

mitochondria do not maintain proper coupling of oxidative and phosphorylative activities and, therefore, are incapable of either actively concentrating ions or of maintaining ion gradients established prior to the damage (Spector 1953).

Oxidation and coupled phosphorylation were measured by a manometric technique utilizing a multiple unit constant pressure respirometer designed and described in detail by Reineke (1961). To the reaction mixture in each standard Warburg reaction vessel was added an aliquot of mitochondrial suspension equivalent to 10 to 15 mg. of mitochondrial protein. The flasks in position on the respirometer were allowed to equilibrate in a water bath at 37°C for 7 minutes, following which side arm vents were closed and readings taken at convenient intervals (5 to 10 minutes) for 20 to 30 minutes.

Immediately prior to affixing flasks to the respirometer, and again upon completion of the incubation period, 0.3 ml. aliquot of the contents of each vessel were transferred to labeled test tubes containing 4.7 ml ice-cold 5%TCA solution. Both zero-time and 30 minute TCA samples were centrifuged and their supernatants recovered in order to determine inorganic phosphate depletion according to the method of Fisk and Subbarow (1925).

Ion Analyses

In order to measure the concentrations of ions in a sample of tissue by use of flame photometry, it is necessary to atomize into a gas flame a solution of the sample. Under proper conditions the flame has sufficient thermal energy to excite electrons of the elements to levels at which they will radiate emissions characteristic for each element.

A Coleman Model 21 flame photometer was used in this study for ion determinations. The instrument was fitted with a direct atomizing oxygen-natural gas burner, and its operation has been described in the Coleman Co. instruction manual "Operating Insturctions for the Model 21 Coleman Flame Photometer D-248." The burner was operated at a pressure of 12 lbs. of oxygen per square inch and five to six cubic feet of natural gas per hour, which is the requirement for city gas flow. This provided the optimum burner conditions according to the manufacturer.

The intensities of the isolated radiations were measured by an auxiliary indicating instrument, the Coleman Model 22 Grav-O-Meter.

Because ion concentrations of liver homogenates and mitochondrial suspensions are not uniformly distributed between solution and suspended particles, it was necessary to extract calcium, potassium and sodium from these samples. It was originally proposed to extract ions from whole liver and liver mitochondria by dry ashing samples

in a muffle furnace at 550°C for 24 hours, and the initial ion extractions were obtained in this fashion. Monib and Evans (1957) have reported that homogenization of tissues in 2% trichloroacetic acid gives results which appear to be reliable and in good agreement with other methods of extracting ions from tissue.

We compared this 2% TCA method with the dry ashing procedure previously proposed. The two methods gave results which were in agreement to within 3%. Since the TCA method proved to be much more conservative of time than the dry ashing procedure, and inasmuch as some of the procedures for other determinations involving these same tissue samples involved the use of TCA, this method was subsequently adopted as the routine method for ion extraction.

A 30% TCA solution (w/v) was made by dissolving 150 gm. of TCA in 500 ml. of de-ionized water. Whole liver samples were accurately weighed and homogenized for 2 to 3 minutes with a Potter Elvehjem motor driven homogenizer. The liver suspensions were then transferred to metal-free polyethylene bottles to which were added 3.3 ml. of 30% TCA and enough de-ionized water to bring the volume to 50 ml. in each. Washed mitochondrial pellets derived from centrifugation of liver homogenates were resuspended directly in sufficient 2% TCA to give a final volume of 10 ml. These final volumes represented approximately 100:1 W/V dilutions of the sample materials.

Following overnight storage at 5°C, the precipitated proteins of the TCA suspensions were separated by centrifugation, and the supernatant solutions filtered through #40 Whatman filter paper and stored in polyethylene vials at -5°C prior to analysis.

Standard solutions of clacium, potassium and sodium were prepared according to Dean (1960). Standard curves were established, based upon analyses of serial dilutions of these standard solutions. Each sample extract was read against the standard solutions and the concentrations of calcium, potassium and sodium were estimated from the standard curves.

Protein Determination

The protein concentration of the samples from which ions were extracted was determined according to the method of Gornall et al. (1949). Gloudiness, resulting from lipid content of livers and liver fractions resulting from absorption of CCl_4, was resolved by shaking the resulting Biuret solution with an equal volume of ethyl ether. After centrifugation, ether was removed by aspiration, and the protein concentration of the resulting clear Biuret solution was determined with the Beckman Model Du spectrophotometer at the wave length of 450 mu.

Carbon Tetrachloride Feeding Experiments

Determination of Maximum Tolerable Dose of CCl_4 Per Feeding on a Sustained Basis

According to Eschenbrenner (1944), the incidence of CCl_4-induced hepatoma in mice increases as the time during which a constant dose is administered increases. In addition, the rate of hepatoma induction increases as the total amount of CCl_4 administered during a constant time interval increases.

A tri-weekly feeding schedule was most convenient for our purposes. The initial investigation was designed to determine the maximum tolerable dose of CCl_4 that could be administered on this basis, in order to develop a satisfactory incidence of tumor as quickly as possible.

Five groups of mice were force-fed 40% CCl_4 in olive oil tri-weekly, while two control groups were force-fed olive oil. Groups (1) and (2) were control groups receiving 0.3 ml and 0.1 ml of olive oil respectively. Groups (3) received 0.5 ml 40% CCl_4; group (4) received 0.3 ml 40% CCl_4; group (5) received 0.2 ml 40% CCl_4; and group (6) received 0.1 ml 40% CCl_4.

Determination of Time Required for Induction of Hepatoma

One of the major objectives of this series of investigations was to identify, if possible, the time interval during which hepatoma induction occurs in mice exposed to repeated feedings of CCl_4. The maximum time

was to be established by noting the time required for gross symptoms of hepatoma to become evident by palpation or at autopsy. Although several investigators have reported 70% to 100% success in inducing hepatomas in mice ranging in age from 1 - 5 months, as a result of administering 0.1 ml 40% CCl_4 solution two or three times weekly to a total of at least 30 feedings, there has been little indication as to the approximate period of time required for the actual induction of hepatomas. Edwards (1941) reported 70% to 98% tumor incidence in several strains of CCl_4-treated mice, with strain-A mice exhibiting the highest incidence. The majority of these mice, however, were autopsied after having received well over 35 feedings and up to 54 feedings. Eschenbrenner (1946) reported 100% tumor induction in strain-A mice surviving 30 tri-weekly feedings of 0.1 ml 40% CCl_4; however, autopsy was not performed until 150 days following the initial feedings.

A group of mice, males and females, was placed on a tri-weekly feeding regime which was extended over a ten-week period during which 30 doses of CCl_4 solution (0.1 ml 40% CCl_4 per dose) were administered. In order to establish the time for development of gross evidence of hepatoma, the mice of this group as well as those used in other experiments of this investigation were examined for evidence of liver tumor post mortem.

Determination of Effects of Sex Hormones on Resistance to CCl_4 Toxicity

The previous experiments regarding the determination of the optimum tolerable dose of CCl_4 for strain-A mice demonstrated that the mortality rate for females is much lower than for males, especially at high dose levels. Our working dose level of 0.005 ml 40% CCl_4 in olive oil per gram of body weight is the highest level at which the mortality rate is approximately the same for both sexes.[1] The differences between mortality rates of males and females with increasing dose levels suggested a possible involvement of sex hormones in the mouse tolerance to CCl_4.

The following experiment was designed to determine (1) whether this assumption was valid, and (2) if so, whether androgens tend to render animals more sensitive to the effects of CCl_4 or whether estrogenic substances offer a degree of protection against the effects of CCl_4.

Male and female strain-A mice were castrated, or sham operated (gonads exposed but not removed) and permitted a 14-day post-operative recovery period before initiation of the CCl_4 regime along with normal control mice, as follows:

Mice were divided into 6 groups of 8 males and 8 females each. Group (A) contained untreated (no hormones

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[1] This dose level was derived from the standard dose of 1 ml. of 40% CCl_4 solution per mouse used routinely by other investigators in tumor induction studies, and is equivalent to 0.1 ml. per 20 gram mouse.

injected), unoperated mice which received olive oil without CCl_4 throughout the experimental period; group (B) contained mice upon which sham operations were performed, and which received olive oil without CCl_4 at each feeding and 0.1 ml corn oil subcutaneously when mice of group E were given hormone injections; group (C) contained untreated mice which received 0.015 ml 40% CCl_4 solution/gm. body weight at each feeding; group (D) contained castrate mice which received 0.015 ml 40% CCl_4/gm. body wt. at each feeding;[2] group (E) contained castrate males which received daily injections of stilbesterol (0.1 ugm in 0.1 ml corn oil) and ovariectomized females which received daily injections of testosterone propionate (60 ugm in 0.1 ml corn oil), and were fed 0.015 ml 40% CCl_4/gm. body wt.; group (F) contained castrated mice which were treated exactly like those of group (E) except that the hormone injections were started 12 days prior to the CCl_4 feeding schedule; group (G) was a group of mice used in a previous experiment in which untreated animals received 0.3 ml 40% CCl_4 per mouse.

The dose level of testosterone propionate for this experiment was set at 60 ugm daily on the basis of reports by Rubenstein and Solomon (1940) that an 11% increase in body weight of rats 26 days of age (average weight 45

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[2]This dose level is equivalent to 0.3 ml 40% CCl_4 solution per average 20 gram mouse.

grams) was produced by daily injections of 50 ugm testosterone propionate, and that six daily injections of 75 ugm of the hormone caused effective regression of the x-zone of the adrenal glands of castrate mice (Starkey & Schmidt 1938). The 0.1 ugm dose level of stilbesterol was established on the basis of reports by Emmens (1939) that this represented the mouse unit for stilbesterol, and by Leighty and Wick (1939) that estrus is induced in spayed mice by 0.066 gamma of stilbesterol.

Oxygen Utilization by Mitochondria in Situ

The metabolism of isolated mitochondria during in vi vitro studies is subject to question, due to difficulties in duplication and maintenance of conditions like those in vivo. It was, therefore, decided to investigate the effects of CCl_4 on the metabolic integrity of mitochondria in mouse liver slices by measuring the rate of oxygen consumption. The tissue slice is thought to represent organized surviving tissue in which observed metabolic activities are qualitatively comparable to those occurring in the original tissue (Umbreit 1964).

Sixteen mice (8 males and 8 females) were given a single oral dose of 0.005 ml 40% CCl_4/gm. body weight, and the oxygen utilization of liver slices of two males and two females was determined at 24, 48, and 72 hours.

A second group of mice of both sexes was given tri-weekly feedings of CCl_4 solution at the dose level indicated

above, and 48 hours following each third feeding (when possible) oxygen utilization by liver slices of two males and two females was determined.

Livers of mice were quickly excised and placed in ice-cold 0.25M sucrose solution. Slices ranging from 0.3 to 0.5 mm in thickness were prepared with a Stadie-Riggs hand microtome, blotted free of excess solution, and weighed. Slices were transferred to the main vessels of a standard Warburg flasks which contained 2.0 ml of 0.01 M glucose in Kreb's Ringers phosphate (pH 7.2) (Umbreit 1964). The center well of each flask contained 0.1 ml 10% KOH absorbed in a filter paper wick. The gas phase was replaced with oxygen, and flasks were attached to a multiple unit constant pressure respirometer. All flasks were incubated at 37°C with constant agitation. The protein content of samples was determined by the Biuret method and metabolic quotients (QO_2) were evaluated on the basis of protein content rather than sample weight.

CHAPTER IV

RESULTS

Histology[1]

Liver sections prepared twenty-four hours following a single dose of 0.1 ml 40% CCl_4 exhibited extensive necrosis of hepatic cells in the central portions of lobules and to a lesser extent in the midzonal regions. Parenchyma of the portal vein-bile duct regions resembled those of control sections in appearance. Necrotic portions of lobules of the central zones contrasted sharply with the adjacent, apparently normal parenchyma of the peripheral zones. Necrotic cells exhibited bright orange to red cytoplasm (H and E stain) with nuclei either missing or in various stages of pycnosis, while non-necrotic cells possessed a granular, purple-stained cytoplasm. The bulk of the necrotic tissue in association with inflammatory changes (infiltration of leucoytes and Kuppfer cells) persisted through the 100 hour observational period.

Regenerative changes were evident at forty-eight hours. Binuculeate cells and mitotic figures were present

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[1]Slides of liver and kidney sections were reviewed by Dr. Pack of the Detroit Cancer Institute.

throughout the intact tissue, but seemed to be most prevalent in areas adjacent to necrotic zones.

At seventy-two hours there is substantial evidence of hepatic cell regeneration as evidenced by recession of necrotic areas, and increasing numbers of mitotic figures and binucleate cells. Our studies indicate considerable overlapping of necrotic and regenerative processes from forty-eight hours through the 100-hour observational period.

Liver sections examined following three, six, and nine doses of CCl_4 exhibit essentially the same pathological features as those associated with the single feeding experiment. However, degenerative changes were more pronounced following the earlier feedings while regenerative phenomena were more prominent following the later feedings. Eosin-hematoxylin-stained sections showed extensive hyaline degeneration of parenchyma in the central vein zone of lobules, accompanied by fatty changes in the remaining liver cells. Normal liver lobular patterns were distorted by the development of slightly more fibrous tissue in the central and peripheral zones. The hepatic cells show marked variations in size and nuclear forms. Nuclei were large and prominent in some cells and pyknotic in others. Mitoses were more numerous than for the comparable period following a single dose of CCl_4. This fact, in addition to numerous cells with double nuclei, was suggestive of active

regeneration. Following the eighteenth feeding, the deposition of fibrous tissue of unknown origin becomes increasingly more evident. A definite pattern of false lobules is produced by deposition of this fibrous tissue accompanied by dense inflitration of pigmented Kuppfer cells.

Following thirty or more doses there were one or more nodules protuding from the surfaces of some of the livers examined at autopsy. These were firm and pink to gray in color, and from a macroscopic point of view, fit the description of CCl_4-induced "hepatomas" reported by others (Edwards 1943). Microscopically there was no evidence of capsule formation between the nodular area and the liver proper. Cells of these masses were highly differentiated and closely resembled hepatic cells. These tissue masses might either constitute benign tumors or non-neoplastic nodules of regeneration, both of which exhibit comparable histological features (Edwards 1943).

Examination of kidney sections at 24, 48, and 72 hours following a single dose of CCl_4, and at 48 hours following each third dose of the multiple-feeding regime, showed no histological changes of significance. With the exception of occasional evidence of mild epithelial swelling, kidney sections of mice exposed to either single or multiple doses of CCl_4 of the concentration used routinely in this series of studies, resembled those of control mice.

