RADIATION-INDUCED CHANGES IN THE PROFILES OF CERTAIN KEY ENZYMES IN RAT MYOCARDIUM AND SERUM: EFFECTS OF EXERCISE AND DIETARY SUPPLEMENTATION WITH VITAMIN E

by

Lyle Dean MacWilliam

B.Sc., (Biochemistry) Simon Fraser University, 1971

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (KINESIOLOGY) in the Department of Kinesiology

LYLE DEAN MACWILLIAM 1974 SIMON FRASER UNIVERSITY April 1974

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NAME: Lyle Dean MacWilliam

DEGREE: Master of Science (Kinesiology)

TITLE OF THESIS: Radiation-induced Changes in the Profiles of Certain Key Enzymes in Rat Myocardium and Serum: Effects of Exercise and Dietary Supplementation with Vitamin E.

EXAMINING COMMITTEE:

Chairman: Dr. T.W. Calvert

Dr. N.M.G. Bhakthan
Senior Supervisor

Dr. A.J. Davison

Dr. K.K. Nair

Dr. E.W. Banister
External Examiner
Professor, Chairman
Department of Kinesiology
Simon Fraser University

Date Approved: 19 April 1994
Levels of lactate dehydrogenase, creatine kinase and glutamate oxaloacetate transaminase in serum show initial elevations within twelve hours of exposure to 2,000 rads of gamma radiation to the thoracic region of rats. Significant decreases of these enzymes in homogenates of heart muscle coincide with initial elevations in the serum and may suggest that enhanced leakage of enzymes is a consequence of radiation injury to heart muscle. However, insignificant alterations in mitochondrial levels of glutamate oxaloacetate transaminase following exposure indicate that in vivo injury to the mitochondria from therapeutic levels of gamma radiation is questionable. The results suggest that ionizing radiation causes alterations in the dynamic permeability of membranes, allowing leakage of biologically active material out of the injured cell.

Levels of lactate dehydrogenase, creatine kinase and glutamate oxaloacetate transaminase in the serum of rats exercised immediately following thoracic exposure to 2,000 rads of gamma radiation become elevated within twelve hours of treatment. Although LDH levels in serum of irradiated, exercised animals were significantly greater than similarly treated sedentary subjects, serum levels of CK and GOT did not differ significantly between the two groups. These observations are interpreted in terms of changes in the permeability of membranes. Temporary reductions in levels of LDH in homogenates of heart corresponded to initial elevations of LDH in serum and suggest that enhanced leakage of the enzyme is a consequence of radiation injury to the heart. In contrast to demonstrated losses of CK and GOT from
homogenates of heart in irradiated sedentary animals, the lesser amount of leakage in exercised subjects suggests that exercise in irradiated, trained subjects may protect against loss of biological material from the damaged system. Levels of GOT in isolated rat heart mitochondria were temporarily decreased twenty-four hours after exposure. However, enzyme levels after irradiation did not differ significantly between exercised and sedentary animals, indicating that exercise, as an added metabolic stress in irradiated rats, did not cause any remarkable alterations in the integrity of mitochondrial membranes.

Comparisons of percentage elevations in serum LDH levels between irradiated rats previously maintained on tocopherol-fortified and standard lab chow diets did not reveal any significant radioprotective action of vitamin E. However, a marked decrease in LDH activity in the serum of both sham-irradiated, dietary groups suggests that α-tocopherol may induce a further stabilization of normal membrane permeability. Although no evidence of formation of lipid peroxides induced by irradiation could be detected, there was a significantly decreased level of TBA reactants in heart homogenates of all tocopherol-fortified animals.
TO my wife, for her encouragement and support.
Be very careful when you are looking for a thing or you will be sure to find it.

Louis Pasteur
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CHAPTER I

INTRODUCTION

Radiation is not a new phenomenon, but is an old hazard that has silently existed through the ages. Upon the initial discovery of X-rays by Roentgen in 1895 and radium by Curie in 1897, new vistas for science and technology were opened and, as a result, new and unknown dangers were exposed.

Ionizing radiation, including alpha and beta particles, gamma rays, X-rays, neutrons and protons, soon became notorious for far reaching harmful effects to biological systems. Although these forms comprise only a portion of the total radiation spectrum, their unique hazard lies in their innate ability to energize matter by ionization. In biological systems this can produce numerous physical and chemical effects which can ultimately lead to massive biochemical breakdown and resultant death of the target tissue.

The reports of Marcuse in 1896 are the first documented descriptions of the injurious effects of X-irradiation on the skin. Lesions consisting of epilation, erythema and radiodermatitis were slow to heal, excruciatingly painful, and were prone to develop skin tumors.

Pierre Curie in 1900 reported radiodermatitis and resultant deep tissue destruction after applying radium to a small area of his forearm, and in handling active products, Mme. Curie developed erythema, dermatitis, and severe blistering of the fingers. Similarly, through the years numerous massive injuries and accidental deaths have occurred from major mishaps.
The interactions of ionizing radiation with matter occurs by direct or indirect action. Direct action is the interaction of radiation with target molecules, resulting in an energy transfer to the molecule. This absorbed energy can raise orbital electrons to higher quantum levels, resulting in highly excited and unstable molecules, or can cause complete ejection of an electron from an orbital shell.

Processes of stabilization may result in dissociation of the molecule into free radicals, in ionization, or in lower levels of excitation, the ultimate result being the formation of stable products which may differ from the original molecule.

Indirect action is the reaction between solute molecules and the reactive species formed by direct action of radiation. In the living cell, which is an extremely complex structure, water is the universal solvent. Therefore, when cells are irradiated, most of the direct energy transfer occurs with water molecules simply because water presents the greatest number of targets. When irradiated, water like any other material is ionized, resulting in the formation of unstable intermediates which quickly dissociate into various radiolytic products, such as the extremely reactive hydrogen and hydroxyl free radicals.