Carbon Tetrachloride Feeding Experiments

The numbers of mice surviving the various dose levels of CCl_4 are summarized in Table 1. Seven of 49 control mice died during the course of the Experiments. None of the males and two of four females receiving 0.5 ml. 40% CCl_4 solution per mouse survived the three-day interval following the initial feeding. The animals of this group were quite sluggish, showed little spontaneous movement, and appeared to have considerable loss of weight at the time of death. (Weight records were not maintained during this experiment). The hair of the anal and pelvic regions was heavily stained with a yellowish brown discharge, either of renal or intestinal origin (the source was not determined). Because of the severe effects of the 0.5 ml dose level, this feeding regime was terminated after administration of the initial dose.

All of the males (12) and seven of 31 females receiving 0.3 ml CCl_4 per mouse (average body weight = 20 grams) died prior to the 18th feeding. Eleven of the 12 males failed to survive the eighth feeding, while only four females died during this period. Fourteen of 31 females were still alive at the termination of this feeding schedule, which involved 36 feedings over a 90-day period.

Of the group receiving 0.1 ml of CCl_4 solution per mouse, thirteen of 24 males and 12 of 20 females survived 53 feedings over a 146-day period.

TABLE 1.--Survival of strain-A mice following repeated oral administration of carbon tetrachloride solution.

Denominators = numbers of mice observed
Numerators = number of mice surviving

	DOSE[1]							
SEX	0.5	Control[2]	0.3	Control[2]	0.2	Control[2]	0.1	Control[2]
M	0/8	4/4	0/12	3/3	0/12	-	13/24	6/9
F	0/4	6/8	14/31	8/8	-	-	12/20	15/17

[1]Milliliters of 40% solution CCl_4 to a total of at least 30 doses for surviving mice per 20 gm. mouse administered triweekly.

[2]Controls received a volume of olive oil equivalent to volume of CCl_4 of experimental dose.

There were no females in the group receiving 0.2 ml CCl_4 solution per mouse. However, the mortality levels of females receiving 0.1 ml and 0.3 ml dosages provides information regarding the probable fate of females exposed to the 0.2 ml dose level. None of the males survived the 71st day of the experiment (18 feedings), and most (9 of 12) died within the first three weeks of the feeding regime.

The survival of mice is shown in Fig. 3, as a function of magnitude of the chronically administered dose. It is evident that the LD_{50} dose for females is greater than 0.005 ml 40% CCl_4 per gm. body weight.

The survival rate of mice at the various dose levels is plotted in Fig. 4 against time (or the total number of doses administered). Again it is evident that females may survive 30 or more feedings at elevated dose levels. However, males do not survive the 30 feedings estimated to be required for tumor induction at dose levels exceeding 0.005 ml. 40% CCl_4 per gm. body weight.

The results of the pooled observations in regard to tumor induction are summarized in Table 2. There was no gross evidence of tumor formation in any of 139 mice autopsied prior to the 23rd feeding. One female that had received 0.3 ml CCl_4 solution per feeding exhibited a small tumor at the twenty-fourth feeding. None of 15 females or of 5 males examined prior to the 30th feeding displayed visible evidence of hepatoma. None of three

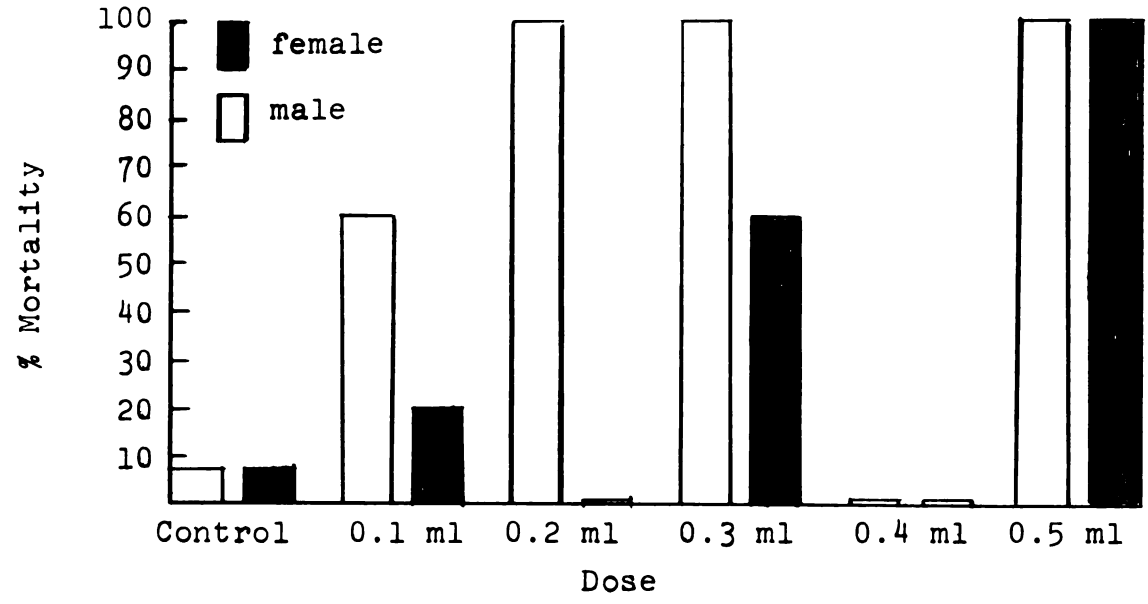


Figure 3.--Plot of mortality data for mice during chronic feeding of 40% CCl_4 solution

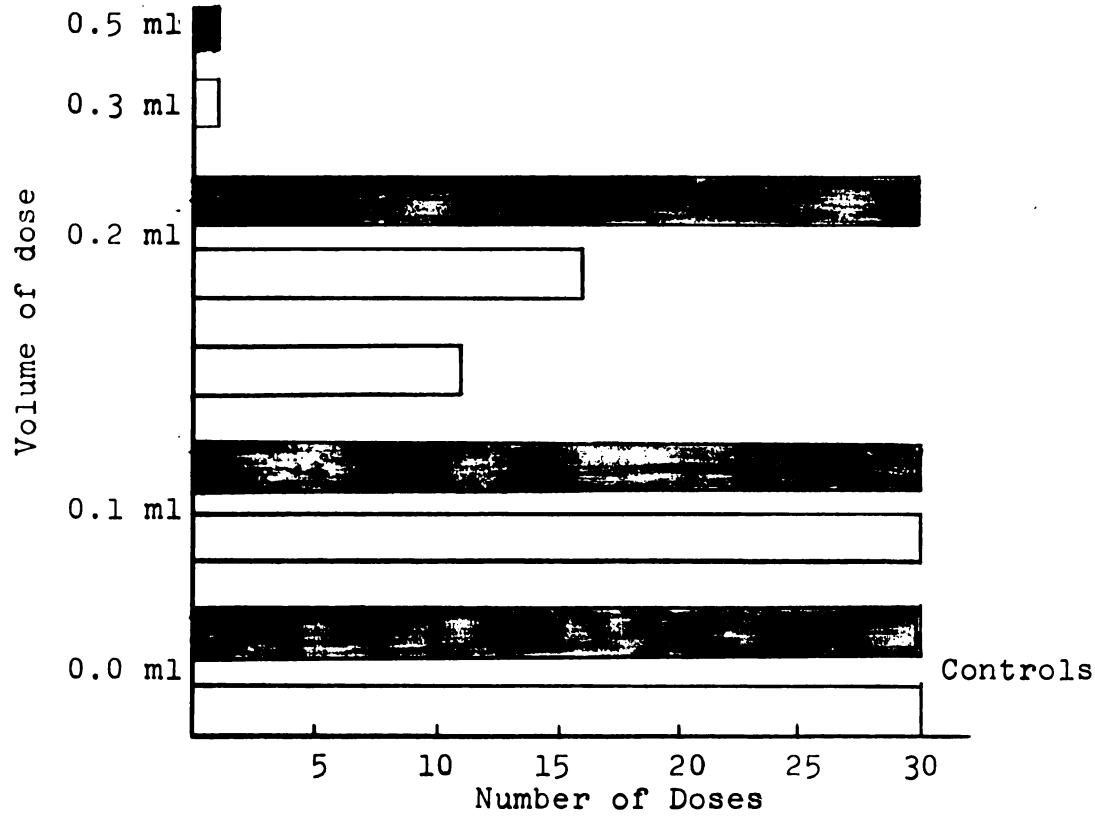


Figure 4.--Total number of tri-weekly administered doses of 40% CCl_4 survived by groups of mice

TABLE 2.--Induction of macroscopic hepatic changes in strain-A mice following chronic feeding of CCl_4.

Numerator = positive occurrences
Denominator = number of mice observed

SEX	Number of Doses				CONTROL
	1-23	24-30	31-40	41-	
M	0/80	0/5	0/3	10/10	0/16
F	0/59	1/16	2/10	23/26	0/33

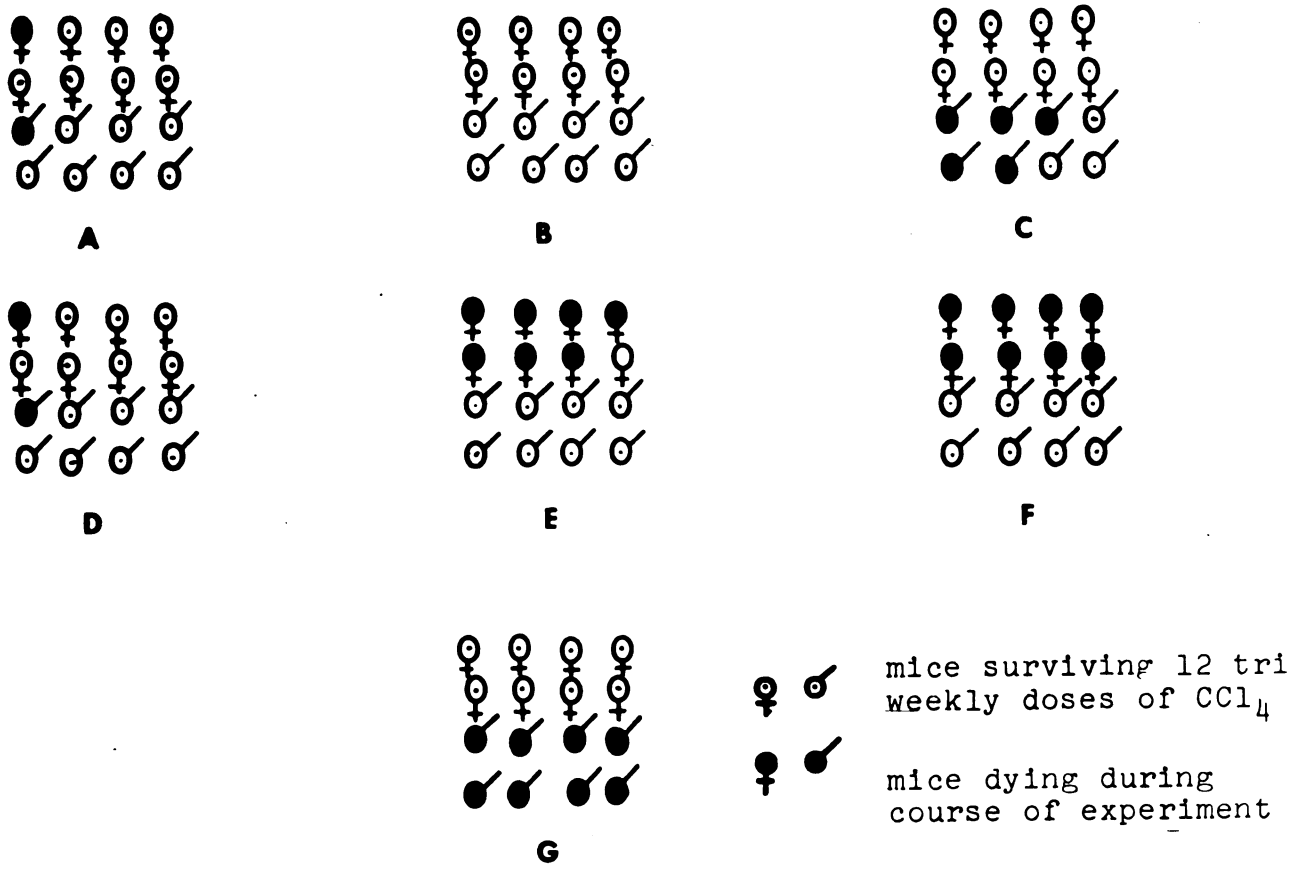
males and 2 of 10 females examined between the 30th and 40th feedings exhibited evidence of tumor. Twenty-three of 26 females receiving 40 or more doses of CCl_4 solution displayed well-defined tumors (multiple in many cases) at the time of autopsy. Ten of ten males that received 40 or more feedings exhibited well-defined tumors at autopsy. There was no evidence of hepatomas in any of the control mice examined post mortem.

Table 3 shows the incidence of death in each of the groups of mice involved in the experiment to determine the effects of sex hormones on resistance to CCl_4 toxicity. One male and one female of the intact control group (A) and none of the sham-operated control mice (group B) died during the course of the experiment. The mortality figures for the castrate males and females receiving CCl_4 do not vary appreciably from those of the control groups. None of the intact females receiving CCl_4 (groups C and G) nor any of the estrogen-treated male castrates receiving CCl_4 (groups E and F) died during the course of the experiment. However, the intact males (groups C and G) and castrate females injected with testosterone (groups E and F) exhibited marked sensitivity to CCl_4.

Ion Distribution Experiments

The values for metal ion concentrations per mg. of protein of whole liver and liver mitochondria are graphed in Fig. 5 as a function of time after the administration

TABLE 3.--Relation of sex hormones to resistance to carbon tetrachloride poisoning.



A: unoperated mice receiving olive oil orally

B: sham-operated mice receiving corn oil injections and olive oil orally

C: unoperated mice receiving 0.015 ml/gm body wt. CCl_4

D: castrate mice receiving 0.015 ml/gm body wt. CCl_4 solution orally

E: castrate mice receiving 0.015 ml/gm body wt. CCl_4 solution orally: males injected with 0.1µg stilbesterol, and females with 60µg testosterone propionate

F: conditions same as those for (E) except that hormone injections started 12 days prior to exposure to CCl_4

G: unoperated mice receiving orally 0.3 ml CCl_4 per mouse

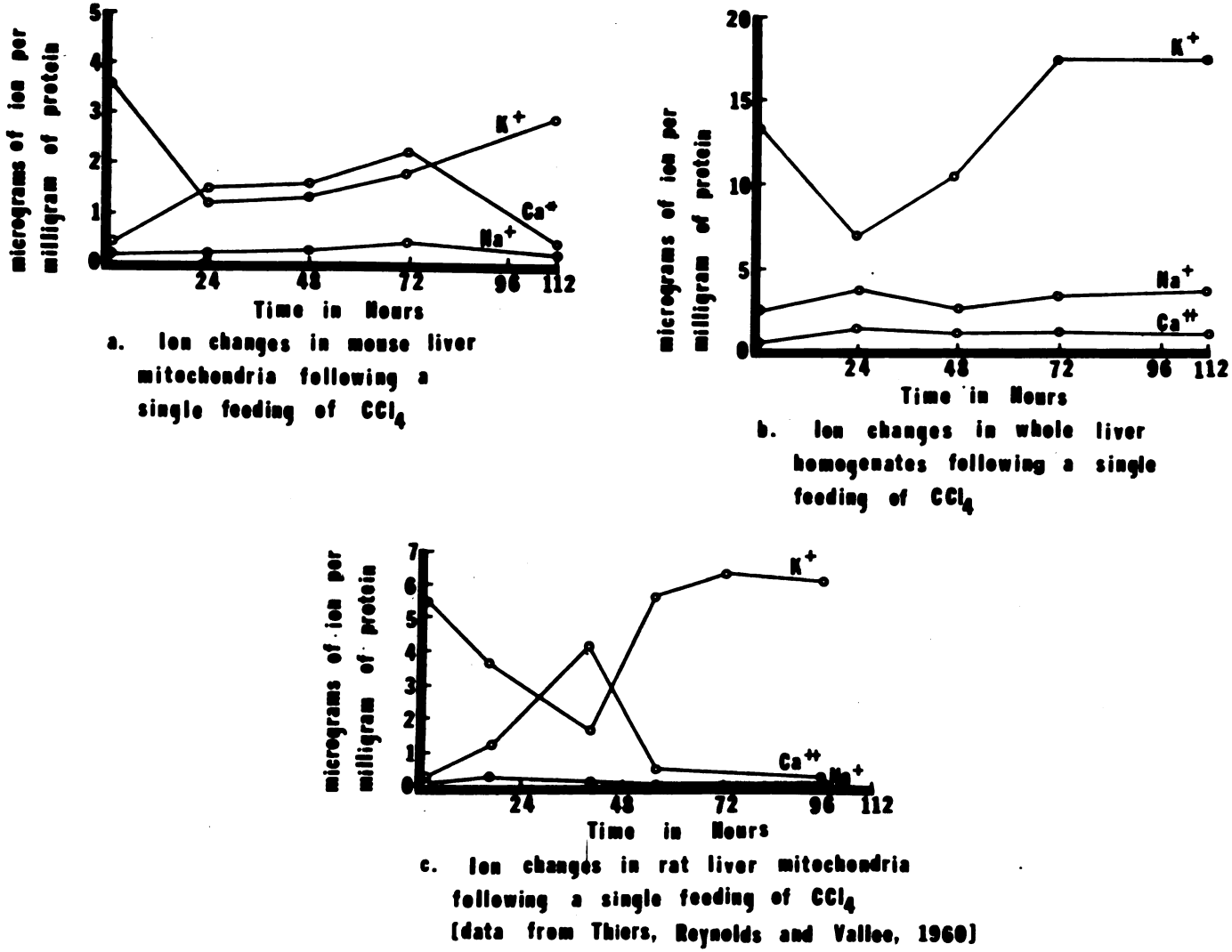


Figure 5.--Time course of changes in a metal ion concentrations in liver and liver mitochondria of strain-A mice following a single dose of CCl_4

of CCl_4. The sodium levels in liver mitochondria after a single CCl_4 dose do not vary markedly from normal values. The calcium content, however, increases to a maximum of five to six fold between 24 and 72 hours, while potassium rapidly decreases to below 50% of the control level. Both of these ion concentrations return to normal levels within 112 hours following feeding.

Thus the comparison of the data for the rat to that of the mouse (Fig. 5a) following a single feeding of CCl_4 shows that the time course of the ion change in mouse liver mitochondria and the direction of these changes are similar in the two species.

Fig. 5b shows the time course of ion changes in whole liver homogenates following a single dose of CCl_4. The maximum calcium and minimum potassium levels occur at times corresponding to those of the mitochondria. The whole liver data no doubt reflect the influence of residual plasma of unperfused livers, note higher Na^+ level, as well as alterations of Ca^{++} and K^+ excretion rates by the kidneys.

Experimental animals exhibited a marked decrease in body weight following the first feeding of CCl_4. Fig. 6 discloses that the average body weights of mice decrease to 90% of the original levels 24 hours following ingestion of CCl_4, and reaches a minimum of 87.4% of the original weight by the third day. It should be noted that the mean weight of females did not decrease to the same extent as

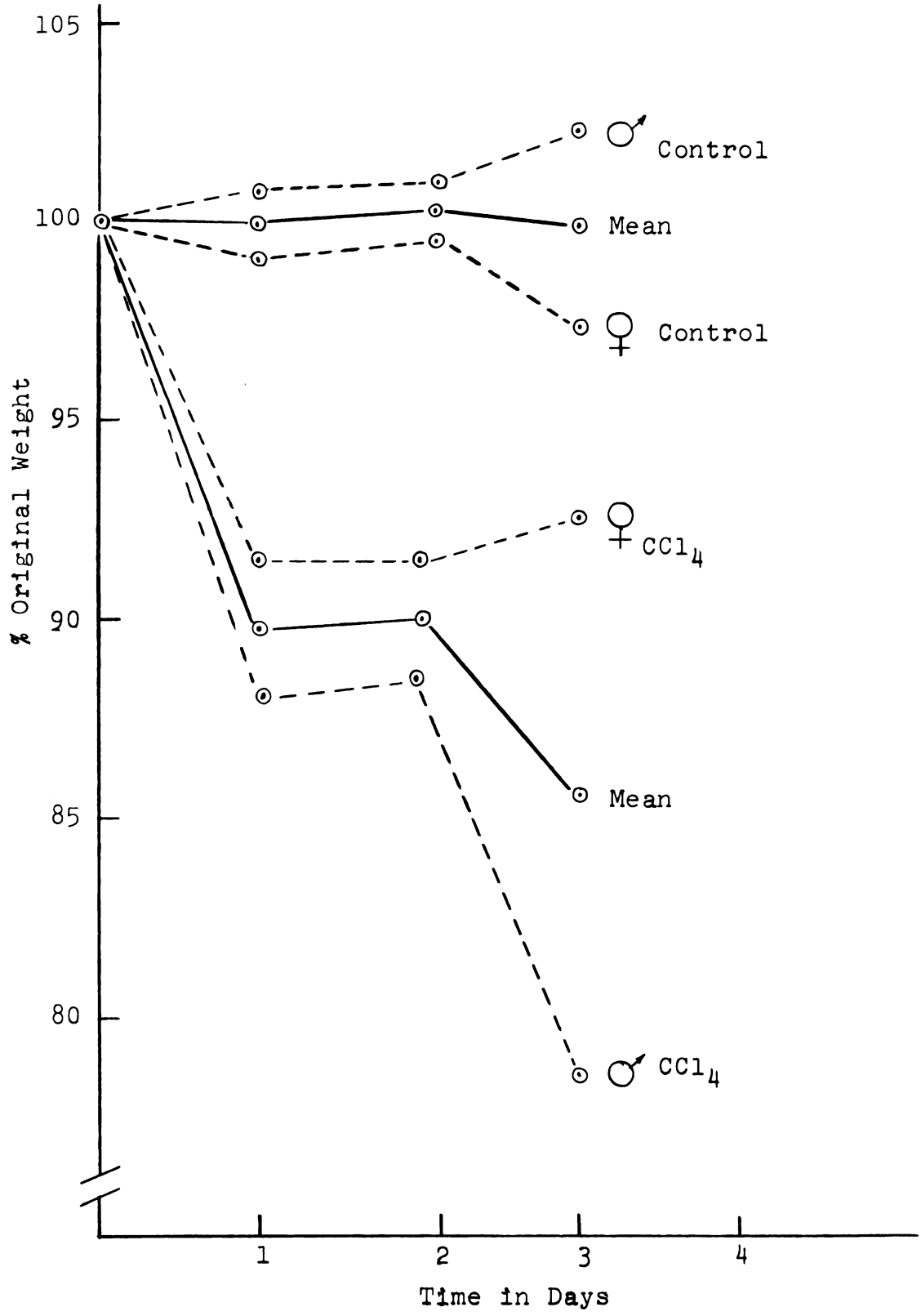


Figure 6.--Changes in body weight of mice following a single dose of CCl_4

did that of males. Moreover, the mean weight of the females actually increased between the second and third day, while that of males continued to decrease.

Observation of livers of experimental animals revealed a marked increase in mean liver weight during the course of the experiment (Fig. 7). Ratios of liver wt. to total body wt. were determined for each animal at the time of death, and a plot of these ratios as a function of time discloses the increase in liver mass over the four-day interval. Liver weight-body weight ratios for normal mice were observed at random during the course of other experiments, the mice ranging in age from six weeks to 12 months. These ratios did not differ significantly from those of the control mice of this experiment. The protein content of the liver and mitochondria was used as a base line for the determination of ion concentrations in terms of unit mass of metabolically active tissue.

In Fig. 8 the values for the protein concentration per gram of liver increase to a maximum of 15% above control values at 48 hours, and decline toward control values from that point. In view of the fact that these values exceed control values during the experimental period when the total liver mass also exceeds control values, we consider this as evidence for a net synthesis of protein during this interval. Since purity of the isolated mitochondrial fraction rather than maximum recovery of the total fraction was of primary

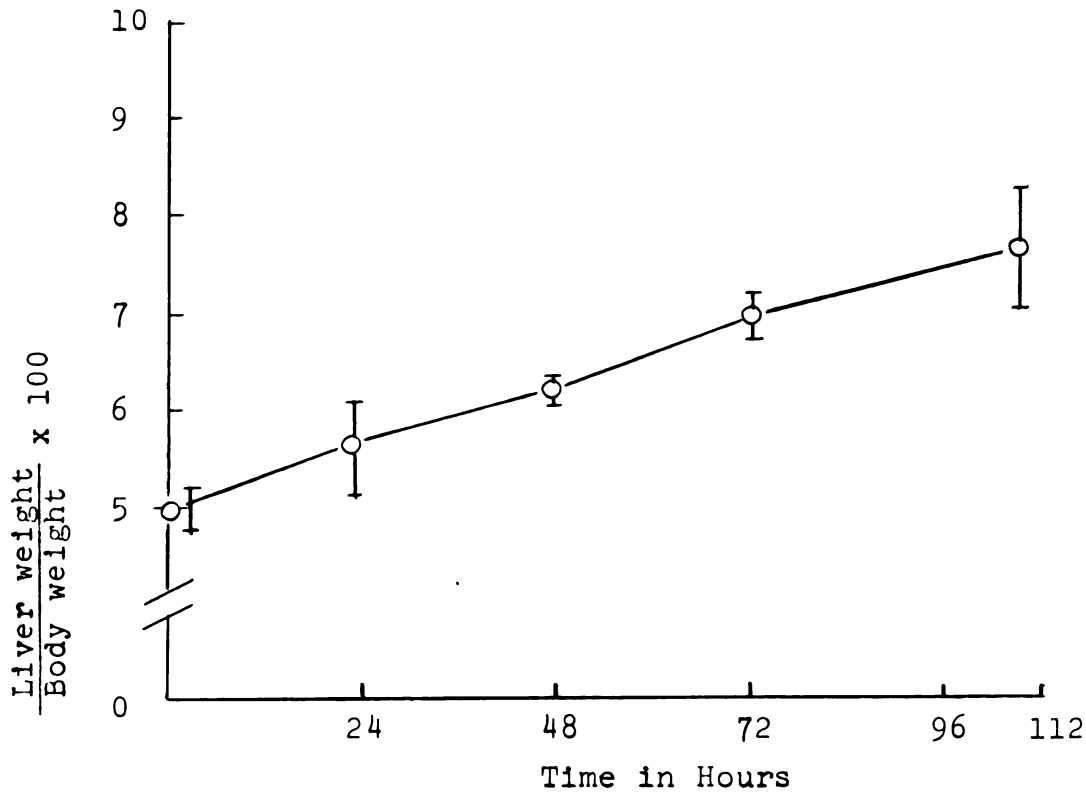


Figure 7.--Time course of changes in liver weight body weight ratios in mice following a single dose of CCl_4 (bars represent standard error of mean)

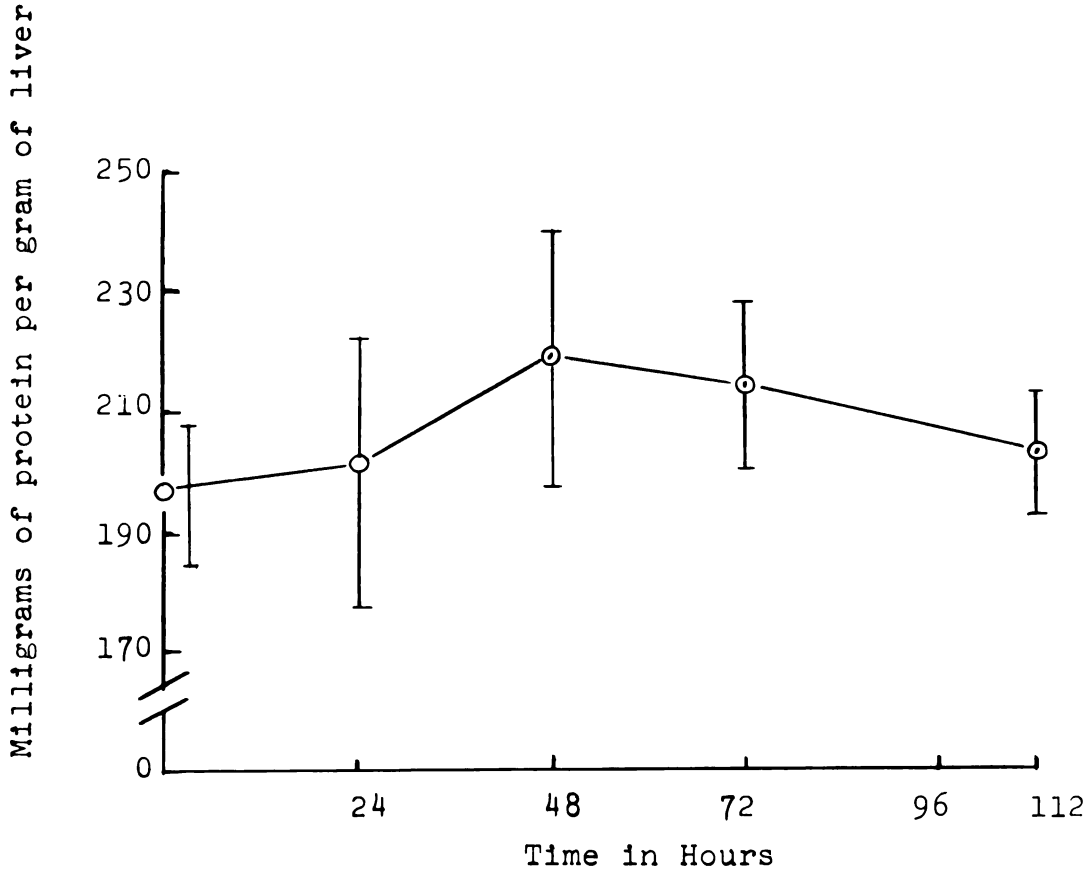


Figure 8.--Changes in protein concentration of mouse liver following a single dose of CCl_4 (bars represent standard error of mean)

importance, data are not available for total mitochondrial protein/unit mass of liver.