The reaction sequence can be expressed as

\[ \text{H}_2\text{O} \rightarrow \text{H}^+ + \text{OH}^- + e^-_{\text{aq}} + \text{H}_2 + \text{H}_2\text{O}_2 + \text{H}_3\text{O}^+ \]

Free radicals, once formed, diffuse through the irradiated system reacting randomly with neighbouring molecules and thereby inflict damage to the intact components of the target cell.
The biologic effects of ionizing radiation represent the efforts of living things to deal with energy left in them after an interaction of one of their atoms with an ionizing ray or particle. For any living system, this energy will be in excess of the system's requirements for normal function; it will be a deviation from the proper energy relationships within that system.¹

Detectible injury to living systems as a consequence of such interactions is the result of a long and complex sequence of events initiated by radiological damage to biologically pertinent molecules, and culminating in the manifestation of major physiological and biochemical alterations. According to Latarjet and Grey (1954) the development of observable injury in an irradiated living cell occurs in four steps. The first step involves the interaction between the ionizing radiation and the component molecules of the cell; the second consists of reactions between short-lived radicals and molecules of biological importance. In the third step chemical chain reactions amplify the primary damage resulting from the previous stages to the point where in the final step "observable" injury is apparent, involving cytological lesions, genetic damage, and major biochemical changes. It is generally felt that this development of expression of the initial injury occurs through the incorporation of the injury into the ongoing metabolism of the cell, and may similarly be compared to the eventual breakdown of a machine which is operated in a slightly damaged condition.

IMPORTANCE OF THE STUDY

Although the heart has often been regarded as one of the most radioresistant organs of the body (Ellinger 1957), the onset of radiation-induced heart disease has been recognized as an important manifestation of thoracic exposure (Stewart and Fajardo 1971). There is evidence that the heart may, in fact, be seriously damaged by ionizing radiation (Stewart 1967). The radiological re-treatment of Hodgkin's disease in the thorax was found by Stewart and Fajardo (1971) to carry an exceedingly high risk of subsequent injury to the heart. Although it has been shown that the myocardium is most frequently injured (Ferri and Dosi 1965), all components of the heart show apparent post-irradiation damage.

Radiation-induced electrocardiographic alterations have been observed in humans (Jones and Wedgwood 1960), dogs (Phillips et al. 1964) and rodents (Fulton and Sudak 1954). Salet (1971) reported evidence of increased pulsation frequencies of rat cardiac cells in tissue culture within minutes of exposure to micro-irradiation by laser. In addition, congestive heart failure induced by large radiological doses in both rabbits (Efskind 1954) and dogs (Bishop et al. 1965) indicates that the heart is indeed susceptible to severe radiation injury.

The clinical indications that the survival of patients undergoing radiation treatment for carcinoma of the left lung was poorer than that of patients treated for right lung cancer, prompted a study by Takaoka (1969) involving the investigation of cardiac damage by therapeutic radiation. The results showed that the degree of myocardial damage was
related to the size of the irradiated area and to the radiological dose given. Similarly, the prognosis of patients receiving intrathoracic radiotherapy may be influenced by the incidentally induced myocardial damage.

Cardiac structural alterations in patients receiving therapeutic radiation to the mediastinum (Fajardo et al. 1968) showed proliferation of connective tissue with pericarditis and extensive fibrosis of the myocardium. In a later study of the mechanism of radiation-induced myocardial fibrosis in rabbits, Fajardo and Stewart (1973) determined that immediate injury causes an acute, transient inflammatory exudate in all tissues of the heart. In addition, the experiments clearly indicated the capillary endothelial cell as an important target of radiation.

An extensive study on the manifestation of chronic cardiac injury induced by radiation has been reported by Burch et al. (1968). The patient, a 37 year old male physician, underwent post-operative radiation treatment for thyroid follicular carcinoma, receiving a dose of 4,800 rads to the thyroid bed with an additional dose of 5,200 rads to the mediastinum through the chest wall. Although there was no recurrence of the carcinoma, the patient died seven years later of congestive heart failure. Microscopic observation revealed disruption of the myofibrils and cell organelles with possible cytoplasmic leaching. Mitochondrial damage was characterized by swelling and reduction of internal cristae concomitant with a loss of orderly arrangement. Although it was not
possible to define a specific subcellular lesion, it appears that the changes seen were related to the initial radiation damage. Evidently large therapeutic radiation doses can cause extensive damage to the heart that may not manifest itself until years after treatment.

The wealth of clinical and pathologic evidence indicating that ionizing radiation can produce myocardial damage shows the importance of the early detection of such injury (Muggia et al. 1970). Since the ultimate manifestation of radiation injury to living systems is related to the degree of initial damage, the quantitative estimates of such damage may provide a useful clinical prediction of the long term effects. Quantitative understanding of the biochemical and morphologic effects of cardiac irradiation is essential for the development of any preventative schemas. Determination of enzyme leakage from the target organ may therefore provide effective means of evaluating the initial damage and thus serve as a useful radiobiological indicator.

At present, there is surprisingly little information relating radiation damage of the heart to elevation of serum enzymes. However, Kubota (1969) has reported elevations of lactate dehydrogenase, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase after subjecting rabbits to precordial beta irradiation of 500 - 4000 R. Similarly, Mochalova et al. (1969) found sharp increases in lactate dehydrogenase, aldolase and creatine kinase in blood serum of rabbits exposed to localized irradiation of the heart. These increases occurred within 60 hours of exposure and were accompanied by marked morphological changes in the myocardial tissue.
The study of radiation-induced leakage of enzymes from the heart should, in addition, yield valuable information on the post-irradiation integrity of cellular and sub-cellular membranes. Differential permeability of cellular membranes controls enzyme leakage into the plasma. Under normal physiological conditions the cell membrane retains biologically active material in high concentrations, the maintenance of which requires a considerable expenditure of energy (Hess 1963). Retention of biologically active molecules may be a result of active transport, the size and geometry of the membrane pores, and the electrostatic charge on the membrane surface. It is therefore clear that pathological manifestations of alterations in permeability characteristics may result either from interruption of the energy supply to the membrane as a result of metabolic lesions (Schade 1953; Wu 1959; Binkley 1961) or from direct damage to the structural components of the membrane. The enzyme-release hypothesis proposed by Bacq and Alexander (1961) suggests that many of the deleterious effects of radiation on cells are caused by damage to the macromolecules constituting the cellular and sub-cellular membranes, resulting in the failure of their selective characteristics, with the consequent leakage of ions, cofactors and other biological material out of the cell and its organelles. Although much effort has been devoted to finding the exact causes of these alterations in membrane permeabilities of irradiated systems, no clearly defined solution is at hand. Much of the evidence accumulated to date implicates lipid peroxidation, and although many workers have reported permeability alterations resulting from in vitro peroxide formation.
(Wills and Wilkinson 1967; Wills 1970), no clear cut evidence has yet been obtained that lipid peroxides are produced in significant amounts in irradiated animals (Mead 1961).

OBJECTIVES OF THE STUDY

The present investigation is an attempt to determine the effects of therapeutic levels of ionizing radiation on the cardiac muscle of rats. The principle objective is to establish whether radiation damage does cause significant serum elevations in certain key enzymes. If so, the enhancement of serum enzyme activities may be related to a concomitant decrease in the tissue levels of the enzymes.