In Fig. 9b the changes in concentrations of sodium, potassium and calcium of mitochondria obtained from livers of mice exposed to tri-weekly feedings of CCl_4 are shown. The general trend of the ion changes during the period of the first 9 feedings is similar to the pattern of ion fluctuations of mitochondria following a single exposure to CCl_4. The calcium content increased to a maximum of nearly 8 times the control value following the third feeding. Following the 9th feeding when the concentration is 7 times the control value, the calcium content steadily declined and approached control values, although remaining significantly (Student's t test) higher throughout the duration of the experiment. The potassium concentration decreased to one-third of the control value following the third feeding, after which time the potassium concentrations were, for the most part, not significantly different from control values throughout the experiment.

Fig. 9a compares the sodium, potassium, and calcium concentrations in whole livers as a function of the cumulative amount of CCl_4 ingested. The potassium and calcium concentrations were quite variable during the course of the first nine feedings and in general varied in the same direction rather than in the reciprocal fashion as seen in the mitochondria (Fig.9b). Alterations in sodium

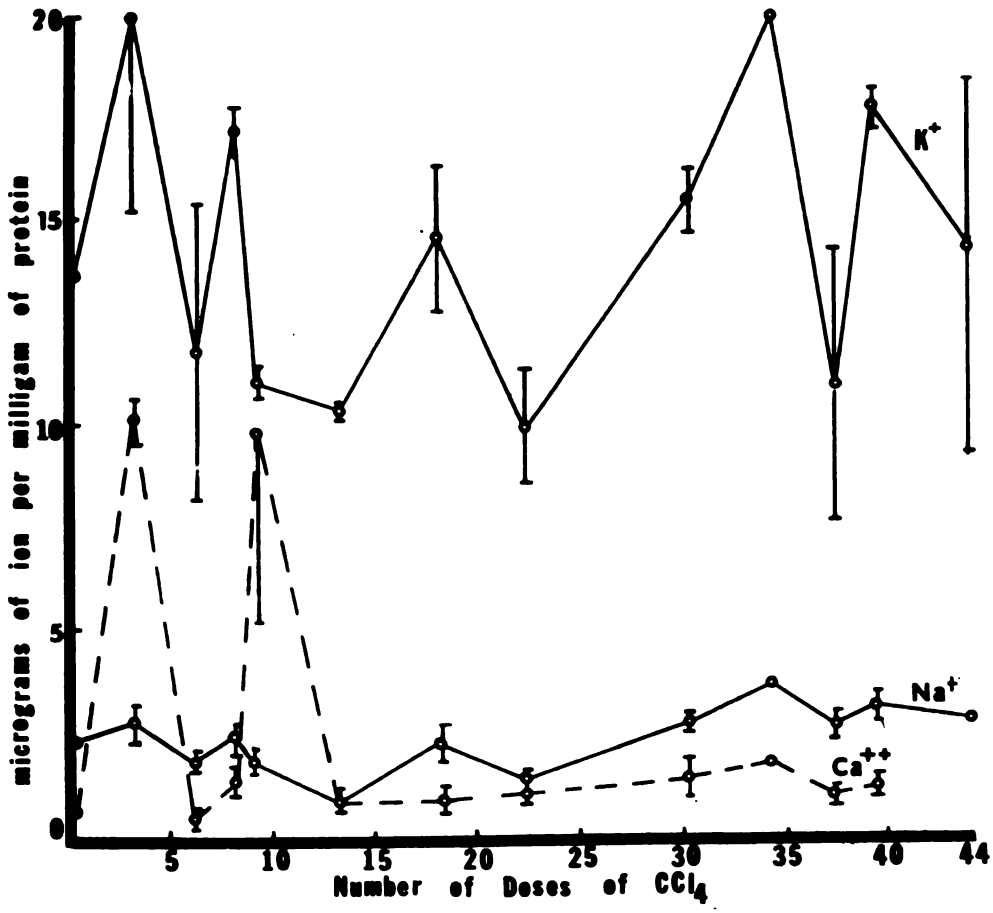


Figure 9a.--Ion changes in whole liver during chornic feeding of CCl_4

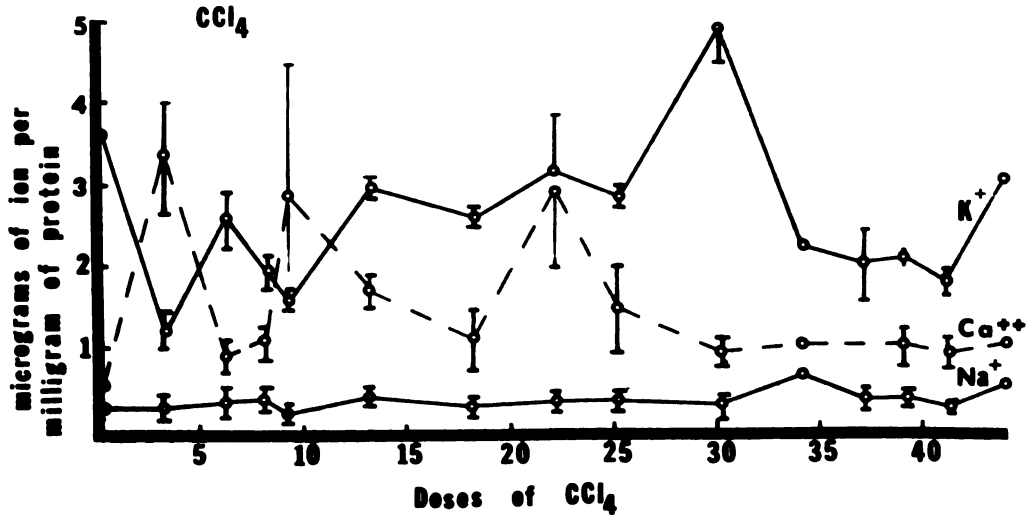


Figure 9b.--Mitochondiral ion changes during chronic feeding of CCl_4. Mice received tri-weekly feedings of 40% CCl_4, dose level 0.005 ml/gm. body wt. (bars represent standard error of mean)

concentrations, although paralleling the directions of potassium and calcium changes, did not vary significantly (Student's t test) from control values during this interval. The ion concentrations vary much less markedly from the ninth through the 30th feedings. The potassium concentrations were near or lower than control values during this interval, while calcium concentrations again were generally not significantly different from control levels.

Weight records were kept for all mice during the course of this experiment. All mice receiving CCl_4 exhibit a 12% to 20% decrease in weight by the second day following the initial feeding. Fig. 10 compares the weight records of control and experimental mice. The average weight of the experimental group exceeds that of the control group by 2% at the beginning of the experiment, while the average weight of the control group exceeds that of the experimental mice by 7% at the end of the 30th feeding. After the initial decrease in weight following the first feeding of CCl_4, the growth record of the experimental group roughly parallels that of the control group, although remaining significantly lower.

There was a general increase in liver mass of CCl_4-fed mice during the course of the experiment. Fig. 11 shows the ratio of liver weight to body weight to increase from 4.99 at the beginning of the feeding regime to 8.03 at the end of 30 feedings. Initially the decrease in

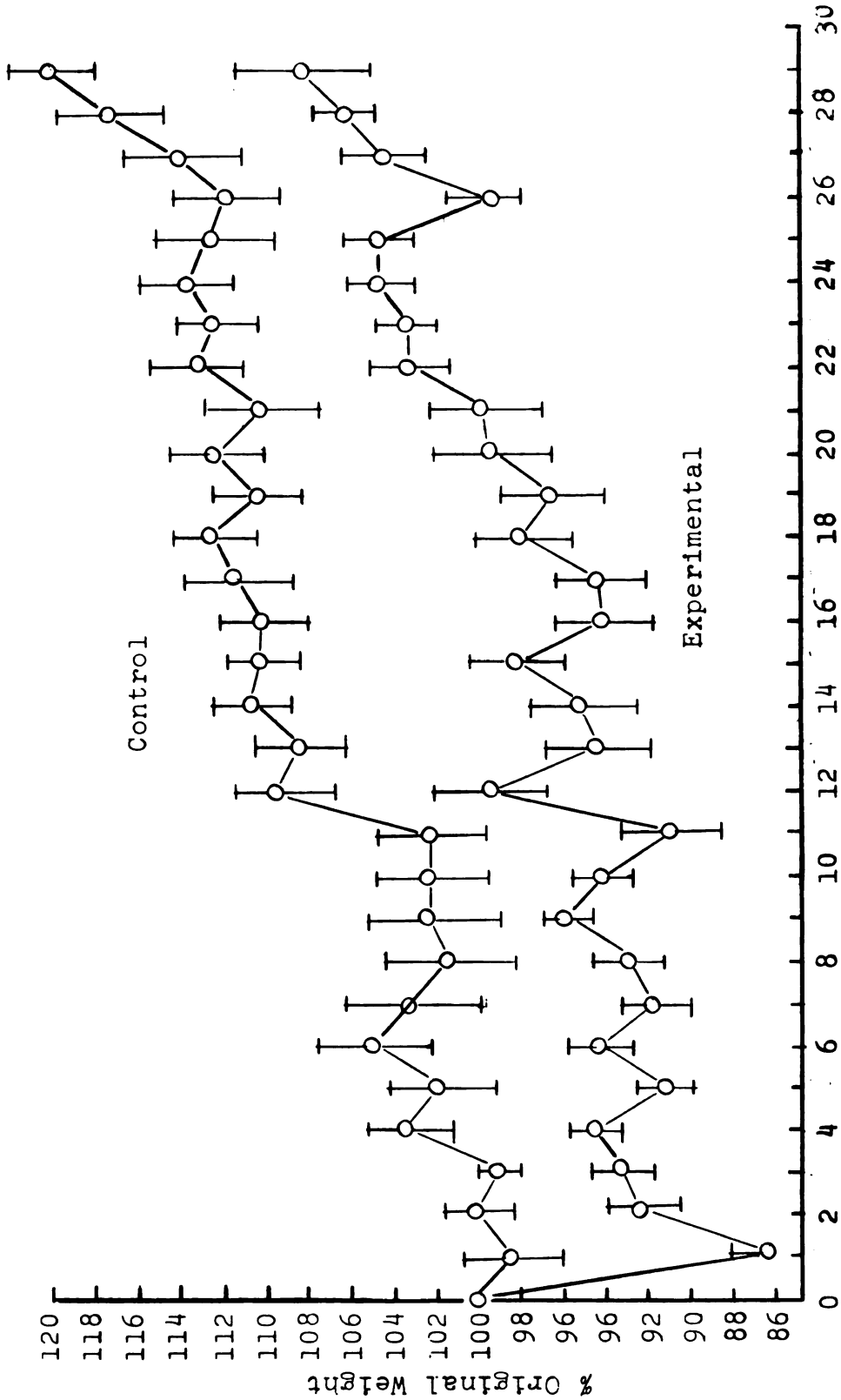


Figure 10.--**Time course of weight changes** in strain-A mice during chronic feeding of CCl_4 (bars represent standard error of mean)

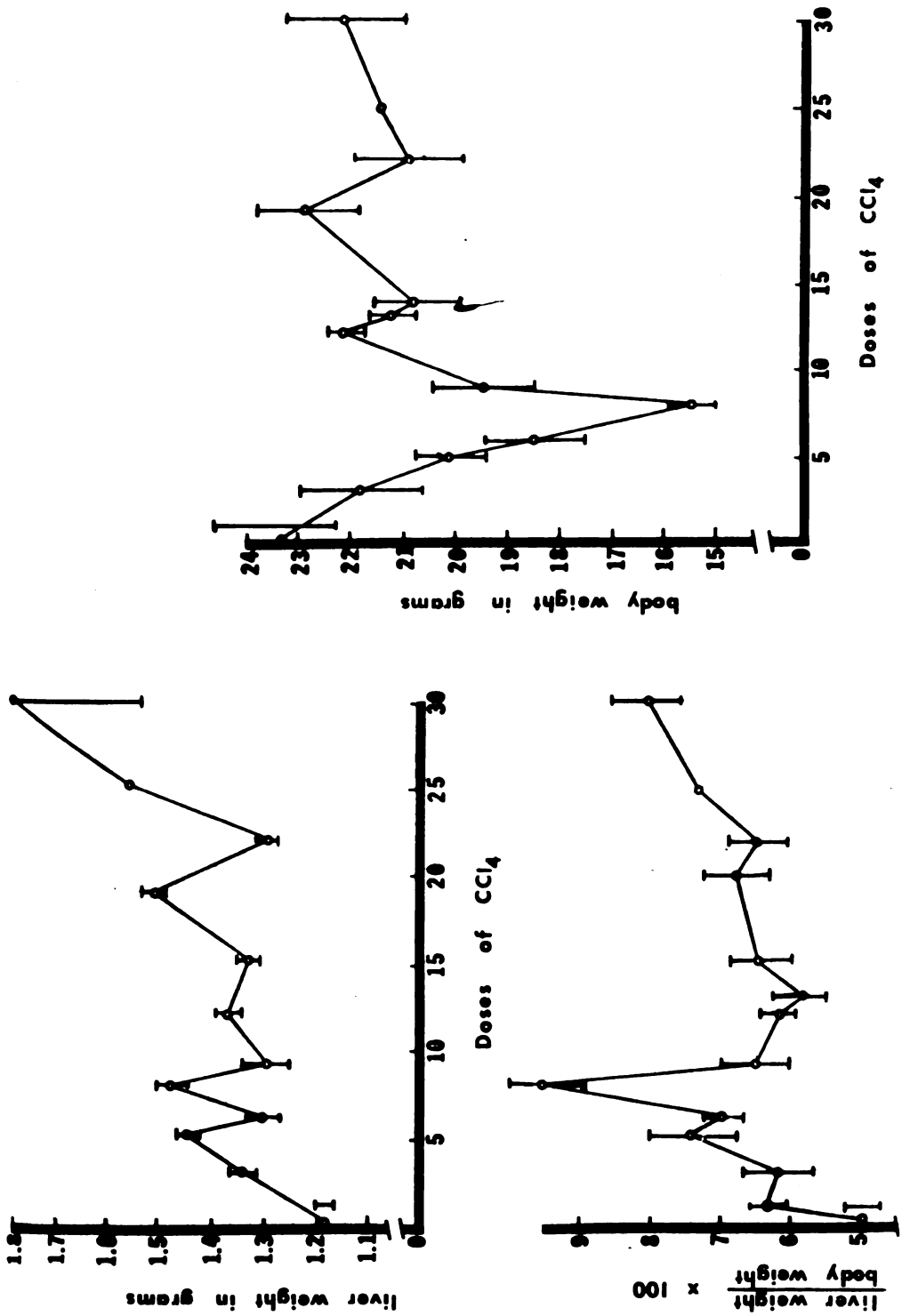


Figure 11.--Comparison of changes in liver weights with changes in body weights of strain-A mice during chronic feeding of CCl_4. Mice received 30 tri-weekly feedings of 40% CCl_4, dose level 0.005 ml/gm. body wt. (bars represent standard error of mean)

body weight may be as important a factor in the elevation of these ratios as an increase in liver mass. The fact that the values for concentrations of protein per gram of liver (Fig. 12) exceed control values from the 9th through the 18th feeding suggests a net synthesis of protein during this time. The fact that the protein concentration per gram of liver decreases from the 18th through the 30th feeding in spite of the fact that total liver weight increased during this interval suggests accumulation of some other liver constituent (possibly lipid or water), and a consequent dilution of protein.

Oxygen Utilization by Mitochondria in Situ

The results of the investigations of the oxygen utilization in relation to the toxic effects of a single dose of CCl_4 are summarized in Fig. 13a. The oxygen uptake was computed on the basis of liver protein concentration. The oxygen utilization is reduced to 30% of the control level at 24 hours and steadily increases to 62% and 72% of control values respectively at 48 and 72 hours.

The O_2 uptake of liver slices obtained from mice to which CCl_4 was administered repeatedly is shown in Fig. 13b. The oxygen utilization falls to approximately 42% of control values on the basis of the liver protein index. From this point there is a generally variable, but always

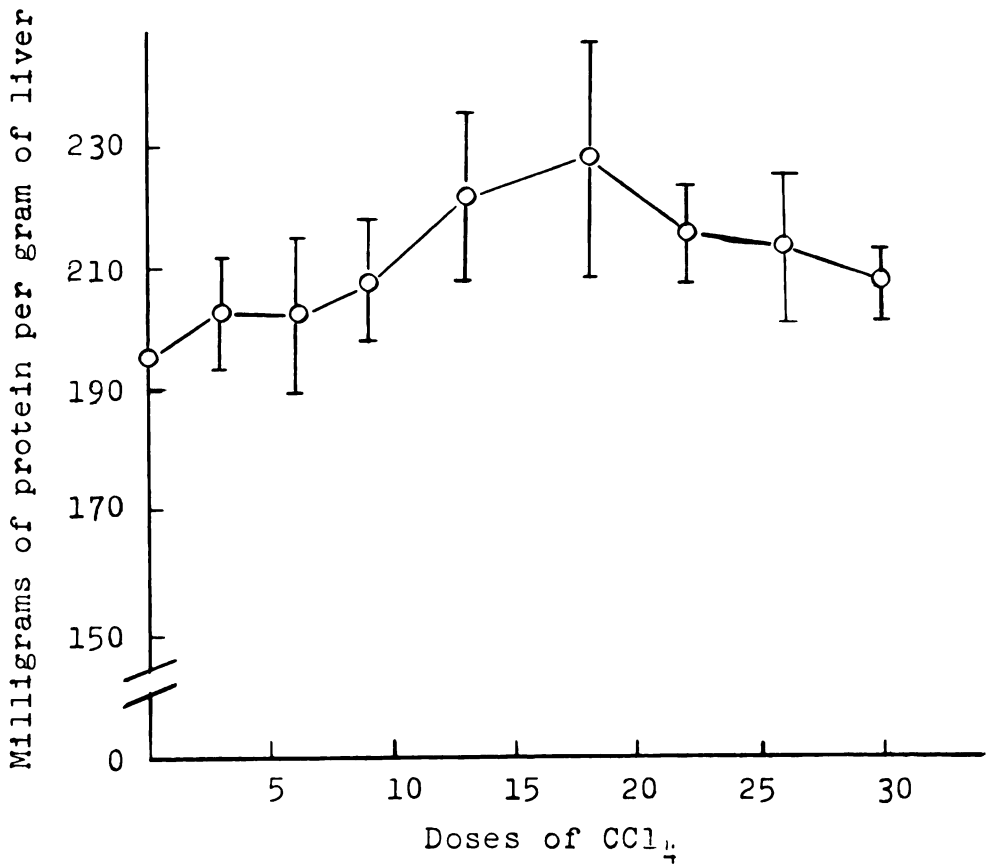


Figure 12.--Changes in protein concentration of mouse livers during chronic feeding of CCl_4 (bars represent standard error of mean)

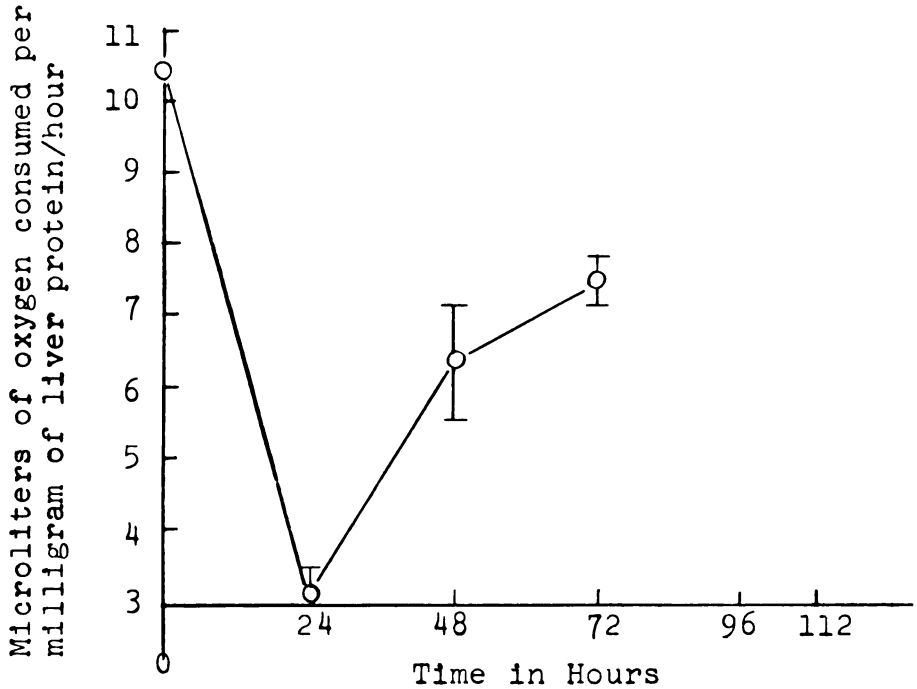


Figure 13a.--Oxygen uptake of liver slices of mice following a single dose of CCl_4 (bars represent standard error of mean)

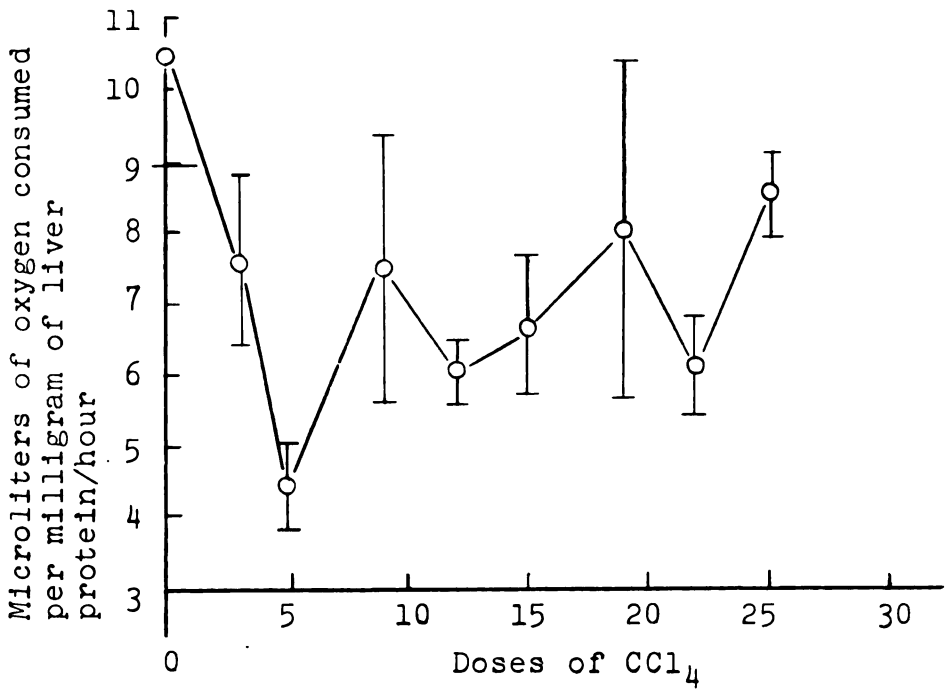


Figure 13b.--Oxygen uptake of liver slices of mice during chronic feeding of CCl_4 (bars represent standard error of mean)

incomplete approach toward recovery of control levels of O_2 utilization during the remainder of the experimental period.

Phosphate fixation-oxygen reduction ratios were not determined routinely for mitochondrial preparations utilized for ion determinations. The results of the determinations that were made at selected intervals, however, indicate that the mitochondria we prepared retained their functional capacity and, thus, are representative of the normal physiological activity of the liver (Table 4). The experimental, mean P/O ratios were 1.47 for succinate preparations (theoretical maximum = 2.0) and 3.26 for ketoglutarate preparations (theoretical maximum = 4.0). The experimental ratios compare favorable with expected, theoretical ratios, and represent acceptable, practical values.

In view of the fact that agents foreign to the physiological systems must be added for protection against swelling and other kinds of degeneration of mitochondria *in vitro*, and that unnatural phosphate traps are utilized to obtain maximum P/O ratios (hexokinase or creatine kinase systems), P/O determinations cannot be utilized to assay the true, specific activities of the enzyme system *in vivo*. However, since the metabolic work of moving ions through membranes, especially against their electrochemical gradients, is of considerable magnitude, it is of importance to remark that those data show that our

TABLE 4.--Reaction system and results for phospate fixation-oxygen reduction determinations.

Substrate			Flasks
PQ_4 Buffer	0.65M	0.5 ml	0.5 ml
Succinate	0.1M	0.2	-
Ketoglutarate	0.1M	-	0.2
Malonate	0.1M	-	0.2
ATP	.015M	0.1	0.1
$MgCl_2$	.015M	0.1	0.1
Hexokinase	1% in 0.1% glucose	0.1	0.1
Mitochondria (equivalent to 20-30 mg Protein)		0.5	0.5
Sucrose (.24M) EDTA (.003M)		0.4	0.2

KOH 10% 0.1 ml in center well of each flask

Results: (means of eight determinations)

Substrate	P/O
Succinate	1.47 ± 0.43
Ketoglutarate	3.26 ± 0.56

preparations are capable of satisfying the needed energy requirements.

CHAPTER V

DISCUSSION

As indicated earlier, the incidence of CCl_4-induced hepatoma increases as the rate at which a measured dose is administered decreases; and the incidence of hepatoma increases with the magnitude of the dose of CCl_4 administered at a constant dose rate. Our study to determine the maximum dose that could be tolerated on the basis of a tri-weekly feeding schedule revealed that the quantity of 0.005 ml of 40% CCl_4 solution per gm. of body weight (0.1 ml per 20 gm. mouse) was the most satisfactory dose that could be administered regularly without increasing the mortality rate.

At a chronic dose level of 0.1 ml, both male and female mice survive reasonably well over a long period of time. With increasing concentrations above 0.1 ml, however, males apparently become increasingly more sensitive to the toxic effects of CCl_4 than females. Our observations indicate that female mice may be maintained over the period of time required for the development of liver tumors at a dose level as high as 0.3 ml per 20 gm. mouse with no histological evidence of kidney derangement. No males

survived the tumor-induction period (30 weeks) at dose levels exceeding 0.1 ml. Those that survived the exposure to the 0.3 ml dose level for several weeks did exhibit renal necrosis.

If there is a close relationship between frequency of hepatoma and dose levels in this series of experiments, it is not evident. Three of nine females receiving 0.3 ml CCl_4 solution and 3 of 15 females receiving 0.1 ml per dose developed tumors prior to the 40th feeding. Six of nine females receiving 42 feedings of 0.3 ml CCl_4 solution and 16 of 16 females receiving 40 to 50 feedings of 0.1 ml per dose exhibited tumors.

The nodules of tissue observed in this study, arising in mice exposed to CCl_4, resembled regenerating liver tissue. The CCl_4-induced neoplastic growths described by Dalton and Edwards (1942) (1943) and others closely resembled regeneration nodules. With eosin-hematoxylin staining, normal and regenerating hepatic cells display an eosinophilic cytoplasm, while CCl_4-induced hepatoma cells frequently show a slight basophilic cytoplasm. Differentiation on this basis, however, is by no means clear-cut and distinct.

If these nodules do indeed consist of hepatoma cells, they are derived from liver parenchyma cells and are not appreciably different from them histologically (Edwards 1943). In any event, the determination of the transition

from normal regenerating liver parenchyma cell to true hepatoma cell will require histochemical, cytochemical, and autoradiographic techniques which can detect the apparently subtle differences between regenerating cells and hepatoma cells.

The time course of the development of a satisfactory incidence of tumor is approximately 100 days. Since these tumors average 5 mm. or less in diameter, additional time would be required for them to develop to sufficient mass to allow recovery of enough sample material for practical chemical and metabolic analysis. Obviously, the actual induction of hepatoma must begin prior to the 40th feeding and would have to be determined on a histological, histochemical, or biochemical basis.