One of the biochemical indicators in acute radiation injury is an elevation of serum lactate dehydrogenase (LDH) activity following total-body exposure (Hori et al. 1968). LDH, found in most organs, entertains an important function in carbohydrate metabolism and under normal conditions remains at a fairly constant level. Glutamate oxaloacetate transaminase (GOT) and creatine kinase (CK) are both found in relatively high concentrations in cardiac muscle. GOT, involved in protein metabolism and CK in the replenishment of the phosphate moiety of ATP for muscular contraction, play important roles as biochemical markers for the clinical detection of myocardial injury (Dreyfus et al. 1960; Schmidt et al. 1965) and were therefore selected as potential indicators of the degree of radiation injury to the cardiac muscle.

Since it is known that the ongoing metabolism of the cell influences
the development of major post-irradiation lesions, the effect of exercise in previously trained animals was also investigated to determine if radiation damage is modified by this stress condition. In addition, lipid peroxidation in heart tissue was measured in vivo, in an attempt to correlate structural damage of the cell membranes to alterations in permeability. The function of vitamin E as a potential radioprotective agent was also examined.

The specific objectives are listed below.

1. Does irradiation of rat cardiac muscle result in increased serum levels of the three metabolic enzymes, lactate dehydrogenase, creatine kinase, and glutamate oxaloacetate transaminase?

2. Is such an increase accompanied by decreases of the enzyme in the target organ or is it caused by increased leakage rates due to elevations of the enzyme within the tissue?

3. Does post-irradiation exercise in trained animals alter the degree of enzyme leakage with respect to irradiated sedentary animals? Does exercise therefore appear harmful or beneficial in exercise-trained, irradiated subjects?

4. Does therapeutic radiation induce in vivo lipid peroxidation in cardiac muscle? If so, can the changes in enzyme efflux in post-irradiated subjects be related to the degree of peroxidation?

5. Does vitamin E, as a dietary supplement, affect the post-irradiation serum enzyme pattern?
CHAPTER II

LITERATURE REVIEW

For the sake of clarity, the following discussion will be divided into four sub-sections. The first section will present contemporary clinical and experimental works involving enzymological determinations of radiation damage, the second section will cover important works relating radiation injury to alterations in membrane permeability, and the third section will propound evidence of the participation of autocatalytic lipid peroxidation in membrane damage. Section four will review literature implicating vitamin E as a biological lipid antioxidant and its possible involvement as a natural radioprotective agent.

Enzymological Considerations

Experimental and clinical investigations have revealed that studies of enzyme activities are useful indicators of the effects of ionizing radiation on living systems. As such, "the estimation of enzymes in tissue homogenates, in the intercellular fluid, in the serum, and in effusions, adds considerably to our knowledge of the effects of ionizing radiation on the homothermal organism."\(^1\)

Ludewig and Chanutin (1950) reported that serum alkaline phosphatase levels decreased following whole-body irradiation of rats. In a study of urinary deoxyribonuclease, Kowlessar and collaborators (1953) observed an

increase in the urine activity in rats exposed to 700 rads of whole-body irradiation. Similarly, Roth and Eichel (1959) observed a general loss of ribonuclease activity from mitochondrial, nuclear and microsomal particles of rat spleen, with a concomitant increase in the cytoplasmic activity. In a biochemical and electron microscopic study of irradiated mitochondria isolated from rat liver, Okada and Peachey (1957) presented evidence in support of the hypothesis that the noted increase in deoxyribonuclease II activity is causally related to structural damage in the mitochondria. Kawasaki and Sakurai (1969), in a study of irradiated mouse liver mitochondria, noted an increase in ATPase activity with a parallel decrease in oxidative phosphorylation within three to six hours after whole-body exposure to sublethal X-radiation. Experiments on E. coli by Billen and co-workers (1953) demonstrated a loss of ATP from the organisms into the media following exposure to radiation. The results indicated that injured cells release more enzymes into the fluid than cells killed instantly. The damaged cells continue to produce metabolites but lose the material through the injured membrane, whereas dead or ruptured cells can release only their immediate contents.

Various workers have investigated the effect of radiation on alterations in the activity of numerous enzymes in concert. Wills and Wilkinson in 1966 noted a release of acid phosphatase, cathepsin and β-glucuronidase from rat liver lysosomes following irradiation in vitro. Concomitant with the release of enzymes, formation of lipid peroxides was noted, indicating that lipid peroxidation may mediate rupture of the lysosome membrane, allowing the release of material. Although
Klein-Szanto and Cabrini (1970) found increases in the mean enzyme concentrations of NADP cytochrome c reductase and glucose-6-phosphate dehydrogenase, lactate dehydrogenase and sorbitol dehydrogenase concentrations decreased in irradiated epidermis. It was suggested that the radiation response might result from widespread metabolic adjustments.

In a recent clinical investigation Muggia and co-workers (1970) studied the serum responses of a series of enzymes in twenty-one patients receiving incidental cardiac exposure in comparison to twenty-four patients receiving non-cardiac irradiation. The serum lactate dehydrogenase (LDH) and glutamate oxaloacetate transaminase (GOT) levels did not appear useful in detecting radiation-induced myocardial injury, due to their frequent inherent abnormalities. Serum creatine kinase (CK) however, appeared elevated in 33% of the patients receiving cardiac irradiation and may reflect radiation damage to the myocardium.

Hori and Nishio (1962) and Hori et al. (1964) found that in rabbits and mice exposed to 300 R of X-rays, plasma LDH activity reached a peak elevation in about six hours, returning to normal by 24 hours. Transient increases were found two and seven days after irradiation and have also been noted by Kürcher and Kato (1964). In a later study Hori et al. (1968) found increased plasma LDH within hours of whole body exposure of mice to X-radiation of 600 - 900 R. The extent of elevation appeared proportional to irradiation exposure in the range below 900 R. Analysis of various organs showed a marked loss of LDH only in thymus and spleen. Radio-resistant organs showed no significant changes, supporting the contention that increased plasma LDH levels are derived from possible cell destruction
and membrane permeability changes in radiosensitive tissues. Further support for the cell destruction hypothesis was obtained by Takamori et al. (1969) using isolated mouse thymocytes and erythrocytes.

Numerous reports of elevations in serum glutamate oxaloacetate transaminase levels as a result of exposure to ionizing radiation have appeared. Milch and Albaum (1956) showed that serum GOT levels rose within a few hours following whole-body exposure of rabbits, and Petersen et al. (1957) found that serum GOT values in rabbits were 223% of normal five hours after exposure to 644 rads. Out of 14 enzymes studied, Albaum (1960) found GOT to be the best early index of radiation injury. However, data from Brent et al. (1958) suggested that the response is varied between species. Although serum transaminase levels in rabbits rose sharply on the first post-irradiation day, no significant elevations could be found in rats either after whole-body exposure or focal irradiation of the liver with 5,000 rads. Becker et al. (1962), in contrast, revealed striking but transient elevations of serum GOT following total-body irradiation of Sprague-Dawley rats. Elevation occurred between the third and ninth hours and appeared to be related to cell destruction in radiosensitive tissues.

From the literature it is apparent that cellular radiation damage does elicit changes in the enzyme patterns within cells and organelles which can cause significant alterations of the serum enzyme levels. Although cell death and disintegration have been attributed as the "raison d'être" for post-irradiation elevations in serum enzymes, it
must be emphasized that many of the studies noted above involved whole-body exposure in which radiation damage would reflect injury to the most radiosensitive organs. In systems such as the spleen and thymus cell disruption may, in fact, play a crucial role in the manifestation of radiation injury. However, biochemical and histological examinations have also confirmed the view that radiation administered in therapeutic levels does cause alterations in membrane permeabilities in subcellular organelles as well as changes in the cellular membrane (Kärcher 1971), which could also account for the noted elevations of enzymes in the blood. Conceivably, both mechanisms are functioning and those cells that are not lethally damaged continue to function in their damaged state, leaking out biologically active material from the cytoplasm.

Evidence of Radiation Injury to Membranes

A number of investigations have revealed that changes occur in membrane permeabilities after exposure to ionizing radiation (Schrek 1948; Lessler 1959; Barer and Joseph 1960; Novack and Shapiro 1960). In 1959 Noyes and Smith observed quantitative changes in rat liver mitochondria following whole-body irradiation. It was concluded that radiation damage involves a preferential fragmentation of small mitochondria, leading to transient depression in the total mitochondrial count. Similar alterations were reported by Bahr in 1971. Hugon and co-workers (1965), in a study of radiation damage to the duodenal crypts in mice, noted expansion, clarification and rupture of the mitochondrial matrix in stem cells within 30 minutes of exposure, and Portela et al.
(1963) proposed that one of the primary effects of radiation exposure is damage to the membrane organization at the level of the mitochondrial cristae.

Although Scaife and Alexander (1961) found no evidence to support the idea of alteration in the permeability of mitochondrial membranes, their conclusion contrasts with findings of Mukerjee and Goldfeder (1972), who noted increased protein and RNA synthesis in mouse liver mitochondria subjected to 2,000 R of X-radiation. Enhanced synthesis was explained by increased transport of RNA and protein precursors due to changes in the mitochondrial membrane permeability following exposure. Work by Desai et al. (1964) revealed that lysosomal preparations exposed to increasing doses of gamma radiation showed release of indicator enzymes, with each enzyme having a different pattern of release and inactivation. Such leakage was attributed to damage and lysis of the lysosomal membrane. Related damage and alterations in the endoplasmic reticulum of hepatic parenchymal cells of mice were observed by Hendee and Alders (1968) within two minutes after exposure to gamma radiation. The rapid development of these changes suggests that membrane damage may be closely related to initial radiation injury. A study of the mechanism of radiation-induced enhancement of sodium accumulation in erythrocytes (Shapiro and Kollman 1968) revealed that membrane sulfhydryl groups appear to be the major target in radiation alteration of cell permeability. There was no direct evidence suggesting the appearance of a membrane hole, but instead, radiation damage appeared to induce a very specific chemical change in the membrane. In contrast, Sutton and Rosen's study in 1968 of the effect
of X-rays on the electrical conductance of phospholipid bilayers produced data which was consistent with a model in which radiation exposure induces the formation of temporary holes through the membrane, large enough to permit the free diffusion of ions present. Petkau (1971), using a model lipid membrane system, reported a scavenging of superoxide ions by the system after radiation treatment. It was evident also that radiation-induced reactions in the aqueous phase were somehow influenced by the organization of the membrane phospholipids.

Much of the recent ultrastructural and cytochemical evidence further elucidates the mode of radiation damage to membranes. Various workers have independently shown alterations in unit membrane characteristics with marked breaks in membranes often occurring in early stages following irradiation (Golfeder and Miller 1963; Hugon et al. 1965; Nair and Bhakthan 1969; Bhakthan and Dharamraj 1972). In vivo and in vitro irradiation of cell organelles has furnished evidence of loss of integrity and changes in the physico-chemical characteristics of the lipoprotein membranes (Desai et al. 1964; Zyss and Michalska-Kaszczynska 1972). Electron microscopic studies (Ghidoni 1967) of irradiated primate liver revealed a loss of integrity and internal rearrangement of the component macromolecules in various structural membranes. Mitochondrial observations revealed consistent alterations, distortion and swelling, with an apparent leaching of matrix material. Similar observations by Hugon and collaborators (1965) support the enzyme release theory as proposed by Bacq and Alexander (1961). Other morphological and architectural studies are consistent with these views (Scherer and Vogell
There is a clear relationship between radiation injury and alterations in structure and permeability of organelle and cell membranes. The extent to which damage to membranes is a result of initial radiation injury or is due to establishment of metabolic lesions is as yet unresolved. However, there has accumulated a wealth of evidence suggesting that radiation-induced lipid peroxidation may be responsible for the observed loss of membrane integrity.

**Lipid Peroxidation and Damage to Membranes**

**A/ General Considerations** Lipid peroxidation is the deteriorative oxidation of polyunsaturated lipids and involves a reaction of molecular oxygen or neighbouring free radicals with the activated allylic hydrogens on the alpha methylene carbons of the unsaturated lipid double bond (Demopoulos 1973; Tappel 1973). Because of their unpaired electrons, lipid free radicals, once formed, will react vigorously, initiating a chain propagation of hydrogen abstractions and addition reactions that can proceed autocatalytically as long as there are available active sites. Termination of this chain reaction can, however, occur by the binding of the free radicals formed or via quenching by antioxidants.

In general, unsaturated fatty acids occur principally in phospholipids which are inherent components of biomembranes. Because peroxidation of the fatty acids of such lipids is influenced not only by their degree of unsaturation but also by the nature of the moiety esterified
to the phosphate, it is clear that the content of the unsaturated lipid, degree of unsaturation and the substitution process present, play an important role in determining the likelihood of peroxidation damage to biological membranes (Demopoulos 1973). Many reports have documented the catalytic effect of metal cations (Cash et al. 1966), especially inorganic iron, on membrane lipid peroxidation (Ottolenghi et al. 1955; Hunter et al. 1963; Wills 1969). Bernheim (1963) suggests that in iron-ascorbate mediated peroxidation the process is initiated by ferrous ions, which in the process are oxidized to ferric ions. Ascorbate oxidation then reduces these back to the ferrous state. It was further proposed by Skrede and Christopherson (1966) that intermediates in thiol oxidation may be involved.