The high mortality of male mice and female castrates injected with testosterone propionate suggests that the androgen is in some fashion responsible for the increased sensitivity to CCl_4. The fact that only one of the castrate males receiving CCl_4, but no hormone and one of the castrate males receiving stilbesterol and CCl_4 died during the course of the experiment is not sufficient evidence that the estrogen, on the other hand, provides a protective effect.

Eschenbrenner (1945), in studying the effects of chloroform intoxication in strain-A mice, observed for high doses general necrosis of renal tubular epithelium (with the exception of the proximal tubule adjacent to

the capsule) in all male mice, but not in females. He reported no evidence of necrosis in female kidney at a dose level of chloroform equivalent in concentration to the dose level of carbon tetrachloride of our experiments. Observations of kidney sections of male and female mice receiving 0.005 ml 40% CCl_4 per gram of body weight in our experiments show no evidence of necrosis in either sex.

Crabtree (1940) reported a high percentage of glomerular capsules lined with cuboidal rather than squamous epithelium in male mice, as opposed to a much lower percentage of such capsules in the female kidney. This sex-linked difference relative to the structure of Bowman's capsule was observed in strain-A mice by Eschenbrenner (1945) and again during this series of investigations. The percentage of cubiodal cell capsules in castrate males was observed by Crabtree (1941) to decrease to approximately the female level for animals of the same age. Administration of testosterone propionate to female castrates resulted in a marked increase in cuboidal capsules. Although it appears that testosterone exerts a characteristic and specific effect on kidney tissue, it is difficult to evaluate this finding. Selye (1939) suggests that the hormone may either have a direct, trophic effect upon the kidney, which would affect its function, or the primary influence may effect general metabolic changes which would necessitate increased kidney function.

From the data regarding the time course of weight loss in mice receiving chronic feedings of CCl_4 a sudden decrease in weight following the initial feeding is suggestive of a fluid loss which is more serious in the male than in the female. The increase in liver protein concentration noted in the early hours of our experiment, when protein synthesis is reported to be depressed (Smuckler 1962), also is suggestive of dehydration. The possibility exists that a CCl_4-induced diuresia in the initial 24-hour period following CCl_4 feeding may be responsible for this phenonmenon. Cornish (1964) indicates that the normal volume of urine produced in 24 hours by one rat (8-10 ml.) is doubled during the first 24 hours following exposure to CCl_4. The urine output returned to normal levels within the second 24-hour interval. The condition should be more pronounced in the male than in the female since the tubular epithelium of the male appears to be more sensitive to CCl_4, or products of CCl_4 metabolism, than that of the female. Urinalyses and glomerular filtration studies should clarify this point.

A damaged intestinal epithelium could serve as another avenue for fluid loss. CCl_4 does produce diarrhea. The relevance of this particular point has not been resolved at the time. There has been no report of structural dimprphism of the gastro-intestinal tract of the mouse. A functional dimorphism of the observed fluid loss is not

excluded on this basis, however. The possibility exists that different products of CCl_4 catabolism might be produced under influence of sex hormones such that the gastrointestinal tract of the male might be exposed to a somewhat more toxic product of CCl_4 catabolism via the bile than that of the female.

Calcium is known to accumulate in tissue cells that are undergoing necrosis. Rouiller (1964), Rees et al. (Gallagher 1956) demonstrated this to be true of rat liver in which necrosis was induced by thioacetamide intoxication. An increase in calcium was found to be associated with necrosis in rat liver resulting from dietary injury (Wachstein et al. 1962), while similar results were shown by Stowell and Lee (1951), (Reynolds 1962), Calvert & Brody et al. (1958), Vallee (Reynolds (1962) in rat livers as a result of exposure to necrotizing doses of CCl_4. This evidence is in agreement with the results reported here regarding accumulation of calcium by livers of Strain-A mice exposed to necrotizing doses of CCl_4. The marked increase of calcium is associated with a decrease of potassium. Vallee (Reynolds 1960) found the increase in calcium to precede the loss of potassium from rat liver mitochondria, and thus suggested an interdependence of the ion fluxes. Such an interdependence could be a manifestation of an exchange of calcium for potassium at a fixed number of mitochondrial binding sites, an

isosmolar exchange of calcium ions for potassium ions, or the fixing of calcium by an increasing number of valences made available through unfolding of protein chains which undergo denaturation during the development of necrosis.

Observations of Hunter (1955) and Gamble (1957) suggest that normal oxidative activity of mitochondria depends upon their capacity to maintain adequate concentrations of potassium. Excess calcium was first shown by Hunter (1955) and Rees et al. (Gallagher 1956) to interfere with normal mitochondrial respiration. Reducation of the calcium level of the medium by chelating agents was shown to diminish the leaching of dehydrogenases from liver slices (Judah 1960). The recovery of in vitro rates of oxidation equal to control levels (Rees et al.) (Gallagher 1956) demonstrated that inhibition of respiration could be attributed directly to the presence of accumulated calcium. The source of the accumulated calcium is uncertain, although observations of Vallee (Reynolds 1962) suggest the serum to be the source.

Calvert and Brody (1958) found the sodium content of hepatic intracellular fluid increases by 60% and the potassium levels decrease some 40% following CCl_4 intoxication. Concomitantly, plasma solium concentrations decrease some 5%, while potassium levels increased by an equivalent amount. Thus, the fact that in our experiments the cations of the whole liver homogenates and

mitochondria behave in the same way, we interpret as evidence that the cations are equilibrating from their higher to their lower concentration compartments, rather than between the mitochondrial and extra-mitochondrial cell fractions. Furthermore, the toxic effects of CCl_4 can be assumed to be the same on the partitioning mechanism (membranes) of the cell and the mitochondria. The less prolonged effect on mitchondria is doubtless the consequence of a less severe initial injury resulting from the diluting effect of the extra-mitochondrial cytoplasmic fraction.

The ion shifts in livers and mitochondria of strain-A mice are comparable in both direction and time to those in rats (Reynolds 1960). The magnitude of the ion shifts is less striking than that obtained by Vallee either because our dose level of CCl_4 was less (0.5 ml 40% CCl_4/ 100 gm. body wt. as opposed to 0.5 ml 50% CCl_4/100 gm. body wt.), or the ion shifts did indeed attain comparable maxima, but at times that did not coincide with our scheduled times of analysis (24, 48, 72 and 112 hours).

The fluctuations in ion concentrations of whole livers and mitochondria during the first few feedings of the long term experiments are as drastic as those resulting from a single exposure; however, these shifts become less pronounced and erratic subsequent to the ninth feeding. This is perhaps a result of a progressively

increasing number of cells which are resistant to the toxic effects of CCl_4. Likewise, histological observations show the degree of necrosis following the first few feedings of the multiple feeding experiment to be about the same as that associated with a single dose. However, the degree of necrosis begins to diminish following the initial exposure. Thus the marked ion shifts due to CCl_4 sensitive cells would become progressively masked by the less pronounced shifts of resitant hepatocytes with time.

The development of a specific resistance of hepatic cells to the necrotizing influence of CCl_4 has been reported by Eschenbrenner (1946). The basis of this resistance is not known, but may be related to the fact that developing neoplasms do not acquire a portal source of circulation (Wilson, 1951), and thus these cells would escape immediate exposure to absorbed CCl_4. The concentration of CCl_4 in hepatic arterial blood would be considerably less than that of portal blood. The fact that the necrotizing effects of CCl_4 are reduced at a time when regenerating cells are exposed to portal circulation suggests that some cellular changes must evolve which are protective in nature.

The effect of CCl_4 on mitochondrial and cellular protein concentrations during the single feeding experiment and generally throughout the multiple feeding experiment can be due either to a stimulation of protein

synthesis or to dehydration. Due to insufficient amount of liver tissue for all types of determination required, dry weights of samples were not obtained on a routine basis. Tsuboi et al.(1951), however, found the moisture levels of livers of mice to be somewhat above control levels throughout a period of 18 days subsequent to a single exposure to CCl_4. During this period during which livers were actually hydrated, protein fractions were found to reach a maximum of 25% above normal values (on a lipid-free dry weight basis) by the sixth day, and to be somewhat above control values during the interval corresponding to the duration of our single feeding experiment. During the course of 20 weeks of exposure to 0.1 ml 40% CCl_4, Stowell et al. (1951) found the nitrogen content of livers to be generally above normal values in non-neoplastic hepatic tissue, although the nitrogen content of neoplastic tissue itself was below control values. These results suggest that the effect of CCl_4 on liver is a stimulation of protein synthesis superimposed upon the protein of necrotic tissue, the bulk of which still exists until the fourth day following a single exposure. Cellular membranes are damaged much earlier by CCl_4 intoxication than are mitochondrial membranes as evidenced by the presence of cytoplasmic dehydrogenases in serum (Gallagher et al. 1960) some 10 to 20 hours prior to detection of mitochondrial enzymes and co-factors in serum. (Rees and

Sinha 1960). Intact, metabolizing mitochondria might actually control their water content at a time when hepatocytes are hydrated. In addition, a decrease in the capacity of mitochondria to bind ions could result in loss of loosely bound ions and the establishment of new ionic and osmotic equilibria during the preparation and centrifugation of mitochondria. Since sodium is much less strongly accumulated and bound by liver mitochondria than either calcium or potassium (Ulrich 1959) Spector (1953), quite likely the bound sodium fraction rather than the total sodium was measured in our experiments (mitochondria were twice washed). Thus, dehydration as a factor in protein concentration cannot be entirely ruled out.

Schwarz et al. (1955) have demonstrated that slices obtained from livers during the onset of necrosis are unable to maintain normal levels of respiration based upon oxygen consumption determinations under standard conditions. Calvert and Brody (1960), among others, have reported that the primary effect of CCl_4, and other chlorinated hydrocarbons, on the liver is a reduction in blood flow resulting from the release of catecholamines from the autonomic nervous system and the adrenal glands. The disruption of biochemical activities of the liver presumably would be a secondary consequence attributable to the ischemia arising from the circulatory impairment. However, it was demonstrated by Brauer et al. (1961) that blood flow through perfused livers exposed to chloroform

exceeds *in vivo* flow rates. This being the case, the effects of chloroform on the liver could be due to no form of anoxia other than possibly histotoxic anoxia. Granted that blood flow rates in isolated liver preparations are higher than *in vivo* rates (Brauer *et al*. 1956) (1959), there is evidence of a chloroform-induced vasodilation in their preparations. If this condition pertains in the intact organism, respiratory derangements would presumably result from a direct effect upon respiratory organelles (mitochondria) and/or respiratory mechanisms.

The results reported here are in agreement with the latter concept. Circulatory phenomena are of no consequence in relation to slices of excised tissue, and could not account for the observed decreases in oxygen uptake by CCl_4-treated tissues. In fact, hepatocytes, in general, are exposed to oxygen tensions no greater than 104 mm Hg., while the oxygen tension at the center of a liver slice of 0.3 mm thickness in an atmosphere of pure oxygen is of the order of 450 mm Hg (Umbreit 1964); thus, availability of oxygen should not have been a limiting factor in this investigation.

Mitochondria vary in number, size, ultra-structure, as well as functional integrity within the liver lobule under normal circumstances. Metabolic changes within physiological limits, and very definitely those of pathological proportions, alter the distribution and the

functional capacity of liver mitochondria. Numbers of functional mitochondria have been found to increase in liver sections examined following partial hepatectomy (Rouiller, 1964). Similar increases in numbers of mitochondria have been observed during the regenerative processes which occur following liver damage by various hepatotoxins, including CCl_4 (Bridgers, 1957).

The decrease in oxygen utilization observed within the first 24 hours of the single feeding experiment undoubtedly represents a decrease in the numbers of functionally intact hepatocytes as well as a considerable decrease in numbers of functionally intact mitochondria resulting from the necrotizing effects of CCl_4. The approach to normal respiratory levels at 48 and 72 hours may be interpreted as a reflection of mitochondrial regeneration and the resynthesis of mitochondrial enzymes and cofactors.

The approach to normal levels of oxygen uptake observed subsequent to the fifth feeding of the multiple feeding experiment presumably reflects less extensive liver damage with increasing exposure to CCl_4, as a consequence of development of increasing numbers of hepatocytes resistant to the toxic effects of the agents.

Inhibition of respiration caused solely by accumulation of calcium has been demonstrated by Gallager et al. (1956). Comparison of Fig.'s 5 and Fig. 9 to Fig.'s 13a

and 13b shows the time courses of ion changes and of oxygen utilization respectively. The maximum depression of respiration is correlated with maximum accumulation of calcium by mitochondria. The approach toward normal levels of oxygen uptake by liver slices parallels closely the decline of calcium concentrations with time.

CHAPTER VI

CONCLUSIONS

The investigation of the possibilities of accelerating tumor induction by manipulation of dose levels of CCl_4 solution administered on a tri-weekly basis has shown that:

1. At a chronic dose level of 0.005 ml 40% CCl_4 solution per gram of body weight, male and female mice survive equally well over a long period of time.
2. With increasing concentration of doses above this level the mortality rate of males is greater than of females.
3. The maximum dose level of 40% CCl_4 per gm. body weight that can be maintained over the period of time required for the development of liver tumors may be as high as 0.015 ml for females but no higher than 0.005 ml for males.
4. Approximately 100 days are required for the development of palpable hepatomas on a tri-weekly feeding regime. Acceleration of tumor development by an increase in dose rate does not seem possible.
5. The high mortality of both male and female castrates injected with testosterone suggests that the presence of androgens is more significant than the absence of estrogens in the expression of sensitivity to CCl_4.

The studies of ion distributions and oxygen utilization subsequent to administration of a single dose of CCl_4, as well as following multiple doses of the toxin, have shown the following:

1. The time course of ion changes in mouse liver mitochondria and the direction of these changes are similar to those reported for the rat following a single exposure to CCl_4.
2. The kinds of ion changes occurring in mouse livers and liver mitochondria following a single feeding persist through the period of 30 feedings required to induce hepatoma. However, following early feedings ion levels approach but do not quite attain control values.
3. Depression of aerobic respiration of liver slices of mice fed CCl_4 solution was shown to parallel calcium accumulation by mitochondria, while recovery paralleled reductions in calcium accumulations with time.
4. Histological changes in liver and kidney tissue were described and difficulties regarding differentiation between hepatoma cells and regenerating liver parenchyma were discussed.

On the basis of the above it was not possible to determine the onset of hepatoma formation, and thus to correlate the alterations in ion concentrations and respiration with the onset and subsequent development of hepatoma cells.

REFERENCES

Bartley, W. and R. E. Davies. 1954. Active Transport of Ions by Sub-Cellular Particles. Biochem. J., 57:37.

Bassi, M. 1960. Electron Microscopy of Rat Liver After CCl_4 Poisoning. Exptl. Cell Research, 20:313.