The importance of haematin compounds, mainly haemoglobin, myoglobin and cytochrome c, as biocatalysts of lipid peroxidation has also been established. Haematin compounds may, in fact, be the primary endogenous catalysts for lipid oxidation in animal tissues (Tappel 1953b). Biomembranes such as occur in mitochondria, endoplasmic reticulum and lysosomes, contain relatively large amounts of unsaturated lipids. Although the active allylic hydrogens of the lipid are buried in the hydrophobic region of the membrane and are thus afforded protection (Demopoulos 1973), the close association with these known biocatalysts arouses interest in the lability of such membranes to possible damage by lipid peroxidation.

In a series of investigations Hunter and co-workers (1963, 1964,
studied the effects of ferrous ion-induced lipid peroxidation in rat liver mitochondria. It was found that changes in the permeability and structural characteristics of the membrane accompanied the onset of lipid peroxidation, leaving little doubt that permeability alterations and swelling were causally related to lipid peroxide formation. McKnight and collaborators in 1965 found that ferrous ion peroxidation of lipids led to a loss of mitochondrial protein and lipid into the surrounding medium. The released material was thought to consist of the bulk of intercristal substance, together with solubilized proteins and elements of the electron transport chain. Similar alterations in the integrity of peroxidizing microsomes were noted by Bidlack and Tappel (1973). Loss of membrane bound NADPH cytochrome c reductase activity and release of protein were found to be related to alterations in the lipophilic region of the membrane.

Evidence of increased permeability in brain microsome preparations induced by lipid peroxide formation has also been observed (Robinson 1965) as documented by changes in turbidity of the system in vitro, and was accompanied by an increase in the reactive sulfhydryl groups in the membrane. Compounds which bound the sulfhydryl groups were found to increase permeability and accelerate lipid peroxidation. This suggests that initiation of peroxidation may involve the alteration of a relationship between these membrane proteins and the double bonds of the phospholipids, unmasking the double bonds and initiating structural changes. A similar relationship between protein sulfhydryl moieties and unsaturated
lipids has been proposed by Scott et al. (1964) to account for similar changes occurring after irradiation.

Peroxidation damage to membrane proteins and enzymes has been well described. Proteins and enzymes in aqueous solutions, when subjected to lipid peroxidation, undergo polymerization, chain scission and chemical changes (Roubal and Tappel 1966a, 1966b). In an attempt to determine the mechanism of damage to protein by lipid peroxidation Desai and Tappel (1963) found considerable damage to cytochrome c as measured by its decreased solubility. Linkages between linolenic acid and the protein were found and identified as mainly peroxyl or ether. This lipid-protein linkage phenomenon was also observed by Chio and Tappel (1969b) in a study of peroxide inactivation of ribonuclease A. Loss of enzyme activity was concomitant with the formation of intra and inter-molecular cross-linkages between the peroxide degradation product, malonaldehyde and ribonuclease A. Crosslinking apparently involves reaction of malonaldehyde with the primary amino groups of amino acids and proteins to form a Schiff base product $\text{RN} = \text{CH} - \text{CH} = \text{CH} - \text{NH} - \text{R}$ which displays fluorescence maxima in the 450 to 470 nm region (Chio and Tappel 1969a). This polymerization of unsaturated lipids with available proteins may be one of the main mechanisms of irreparable damage to enzymes and membranes.

Visualize the molecular havoc that occurs when enzymes cross-link with their molecular neighbours in such a random destructive reaction! The normal precision arrangement of proteins and enzymes in subcellular membranes and organelles would be badly disrupted and their biological activities -
would be lost or impaired.²

**B/ Radiological Considerations** The possibility that ionizing radiation may induce autoxidation of lipids in the living organism has occupied investigators interested in the effects of radiation exposure on animals. In studies with tissue homogenates and isolated systems it has been observed that in exposure to ionizing radiation peroxides are formed that can cause extensive destruction of lipids (Haugaard 1968). However, the ability of the living organism to destroy peroxides has made the search difficult and of many attempts to find peroxides in living organisms, few have been successful.

Propagative oxidation of lipids induced by irradiation is unique only in the details of initiation and termination (Mead 1961). Promotion of autoxidation by radiation may proceed via direct free radical formation of the target molecule or through extraction of allylic hydrogens from free radicals formed in the cell water.

\[ \text{INITIATION: } \text{RH} \xrightarrow{A} \text{R}^* + \text{H}^* \]

\[ \text{PROPAGATION: } \text{R}^* + \text{O}_2 \xrightarrow{B} \text{ROO}^* \]

\[ \text{ROO}^* + \text{RH} \xrightarrow{C} \text{ROOH} + \text{R}^* \]

AUTOCATALYTIC

\[ \text{TERMINATION: } \text{R}^* + \text{R}^* \xrightarrow{D} \text{R:R} \]

or \[ \text{R}^* + \text{H}^* \xrightarrow{\text{from Harmon 1968}} \text{R:H} \]

In the above diagram reaction A is initiated by either direct or indirect action of ionizing radiation, forming a lipid free radical which

combines with oxygen (reaction B) to form a lipid peroxide radical. This species can then react with neighbouring lipids to form lipid peroxide and newly formed free radicals which can serve to catalyze the propagation sequence again, unless terminated by free radical binding or hydrogen quenching (reaction D).

Work by Dubouloz and collaborators (1950, 1952) has shown that lipid peroxides are formed in rat skin following X-irradiation. However, the fact that the peroxides were not detected until the fourth day suggests that they could not have been primary products of irradiation. Barber and Ottolenghi (1957), in demonstrating the protective action of EDTA in irradiated mitochondria, showed that this protective ability was proportional to the inhibition of peroxide formation. An interesting suggestion was that radiation may release cations, known for their catalytic properties, from normal biological chelating agents, thus initiating the chain propagation. Earlier experiments on mice by Horgan and Philpot (1954, 1955) indicating that autocatalytic peroxide production may be operating in the post-irradiated animal were supported by Bernheim and collaborators (1956), who found that exposure of rabbits to 1400 rads of X-radiation produced significant elevations in peroxide values in the bone marrow. In addition, evidence of partial destruction of antioxidants supports the contention that antioxidant destruction may be involved in radiation injury. Alteration of the membrane lipids leading to increased sensitivity to peroxidation, or an increased catalytic ability of biological pro-oxidants such as non-haem iron, have also
been proposed by Wills (1970) as alternate modes inducing peroxidation.