Bensley, R. R. and N. L. Hoerr. 1934. Studies on Cell Structure by the Freezing-Drying Method. VI. The Preparation and Properties of Mitochondria. Anat. Record, 60:449.

Bernhard, W. and C. Rouiller. 1956. Close Topographical Relationship Between Mitochondria and Ergastophasm of Liver Cells in a Definite Phase of Cellular Activity. J. Biophysic. Biochem. Cytol. 2, No. 4 Suppl., 73.

Brachet, J. and A. E. Mirsky. 1961. The Cell, Vol. II. Cells and Their Component Parts. Academic Press, New York.

Brauer, R. W., G. F. Leong, R. F. McElroy and R. J. Holloway 1956. Am. J. Physiol., 186:537.

Brauer, R. W., R. J. Holloway and G. F. Leong. 1959. Am. J. Physiol., 197:681.

Brauer, R. W., G. F. Leong and R. J. Holloway. 1961. Liver Injury in Isolated Perfused Rat Liver Preparations Exposed to Chloroform. Am. J. Physiol. 200:548.

Brierley, G. P., E. Bachman, and D. E. Green. 1962. Active Transport of Inorganic Phosphate and Mg^{++} by Beef Heart Mitochondria. Proc. Natl. Acad. Sci. U.S. 48:1928.

Bridgers, W. F., D. Abele and F. E. Hunter Jr. 1957. Action of Carbon Tetrachloride on Liver Mitochondria. Federation Proc., 16:158.

Buchner, N. L. R., J. J. Scott and J. C. Aub. 1951. Regeneration of Liver in Parabiotic Rats. Canc. Res., 11:457.

Calvert, D. N. and T. M. Brody. 1958. Biochemical Alterations of Liver Function by Halogenated Hydro-carbons. J. Pharm. Exptl. Therap., 124:273.

Calvert, D. N. and T. M. Brody. 1960. Role of Sympathetic Nervous System in CCl_4 Hepatotoxicity. Amer. J. Physiol., 198:669.

Chernick, S. S., J. E. Moe, G. P. Rednan and K. Schwarz. 1955. A Metabolic Lesion in Dietary Necrotic Liver Degeneration. J. Biol. Chem., 217:829.

Christie, G. S. and J. D. Judah. 1954. Mechanism of Action of Carbon Tetrachloride on Liver Cells. Proc. Roy. Soc., B142:241.

Cornish, H. H. and R. C. Ryan. 1964. A Study of Carbon Tetrachloride. VI. Aminoaciduria in Response to Carbon Tetrachloride Inhalation. Toxicology and Pharmacology, 6:96.

Crabtree, C. 1940. Sex Differences in the Structure of Bowman's Capsule in the Mouse. Science, 91:299.

Crabtree, C. 1941. The Structure of Bowman's Capsule as an Index of Age and Sex Variations in Normal Mice. Anat. Rec., 79:395.

Crane, F. L., J. L. Glenn and D. E. Green. 1956. Studies on the Electron Transfer System. IV. The Electron Transfer Particle. Biochem. et Biophys. Acta, 22:475.

Dean, J. A. 1960. Flame Photometry. McGraw-Hill, New York.

Deane, H. W. 1954. "Liver and Gall Bladder" in R. O. Greep (ed.), Histology, Blakiston Co., New York.

Devlin, T. M. and A. L. Lehninger. 1958. Preparation of Phosphorylating Subfragments of Rat Liver Mitochondria with Digitonin. J. Biol. Chem., 233:1586.

Drill, V. A. 1958. Pharmacology in Medicine. McGraw-Hill, New York.

Edwards, J. E. 1941. Hepatomas in Mice Induced with Carbon Tetrachloride. J. Nat. Canc. Inst., 2:197.

Edwards, J. E., W. E. Heston and A. J. Dalton. 1942. Induction of the CCl_4 Hepatoma in Strain-L Mice. J. Nat. Canc. Inst., 3:297.

Edwards, J. E. and A. J. Dalton. 1943. Induction of Cirrhosis of the Liver and of Hepatomas in Mice with Carbon Tetrachloride. J. Nat. Cancer Inst., 3:19.

Emmens, C. W. 1939. Duration of Action of Certain Natural and Synthetic Oestrogens When Administered Orally or by Injection. J. Endocrinol., 1:142.

Eschenbrenner, A. B. 1944. Studies on Hepatomas. I. Size and Spacing of Multiple Doses in the Induction of Carbon Tetrachloride Hepatomas. J. Nat. Cancer Inst.

Eschenbrenner, A. B. and E. Miller. 1945. Induction of Hepatoma in Mice by Repeated Oral Administration of Chloroform, With Observations on Sex Differences. J. Nat. Canc. Inst., 5:251.

Eschenbrenner, A. B. and E. Miller. 1946. Liver Necrosis and the Induction of Carbon Tetrachloride Hepatomas in Strain-A Mice. J. Nat. Canc. Inst., 6:325.

Fernandez-Moran, H. 1962. Cell Membrane Ultrastructure. Circulation, 26:1039.

Fiske, C. H., and Y. Subbarow. 1925. The Colorimetric Determination of Phosphorus. J. Biol. Chem., 66:375.

Frederick, J. 1956. Action of Various Substances on Mitochondria of Living Cells Cultivated in Vitro. Ann. N. Y. Acad. Sci., 58:1246.

Gallagher, C. H., D. N. Gupta, J. D. Judah and K. R. Rees. 1956. Biochemical Changes in Liver in Acute Thioacetamide Intoxication. J. Path. Bact., 72:193.

Gamble, J. L. Jr. 1957. K^+ Binding and Oxidative Phosphorylation in Mitochondria and Mitochondrial Membrane Fragments. J. Biol. Chem., 228:955.

Glinos, A. D. and G. O. Gey. 1952. Humoral Factors Involved in the Induction of Liver Regeneration in the Rat. Proc. Soc. Exper. Biol. & Med., 80:421.

Glinos, A. D. 1956. Mechanism of Growth Control in Liver Regeneration. Science 123:673.

Goodman, L. and A. Gillman. 1952. The Pharmacological Basis of Therapeutics. MacMillan, New York.

Gornall, A. G., C. J. Bardawill and N. M. David. 1949. Determination of Serum Proteins by Means of the Biuret Reaction. J. Biol. Chem., 177:751.

Green, D. E., P. V. Blair, and T. Oda. 1963. Isolation and Characterization of the Unit of Electron Transfer in Heart Mitochondria. Science, 140:382.

Green, D. E. 1964. The Mitochondrion. Sci. American, 210(1):63.

Greep, R. O. 1954. Histology. Blakiston Co., New York.

Hogeboom, G. H., W. C. Schneider and G. E. Palade. 1948. Cytochemical Studies of Mammalian Tissues. J. Biol. Chem., 172:619.

Hunter, G. E., and L. Ford. 1955. Inactivation of Oxidative and Phosphorylative Systems in Mitochondria by Preincubation with Phosphate and other ions. J. Biol. Chem., 216:357.

Judah, J. D. 1960. Effects of Antihistamines on Mitochondrial Swelling and Liver Injury. Nature, 185:390.

Keilin, D. and E. F. Hartee. 1947. Activity of the Cytochrome System in Heart Muscle Preparations. Biochem. J., 41:500.

Kennedy, E. P. and A. L. Lehninger. 1949. Oxidation of Fatty Acids and Tricarboxylic Acid Cycle Intermediates by Isolated Rat Liver Mitochondria. J. Biol. Chem., 179:957.

Kielley, W. W. and J. R. Bronk. 1958. Oxidative Phosphorylation in Mitochondrial Fragments Obtained by Sonic Vibration. J. Biol. Chem., 230:521.

Lehninger, A. L. 1959. Reversal of Various Types of Mitochondrial Swelling by Adenosine Triphosphate. J. Biol. Chem., 234:2465 1.

Lehninger, A. L. 1962. A Heat-Labile Factor Required for Extrusion of Water from Mitochondria. J. Biol. Chem., 237:946.

Lehninger, A. L. 1962. Mitochondrial Swelling and Contraction. Physiol. Rev., 42:467.

Lehninger, A. L., C. S. Rossi and J. W. Greenawalt. 1963. Respiration-dependent Accumulation of Inorganic Phosphate and Ca by Rat Liver Mitochondria. Biochem. Biophys. Res. Commun., 10:444.

Lehninger, A. L. 1964. The Mitochondrion. W. A. Benjamin Inc., New York.

Leighty, J. A. and J. J. Wick. 1939. Diethylstilbesterol Compared to Estrone in Causing Estrus in Spayed Mice. Endocrinology, 25:597.

Lewis, M. R. and W. H. Lewis. 1914. Mitochondria (and Other Cytoplasmic Structures) in Tissue Cultures. Am. J. Anat., 17:339.

Little, M. J. 1961. An Introduction to the Experimental Method. Burgess Publishing Co., Minneapolis, Minn.

Loewy, A. G. and P. Siekevitz. 1963. Cell Structure and Function. Holt, Rinehart & Winston.

MacFarlane, M. G. and A. G. Spencer. 1953. Changes in the Water, Sodium and Potassium Content of Rat-Liver Mitochondria During Metabolism. Biochem. J., 54:569.

Meglitsch, P. A., R. R. Mote and L. P. Johnson. 1959. Some Aspects of Quantitative Histology of the Liver of A-jax Mice Before and During Tumor Induction with Carbon Tetrachloride. Iowa Acad. Sci., 67:511.

Michaelis, L., 1900. Die Vitale Farbung, Eine Darstellungsmethode der Zellgranula, Arch. Mikroskop. Anat. 55:558.

Mercer, F. 1960. The Submicroscopic Structure of the Cell. Ann. Rev. Plant Physiol., 11:1.

Monib, M. S. and J. V. Evans. 1957. Comparison Between Three Methods Used for the Preparation of Tissues for Determination of Sodium and Potassium. Analyst. 82:522.

Morrison, G. R., F. E. Brock, I. E. Karl, and R. E. Shank. 1965. Quantitative Analysis of Regenerating and Degenerating Areas within the Lobule of the Carbon Tetrachloride-Injured Liver. Arch. Biochem. and Biophys., 111(2):448.

Neifakh, S. A. and T. B. Kazakova. 1963. Actomyosin-like Protein in Mitochondria of Mouse Liver. Nature, 197:1106.

Nomiya, T. 1960. Histochemical and Manometric Studies on the Liver of Mice in Carbon Tetrachloride Intoxication. I. Histological and Histochemical Studies. Sapparo Medial Journal, 17(6):417.

Novikoff, A. B. 1956. Preservation of the Fine Structure of Isolated Liver Cell Particulates with Polyvinylpyrrollidone-sucrose. J. Biophys. Biochem. Cytol. 2, No. 4, Suppl. :65.

Palade, G. E. 1953. An Electron Microscope Study of the Mitochondrial Structure. J. Histochem. Cytochem., 1:188.

Palade, G. E. 1956. Electron Microscopy of Cytoplasmic Structures in O. H. Gaebler (ed), Enzymes: Units of Biological Structure and Function, Acad. Press, New York.

Parsons, D. F. 1963. Mitochondrial Structure. Two Types of Subunits on Negatively Stained Mitocondrial Backgrounds. Science, 140:985.

Pullman, M. E., H. Penefsky and E. Racker. 1958. A Soluble Protein Fraction Required for Coupling Phosphorylation to Oxidation in Submitochondrial Fragments of Beef Heart Mitochondria. Arch. Biochem. Biophys., 76:227.

Rees, K. R., K. P. Sinha and W. G. Spector. 1961. The Pathogenesis of Liver Injury in Carbon Tetrachloride and Thioacetamide Poisoning. J. Path. Bact., 81:107.

Reineke, E. P. 1961. A New Multiple-Unit Constant Pressure Microrespirometer. J. Appl. Physiol, 16(5):944.

Reynolds, E. S., R. E. Thiers and B. L. Vallee. 1962. Mitochondrial Function and Metal Ion Content in Carbon Tetrachloride Poisoning. J. Biol. Chem. 237:3546.

Robbins, B. H. 1929. Carbon Tetrachloride Distribution and Elimination. J. Pharmacol. Exper. Therap., 37:203.

Robertson, J. D. 1962. The Membrane of the Living Cell. Sci. American, 206(4):64.

Rouiller, C. 1960. Physiological and Pathological Changes in Mitochondrial Morphology. Intern. Rev. Cytol., 9:227.

Rouiller, C. 1964. The Liver Morphology, Biochemistry, Physiology. Vol. II. Academic Press, New York.

Rubenstein, H. S., and M. L. Solomon. 1940. Effect on Body Growth of Small Doses of Testosterone Propionate Administered at Different Seasons. Proc. Soc. Exper. Biol. & Med., 45:745.

Schneider, W. C. 1948. Intracellular Distribution of Enzymes. III. The Oxidation of Octanoic Acid by Rat Liver Fractions. J. Biol. Chem., 176:259.

Schneider, W. D. 1959. "Mitochondrial Metabolism" Advan. Enzymol., 21:1

Schneider, W. D. and V. R. Potter. 1948. The Distribution of Oxalloacetic Oxidase Activity in Rat Liver and Rat Kidney Fractions. J. Biol. Chem. 177:893.

Schneider, W. C. and G. H. Hogeboom. 1956. Biochemistry of Cellular Particles. Ann. Rev. Biochem., 25:201.

Schwarz, K., S. S. Chernick, J. G. Moe and G. P. Rodnan. 1955. A Metabolic Lesion in Dietary Necrotic Liver Degeneration. J. Biol. Chem. 217:829.

Selye, H. 1939. The Effect of Testosterone on the Kidney. J. Urol., 42:637.

Share, L. and R. Recknagel. 1959. Effect of Carbon Tetrachloride Poisoning on Potassium, Sodium, and Water Content of Liver Mitochondria. Am. J. Physiol., 197(1):121.

Sjöestrand, F. S. 1953. Electron Microscopy of Mitochondria and Cytoplasmic Double Membranes. Nature, 171:30.

Smuckler, E. A., O. A. Iseri and E. P. Benditt. 1962. An Intracellular Defect in Protein Synthesis Induced by Carbon Tetrachloride. J. Exp. Med., 116:55.

Spector, W. G. 1953. Electrolyte Flux in Isolated Mitochondria. Proc. Roy. Soc., London, Ser. B., 141:268.

Starkey, W. F. and E. C. H. Schmidt. 1938. The Effect of Testosterone-propionate on the X-Zone of the Mouse Adrenal. Endocrinol., 23:339.

Stich, R. F. and M. L. Florian. 1958. The Presence of A Mitosis Inhibitor in the Serum and Liver of Adult Rats. Can. J. Biochem. & Biophys.