Studies by Wills and Wilkinson (1967) on the radiation effect in subcellular fractions of rat liver in vitro provide further evidence of radiation induced lipid peroxidation. The authors suggested that the formation of lipoidal peroxides may be responsible for the primary biochemical lesion in irradiated animals. Similar results were obtained by Wills (1970) in irradiated microsomal systems in which post-irradiation peroxide formation produced extensive degradation of the microsomal lipid, accompanied by a release of malonaldehyde or related di-aldehydes.

Although Bacq and co-workers detected peroxides in the xylene extracts of mouse and rat depot fat following irradiation (1951), the extremely small values obtained do not give conclusive evidence that injury in living systems initiates in vivo peroxidation of lipids. Furthermore, only negligibly small increases in lipid peroxides occurred in vivo in various organs of rats in response to whole-body irradiation, in contrast to significant amounts of peroxides formed in incubated tissue homogenates (Ichii et al. 1968). These results indicate that lipid peroxidation in vitro does not represent the amount formed in vivo, and makes the extrapolation of in vitro evidence to the in vivo situation very tenuous indeed.

Although there is no clear cut evidence that peroxides are formed in damaging amounts in irradiated animals, it is possible that even extremely small amounts of peroxides may produce a considerable radiological effect, as was shown by Bernheim and co-workers (1952), so that
the question of radiation-induced peroxide damage remains open.

**Vitamin E: Antioxidant or Metabolic Intermediate?**

The biological function of vitamin E has been a topic of intensive research for over 30 years and to date there is still no agreement on its true biochemical role. Two schools of thought have, however, dominated the literature. The antioxidant hypothesis, which has received the widest support, contends that vitamin E acts solely as a physiological lipid antioxidant, active in terminating the autocatalytic propagation of free radical intermediates. The second hypothesis considers that α-tocopherol has some other biological role(s) distinctly separate from its possible antioxidant activity.

Although Dam (1957) has summarized evidence that vitamin E functions entirely as an *in vivo* antioxidant, there is much accumulated evidence suggesting that vitamin E does not prevent autoxidation of lipids *in vivo* (Green *et al.* 1967; Diplock *et al.* 1968). Recent experimental work has been presented by Diplock *et al.* (1971) which indicates that the function of vitamin E may be to protect a reduced form of selenium from bio-oxidation. Another idea contends that, instead of functioning as an antioxidant, vitamin E may fill a physiological role in membrane stabilization by virtue of specific physico-chemical interactions between its phytanyl side chain and the fatty acid acyl chains of polyunsaturated lipids. Different conclusions were drawn, however, by McCay and co-workers (1972) in a study involving lipid degradation in membranes resulting from free-energy changes in electron transport systems. It was found that α-tocopherol
preserved membrane integrity by scavenging free radicals formed as a result of electron transport and thus afforded protection against peroxidative chain cleavage by virtue of its antioxidative capabilities. In contrast, Levander and Morris (1971) suggest that α-tocopherol may play a role in the maintenance of inter-cellular cation gradients. To add to the confusion, there is now a large body of evidence suggesting that vitamin E plays a key role in the regulation of haem biosynthesis, presumably by controlling the mechanisms of induction-repression at the enzymatic steps in the synthesis of α-aminolevulinic acid (ALA) and porphobilinogen (PBG) (Nair 1972). In an effort to find common ground between the two viewpoints, Nair (1972) has proposed that the regulation of haem biosynthesis by α-tocopherol inherently controls the conjugation of haem with the apoprotein of catalase, which in turn carries out the catalytic scavenging of peroxides formed by the biological oxidation of lipids. Hence, a metabolic function of vitamin E is proposed which is manifested in its antioxidant activity. However, Horwitt states that

Since the mammalian organism will survive in the absence of α-tocopherol if some other antioxidant ... is subjected, it is most illogical to continue to attach any major importance to reports about α-tocopherol being an integral part of a critical enzyme system.3

Although many workers disagree with the biological antioxidant concept of vitamin E (Green et al. 1967; Diplock et al. 1968; Green 1972; Lucy 1972; Schwarz 1972; Diplock and Lucy 1973), many workers have produced considerable evidence in support of the proposal. An investigation

by Tappel (1952a) of the oxidation of unsaturated fatty acids catalyzed by haematin compounds revealed that the reaction could be inhibited by vitamin E and other antioxidants, indicating that α-tocopherol may function in vivo to inhibit haematin-catalyzed oxidation of fat. In addition, changes in catalytic and structural proteins due to vitamin E deficiency are thought by Tappel (1955) to result from the reaction of lipid peroxide products with the protein, and support the contention that vitamin E functions principally as an antioxidant. It is likely that vitamin E acts as a chain-breaking antioxidant, inhibiting the free-radical peroxidation via hydrogen abstraction or through the formation of a lipid peroxy radical-tocopherol complex (Tappel 1972). However, in studies of α-tocopherol oxidation products isolated from animal tissue, the abstraction of hydrogen appears to be the dominant process (Tappel 1972).

A study of the effects of radiation and peroxidation-induced radicals on α-tocopherol showed that radiation of vitamin E in isooctane produced many products. However, 5-exomethylenetocopher-6-one, derived from the extraction of two hydrogen atoms, appeared to be the major product formed (Knapp and Tappel 1961). The conversion of α-tocopherol to this array of degradation products is consistent with observed partial destruction of tocopherol in living tissues (Mead 1961). Furthermore, the dehydrogenation of vitamin E to the conjugated ketone product from radiation suggests that the "modus operandi" of radiation damage may involve destruction of the antioxidant in living tissue, allowing uncontrolled lipid peroxidation to ensue. Such has been proposed by
Myers and Bide (1966), who suggested that radiation-induced destruction of the membrane antioxidant is causally related to the formation of lipid peroxides in erythrocyte membranes. In addition, the irradiation of blood from mice placed on various diets supplemented with the vitamin (Prince 1973) has shown that tocopherol fortification not only reverses haemolytic effects in tocopherol deficient erythrocytes but also decreases haemolysis and loss of potassium from the cells. It was furthermore apparent that dietary levels of antioxidant may be an important factor in the radiosensitivity of erythrocytes.

Wills (1970) found vitamin E to be an effective inhibitor of in vitro peroxidation of irradiated rat liver microsomes, and in a later study Dawes and Wills (1972) showed that supplementation of the diet with vitamin E depressed the rate of peroxidation in tissue homogenates of mice following exposure to ionizing radiation. It was concluded that the antioxidant concentrations at the cellular and subcellular level play a major role in the regulation of post-irradiation peroxidation of lipids.

Although many investigations in vitro support the antioxidant hypothesis, the lack of conclusive evidence of in vivo peroxide formation or the presence of peroxides in tissues (Schwarz 1972) has severely hampered study of the antioxidant activity of vitamin E in the intact, living organism. Perhaps confusing the issue is the fact that in vivo peroxidation of polyunsaturated lipids is suppressed by many other mechanisms in addition to vitamin E (Tappel 1972). Strong evidence of the antioxidant ability of vitamin E in intact, living systems will be required in the final resolution of this controversy.
CHAPTER III

RADIATION-INDUCED ENZYME EFFLUX FROM RAT HEART: I. SEDENTARY ANIMALS

INTRODUCTION

One of the biochemical indicators of acute radiation injury is an increase in serum levels of lactate dehydrogenase (LDH). This has been documented by Hawrylewicz and Blair (1966), Hori et al. (1968, 1970) and Kärcher (1971), who have independently shown transient elevations of total LDH and the various isoenzymes following irradiation. In addition, research involving serum glutamate oxaloacetate transaminase (GOT) as a radiobiological marker has shown that in some mammalian species sub-lethal exposures led to significant, transient increases in serum activities (Milch and Albaum 1956; Petersen et al. 1957; Brent et al. 1958; Becker et al. 1962; Almonte et al. 1965). The data from Brent et al. (1958) suggests that the response is varied between species. Irradiated rabbits exhibited significant elevations of serum GOT within twenty-four hours of treatment, while rats subjected to either whole-body or local irradiation of the liver did not show any substantial GOT elevation after one day. Although little is known about the potential of creatine kinase (CK) as a radiobiological marker, observable serum elevations occurred in patients (post-treatment) receiving incidental cardiac irradiation (Muggia 1970). Since CK is located in skeletal and cardiac muscle in high concentration, its appearance in the blood may indicate radiation injury to these tissues.
Much of the work to date has involved the enzymological effects of whole-body irradiation, where cell death and disintegration of radiosensitive tissues may contribute greatly to the observed serum elevation. Little work, however, has been published outlining the effects of localized irradiation of radioresistant tissues, where therapeutic doses do not involve massive cell disruption. The present study was undertaken to investigate possible changes in serum levels of LDH, GOT and CK as possible indicators of radiation injury to the heart, and to attempt to correlate these changes with alterations in tissue levels of the enzyme.

MATERIAL AND METHODS

Irradiation Procedure

Sprague Dawley, male rats of 150 to 200 grams were placed under light ether anaesthesia twenty-four hours prior to irradiation and marked laterally across the top of the scapula to define the target area. At the time of irradiation the animals were placed into ventilated plexiglass tubes to restrict movement. The field exposure area was adjusted to cover 3 cm posterior to the scapula markings, as this was found to be optimal in minimizing radiation damage to peripheral tissues and organs, while allowing irradiation of the entire heart. Rats were irradiated in groups of four, using an Eldorado 8 (Atomic Energy of Canada) therapeutic cobalt unit. The dose rate was 281 rads per minute at the surface and exposure time was corrected for an absorbance loss of 15.7% at an average depth of 3 cm to give an acute dosage of 2,000 rads. Sham-irradiated controls were
treated in an identical manner to the experimental animals. All animals were treated in accordance with the guidelines established in *Care of Experimental Animals - A Guide for Canada*, published by the Canadian Council on Animal Care, Ottawa.

**Sampling Procedure**

In order to minimize serum turbidity the rats were fasted for six hours prior to sacrifice. At pre-determined intervals rats were placed under mild ether anaesthesia and an incision was made across the abdomen. Blood was withdrawn from the inferior vena cava using a non-heparinized syringe and immediately transferred to sealed centrifuge tubes. Samples were allowed to clot for 30 minutes at 25°C and were then placed on ice for approximately 30 minutes until centrifuging at 2,000 x g for 15 minutes (Wisniewska 1970).

Serum was collected and divided into individual vials for the enzyme assays. All samples for lactate dehydrogenase (LDH) determinations were stored at 4 - 6°C and assays were run within 48 hours. LDH samples were not frozen due to known inactivation of the enzyme (Soliman and Van Den Berg 1971). Samples for creatine kinase (CK) and glutamate oxaloacetate transaminase (GOT) determinations were frozen in liquid nitrogen and stored on dry ice until assayed. It has been shown that CK and GOT activity levels remain stable for several weeks when stored at -20°C (Lederle Diagnostics, American Cyanamid Co., Bulletin 27819).
Heart Muscle Preparation

Rat hearts were excised, trimmed of fatty and connective tissue and plunged into ice-cold 0.15 M KCl. After rinsing, the hearts were quickly blotted dry and weighed. The tissue was finely minced with scissors and washed five times in ice-cold 0.15 M KCl and twice in a multiple salts buffer modified from Chappell and Perry (1954). This medium contained 0.1 M KCl, 0.005 M MgSO₄, and 0.001 M ethylenediaminetetraacetate (EDTA) in 0.05 M TRIS HCl buffer, pH 7.4. The minced muscle was then homogenized in two volumes of the above medium using a ground-glass, Ten-Broeck type homogenizer, and appropriately diluted to make a 10% homogenate. Homogenate samples were centrifuged at 650 x g for five minutes after which the supernatant was collected and the operation repeated to remove cellular debris (Ernster and Nordenbrand 1967). The resultant supernatant was centrifuged at 6,000 x g for 15 minutes to obtain a crude mitochondrial pellet, which was re-suspended in fresh buffer and centrifuged as before. Although higher centrifuge speeds increase the product yield, it was found that 6,000 x g offered considerably purer fractions. This has been independently shown by Holton et al. (1957) and Hedman (1965). Supernatant resulting from the first mitochondrial fraction was collected, divided into individual vials and stored in the same manner as were serum samples. The washed mitochondrial pellet was rinsed three times in 0.15 M KCl and suspended in 1.0 ml of this solution. The final mitochondrial suspension was frozen in liquid nitrogen and stored on dry ice until assayed.
In the above procedure all manual operations were carried out on ice and centrifugations were performed at 0°C in a Beckman model J21 centrifuge using a JA-20, fixed-angle rotor.