Stoeckenius, W. 1963. Some Observations on Negatively Stained Mitochondria. J. Cell Biol., 16:483.

Stowell, R. E., C. S. Lee, K. K. Tsuobi and A. Villasana. 1951. Histochemical and Microchemical Changes in Experimental Cirrhosis and Hepatoma in Mice by Carbon Tetrachloride. Canc. Res., 11:345.

Swann, M. M. 1958. The Control of Cell Division. II. Special Mechanisms. Canc. Res. 18:1118.

Tedeschi, H. 1959. The Structure of the Mitochondrial Membrane: Inferences from Permeability Properties. J. Biophys. Biochem. Cytol., 6:241.

Thiers, R. E., E. S. Reynolds and B. L. Vallee. 1960. The Effect of Carbon Tetrachloride Poisoning on Subcellular Metal Distribution in Rat Liver. J. Biol. Chem., 235:2130.

Tobioka, M. and J. J. Biesle. 1956. Mitochondria in Living Cells: An Analysis of Movements. J. Biophys. Biochem. Cytol., 2, Suppl. 319.

Tsuboi, K. K., R. E. Stowell and C. S. Lee. 1951. Chemical Alterations Induced in Mouse Liver Following A Single Feeding of Carbon Tetrachloride. Canc. Res., 11:87.

Ulrich, F. 1959. Ion Transport by Heart and Skeletal Muscle Mitochondria. Am. J. Physiol., 197:997.

Umbreit, W. W., R. H. Burris and J. F. Stauffer. 1964. Manometric Techniques, fourth ed. Burgess Publ. Co., Minneapolis, Minn.

Wachstein, M. E. Meisel and C. Falcon. 1962. Enzyme Histochemistry in the Experimentally Damaged Liver. Am. J. Pathol., 40:219.

Wakim, K. G. and F. C. Mann. 1942. The Intrahepatic Circulation of Blood. Anat. Rec., 82:233.

Webster, G., and D. E. Green. 1964. The Enzymes and Enzyme-Catalyzed Reactions of Mitochondrial Oxidative Phosphorylation. Proc. Natl. Acad. Sci. U. S., 52(5):1170.

Wilson, J. W., 1951. Hepatomas in Mice Fed a Synthetic Diet Low in Protein and Deficient in Choline. Canc. Res., 11:290

Wilson, M. W., R. E. Stowell, H. O. Yokoyama and K. K. Tsuboi. 1953. Cytological Changes in Regenerating Mouse Liver. Canc. Res., 13:86.

Yokoyama, H. O., M. E. Wilson, K. K. Tsuboi and R. E. Stowell. 1953. Regeneration of Mouse Liver After Partial Hepatectomy. Canc. Res., 13:80.

Ziegler, D. M., A. W. Linnane, D. E. Green, C. M. S. Dass, and H. S. Ris. 1958. Studies on the Electron Transport System: XI. Correlation of the Morphology and Enzyme Properties of Mitochondrial and Submitochondrial Particles. Biochim. et Biophys. Acta, 28:524.

Ziegler, D. M. and A. W. Linnane. 1958. Mitochondrial Structure and Dehydrogenase Activity in Isolated Mitochondria. Biochem. Biophys. Acta. 29:630.

Zimmerman, M. and E. Cellozi. 1960. Stimulation of Cell Division in Normal Rat Liver by a Factor in Serum from Hepatectomized Rats (Abstract). Federation Proc., 19:139.

APPENDICES

TABLE A.--Effects of single dose of CCl_4 on metal ion content of liver and liver mitochondria.

n	Time after CCl_4	Na+	K+	Ca++
Whole Liver:		micrograms of ion/mg. protein		
4	control	2.30 ± 0.27	13.38 ± 0.74	0.45 ± 0.12
4	24 hrs.	3.68 ± 0.50[b]	6.99 ± 0.49[a]	1.54 ± 1.12
4	48	2.76 ± 0.06	10.55 ± 0.94[b]	1.19 ± 0.13
4	72	3.54 ± 0.68[b]	17.53 ± 2.63	1.25 ± 0.22[a]
4	112	3.76 ± 0.50[b]	17.50 ± 1.31	1.19 ± 0.36[b]
Mitochondria:				
4	control	0.26 ± 0.02	3.64 ± 0.12	0.40 ± 0.03
4	24 hrs.	0.28 ± 0.06	1.29 ± 0.03[a]	1.54 ± 0.73[a]
4	48	0.31 ± 0.09	1.35 ± 0.24[a]	1.60 ± 0.24[b]
4	72	0.45 ± 0.08[b]	1.80 ± 0.12[b]	2.30 ± 0.60[b]
4	112	0.20 ± 0.19	2.93 ± 0.30	0.40 ± 0.12

[a] 1% significance

[b] 5% significance

Mean values presented in thesis Fig. 5.

TABLE B.--Liver weight* bodyweight relationship in mice following a single dose of carbon tetrachloride.

Time after CCl_4	n	Body weight[a]	Liver weight[a]	$\frac{\text{Liver wt.}}{\text{Body wt.}} \times 100$
control	4	23.61 ± 1.09	1.19 ± 0.10	4.99 ± 0.26
24 hours	4	24.45 ± 1.31	1.34 ± 0.14	5.70 ± 0.55
48 hours	4	22.69 ± 1.59	1.42 ± 0.29	6.26 ± 0.14[b]
72 hours	4	21.62 ± 0.68	1.55 ± 0.05	7.19 ± 0.27[b]
112 hours	4	20.82 ± 1.34	1.59 ± 0.05	7.77 ± 0.58[b]

[a] ± standard error of mean

[b] 1% significance

Liver weight-body weight ratios presented in thesis Fig. 7.

TABLE C.--Effect of chronic feeding of CCl_4 on metal ion content of mouse liver homogenates.

Doses of CCl_4	n	Na^+[a]	K^+[a]	Ca^{++}[a]
Control	4	2.30 ± 0.27	13.38 ± 0.74	0.45 ± 0.12
3	4	2.80 ± 0.36	19.93 ± 3.78[b]	10.06 ± 0.47[c]
6	4	1.81 ± 0.08	11.61 ± 3.72	0.49 ± 0.05
8	3	2.48	17.05	1.26
9	4	1.79 ± 0.26[c]	10.99 ± 0.41[b]	9.90 ± 4.87[c]
13	4	0.79 ± 0.21[b]	10.30 ± 0.12	0.74 ± 0.23
18	4	2.21 ± 0.47	14.49 ± 1.97	0.79 ± 0.28
22	4	1.28 ± 0.25[b]	9.87 ± 1.37[b]	1.04 ± 0.26[c]
30	4	2.69 ± 0.19[c]	15.45 ± 0.90[b]	1.39 ± 0.50[c]
34	3	2.71	19.66	1.63
37	2	3.06	10.82	0.99
39	2	2.86	17.64	1.07

[a] ± standard error of mean

[b] 1% significance

[c] 5% significance

Mean concentrations presented in thesis Fig. 9a.

TABLE D.--Effect of chronic feeding of CCl_4 on metal ion concentration of mouse liver mitochondria.

Doses of CCl_4	n	Na^+[a]	K^+[a]	Ca^{++}[a]
control	4	0.26 ± 0.02	3.64 ± 0.12	0.41 ± 0.03
3	4	0.25 ± 0.05	1.19 ± 0.05[b]	3.35 ± 0.65[b]
6	4	0.29 ± 0.21	2.58 ± 0.37[b]	0.82 ± 0.12[b]
8	4	0.38	1.92	1.15
9	4	0.20 ± 0.02[b]	1.56 ± 0.02[b]	2.84 ± 1.67[c]
13	4	0.28 ± 0.01	2.94 ± 0.23[b]	1.70 ± 0.20
18	4	0.27 ± 0.02	2.61 ± 0.32[b]	1.12 ± 0.39[b]
22	4	0.27 ± 0.02	3.22 ± 0.49	2.91 ± 0.85[b]
25	3	0.28	2.81	1.49
30	4	0.34 ± 0.02[b]	4.90 ± 0.37[b]	0.93 ± 0.15[c]
34	3	0.75	2.25	1.02
37	2	0.34	2.08	
39	2	0.34	2.37	1.08

[a] ± standard error of mean

[b] 1% significance

[c] 5% significance

Mean concentrations presented in thesis Fig. 9b.

TABLE E.--Weight records of mice during the course of administration of 30 feedings of olive oil on a tri-weekly basis.

Doses of oil	n	Mean* weight	%Original weight	Doses of oil	n	Mean* weight	%Original weight
0	24	19.53 ± 0.42	100.0	15	22	21.37 ± 0.40	109.4
1	24	19.30 ± 0.43	98.8	16	22	21.36 ± 0.51	109.4
2	23	19.87 ± 0.25	101.7	17	22	21.83 ± 0.48	111.8
3	23	19.35 ± 0.36	99.1	18	22	22.05 ± 0.42	112.9
4	23	20.28 ± 0.42	103.8	19	22	21.56 ± 0.42	110.4
5	23	19.94 ± 0.48	102.1	20	22	21.98 ± 0.45	112.5
6	23	20.54 ± 0.48	105.2	21	22	21.54 ± 0.59	110.3
7	23	20.23 ± 0.59	103.6	22	21	22.15 ± 0.57	113.
8	23	19.87 ± 0.65	101.7	23	21	22.00 ± 0.52	112.7
9	23	19.98 ± 0.57	102.3	24	21	22.24 ± 0.49	113.9
10	22	19.99 ± 0.47	102.4	25	21	22.01 ± 0.54	112.7
11	22	20.40 ± 0.47	104.5	26	21	21.87 ± 0.64	111.9
12	22	21.45 ± 0.47	109.8	27	21	22.27 ± 0.56	114.0
13	22	21.23 ± 0.43	108.7	28	21	22.92 ± 0.44	117.4
14	22	21.67 ± 0.42	110.9	29	21	22.86 ± 0.62	120.1
				30	21	22.60 ± 0.54	115.1

*± standard error of mean

Weight percentages presented in thesis Fig. 10.

TABLE F.--Weight records of mice during the course of administration of 30 feedings of CCl_4 on a tri-weekly basis.

Doses of CCl_4	n	Mean* weight	%Original weight	Doses of CCl_4	n	Mean* weight	%Original weight
0	98	19.90 ± 0.27	100.00	15	74	19.59 ± 0.46	98.44[b]
1	98	17.24 ± 0.27[a]	86.63	16	74	18.72 ± 0.47	94.07[a]
2	97	18.40 ± 0.23	92.46	17	74	18.85 ± 0.39	94.72[a]
3	96	18.57 ± 0.26	93.32	18	71	19.53 ± 0.48	98.14[a]
4	92	18.88 ± 0.15[a]	94.88	19	68	19.28 ± 0.48	96.88[b]
5	90	18.13 ± 0.25[a]	91.1	20	67	19.84 ± 0.58	99.7 [b]
6	89	18.84 ± 0.33[b]	94.7	21	65	20.02 ± 0.55	100.6
7	85	18.29 ± 0.31[a]	91.9	22	62	20.57 ± 0.39	103.4 [b]
8	84	18.56 ± 0.34	93.2	23	61	20.63 ± 0.25	103.7 [b]
9	80	19.11 ± 0.23	96.0	24	56	20.89 ± 0.25	104.9 [b]
10	80	18.78 ± 0.19[a]	94.4	25	55	20.88 ± 0.33	104.9
11	80	18.12 ± 0.38[a]	91.1	26	55	19.82 ± 0.34	99.6 [a]
12	78	19.85 ± 0.46	99.7	27	55	20.86 ± 0.36	104.8 [b]
13	74	18.80 ± 0.44[a]	94.5	28	53	21.17 ± 0.32	106.4 [a]
14	74	18.97 ± 0.46[a]	95.3	29	53	21.58 ± 0.77	108.4
				30	53	21.09 ± 0.26	105.4 [b]

*± standard error of mean

[a]1% significance

[b]5% significance

Weight percentages presented in thesis Fig. 10.

TABLE G.--Liver weight-body weight relationship in mice during chronic feeding of carbon tetrachloride.

Doses of CCl_4	n	Body Weight	Liver Weight	$\frac{\text{Liver wt.}}{\text{Body Wt.}} \times 100$
0	4	23.61 ± 1.09	1.10 ± 0.10	4.98 ± 0.21
		4.45 ± 1.31	1.34 ± 0.14	5.70 ± 0.55
3	4	21.85 ± 1.18	1.34 ± 0.04	6.23 ± 0.34[a]
5	4	20.05 ± 0.70	1.44 ± 0.10	7.31 ± 0.70[a]
6	3	18.67	1.30	6.94
8	3	15.40	1.47	9.53
9	4	19.95 ± 0.50	1.29 ± 0.13	6.41 ± 0.49[a]
12	4	22.22 ± 0.26	1.37 ± 0.07	6.18 ± 0.23[a]
13	4	21.25 ± 0.44	1.23 ± 0.10	5.80 ± 0.39[b]
15	4	20.80 ± 0.86	1.33 ± 0.06	6.43 ± 0.38[a]
19	4	22.95 ± 0.87	1.55 ± 0.05	6.75 ± 0.24[a]
22	4	20.87 ± 0.93	1.33 ± 0.04	6.49 ± 0.17[a]
25	3	21.43	1.56	7.29
30	4	22.12 ± 1.21	1.80 ± 0.29	8.04 ± 0.58[a]

[a] 1% significance

[b] 5% significance

Liver weight-body weight ratios presented in thesis Fig. 11.

TABLE H.--Oxygen utilization of liver slices of mice following single feeding of CCl_4.

Time after CCl_4	n	Q_{O_2}*
control	4	[a]10.44 ± 1.51
24 hours	4	3.01 ± 0.54[b]
48 hours	4	6.43 ± 0.84[b]
72 hours	4	7.51 ± 0.22[c]

*microliters of O_2 per mg. liver protein per hour

[a] ± standard error of mean

[b] 1% significance

[c] 5% significance

Mean values presented in thesis Fig. 13a.

TABLE I.--Oxygen utilization of liver slices of mice during chronic feeding of CCl_4.

Doses of CCl_4	n	Q_{O_2}[a]
control	4	10.44 ± 1.51
3	4	7.57 ± 1.28
5	4	4.38 ± 0.70[b]
9	4	7.56 ± 1.83
12	4	6.03 ± 0.40[b]
19	4	8.05 ± 2.34
22	4	6.02 ± 0.71[b]
25	3	8.51

[a] microliters of O_2 per mg. liver protein per hour

[b] 1% significance

Mean values presented in thesis Fig. 13b.