**Enzyme Assays**

Creatine kinase assays employed the forward reaction or Rosalki technique (Rosalki 1967) using the following reagents (given as final concentrations in the assay medium): 5 x 10⁻² M PIPES buffer, 11.5 x 10⁻³ M magnesium salt, 1.45 x 10⁻² M glucose, 9.1 x 10⁻³ M AMP, 1.8 x 10⁻² M creatine phosphate, 1.1 x 10⁻³ M ADP, 8.67 x 10⁻³ M reduced glutathione, 5.0 x 10⁻⁴ M NADP, 3 IU per assay of hexokinase, 1 IU per assay of glucose-6-phosphate dehydrogenase (All reagents were pre-packaged by Calbiochem, San Diego). Serum assays were run using freshly thawed, undiluted samples. Homogenate samples were diluted to 0.2% after thawing, using modified Chappell-Perry medium. Measurement of background utilized NADP, using creatine phosphate-free control solutions, (Calbiochem) was pre-determined for both serum and homogenate preparations, and was found to be negligible. Since turbidity errors for both assays were also found negligibly small, assays were calibrated against a distilled water blank at 340 nm and run for twelve minutes at 30°C by addition of 10 - 20 μl of enzyme solution to 3.0 ml of assay mixture.

Lactate dehydrogenase assays were performed using the reverse reaction developed by Wroblewski and LaDue (1955) in which the substrate, pyruvate, is reduced to lactate. The assay system contained 0.08 M potassium phosphate buffer, pH 7.5, 7.5 x 10⁻⁴ M sodium pyruvate, and
1.1 x 10^{-4} M NADH. Prepared reagents were purchased from Calbiochem. Serum was assayed in undiluted form; homogenate samples were diluted to 1.0% with modified Chappell-Perry buffer. Background error was predetermined, using a substrate-free control solution of 1.1 x 10^{-4} M NADH (Calbiochem) in 0.08 M potassium phosphate buffer, pH 7.5. The analysis showed no detectible utilization of NADH, indicating no need for parallel control runs. Turbidity errors in serum and homogenate were found to be negligibly small, allowing assays to be calibrated at 340 nm, against a distilled water blank. Assays were run for 2 - 3 minutes at 30°C, after adding 10 - 20 µl of enzyme solution to 3.0 ml of assay mixture.

Glutamate oxaloacetate transaminase was determined by the ultraviolet method of Karmen (1955) on serum, homogenate and heart mitochondrial fractions. The assay system contained 0.1 M phosphate buffer, pH 7.4, 4.0 x 10^{-2} M L-aspartate, 1.2 x 10^{-2} M NADH, 0.25 mg malate dehydrogenase per ml, 0.25 mg lactate dehydrogenase per ml and 0.25 M α-oxoglutarate. Prepared reagents were purchased from Boehringer Mannheim Corporation. To 3.2 ml of assay mixture was added 0.5 ml of enzyme solution, and the degradation of NADH was followed at 366 nm for 4 to 6 minutes at 25°C. Serum was assayed in undiluted form; homogenate samples, however, were diluted with buffer to 0.1%. Due to the impermeability of biological membranes to many substrates and cofactors (Pycock and Nahorski 1971), mitochondrial samples were ruptured by addition of 1% TRITON X-100 in 0.1 M tris-acetate buffer, pH 6.8 (Abood and Alexander 1957; Greville and Chappel 1959; Wainio 1970).
Although this assay employs an ammonia-free system, possible contamination by glutamate dehydrogenase (GLDH) in homogenate and mitochondrial preparations was checked. Enzyme analysis by the method of Schmidt (1965) revealed only negligible levels of the enzyme in rat heart. This has been further shown by Kochakian et al. (1959), who found attempts to determine GLDH levels in mice and rat hearts yielded questionable results. Background degradation of NADH, using substrate-free controls for the GOT system, indicated an average error of less than 3% in serum. No detectable background activity was found in homogenate and mitochondrial preparations. Due to considerable serum turbidity, samples were calibrated against a serum blank containing 0.50 ml undiluted serum in 3.2 ml of 0.1 M phosphate buffer, pH 7.4. Because turbidity errors for homogenate and mitochondrial samples were negligible, transmittance was calibrated against a blank of 0.1 M phosphate buffer.

**Protein Determination**

In order to relate the calculated mitochondrial enzyme activities to the concentration of mitochondria, determinations of total mitochondrial protein were obtained using the biuret method (Layne 1957). To 0.4 - 0.8 ml of mitochondrial suspension, 0.6 - 0.2 ml of 5% deoxycholate (Jacobs et al. 1956) was added to remove turbidity. After ten minutes 4 ml of biuret reagent (Gornall et al. 1949) was added and the mixture was allowed to stand for 30 minutes at room temperature. Absorbance values were recorded at 550 nm against a blank containing 4 ml of biuret reagent with 1.0 ml of distilled water. Protein concentrations were
obtained by reference to a calibration curve established by serial
dilution of bovine serum albumin.

Data Analysis

Reaction order kinetics for each enzyme system were previously
determined and reaction rates were followed on a Beckman DB-GT spectro-
photometer fitted with a Phillips model PM 8100 flat-bed recorder.
Reaction rate data was then fitted to the equation for the corresponding
order, and rates were computed by a least squares fit using a Digital
Equipment Company PDP-8 computer. Mean values and individual variances
were determined for each sample group after subjecting the samples to a
Q-test analysis. Application of the F-test for an analysis of variance
between groups showed no significant differences in the population
variances, and warranted the determination of a pooled estimate of
variance (Dixon and Massey 1957). T-tests between control and experi-
mental values were run for a one-tailed, directional hypothesis, using
the pooled estimate of variance (Downie and Heath 1970).

RESULTS

Serum Assays

In all cases serum enzyme levels reached maximal values within
twenty-four hours following thoracic irradiation. Peak elevations arose
six to twelve hours post-irradiation, after which was noted a general
trend to return to normal levels. These results are in agreement with
other studies involving whole-body irradiation (Becker et al. 1962;
Lactate dehydrogenase (LDH) serum levels rose 139% above control levels six hours after exposure (Fig. 1). Statistical analysis showed this increase to be significant ($p < .05$). Twelve and twenty-four hours after exposure, serum LDH activity decreased respectively to 48% and 21% above control level. Although the twelve hour sample shows significant elevation, the twenty-four hour level is not statistically different from control level. On day three a second increase in serum LDH activity to 52% above control showed marginal significance at the 5% level, and returned to normal on days six and nine. This general pattern has also been observed by Hori and Nishio (1962). A third transient increase to 79% above control activity occurred twelve days after irradiation. The mean LDH activity on day fifteen does not show significant deviation from the control (Table 1).

Creatine kinase (CK) serum activity rose 131% above the control value six hours after irradiation ($p < .05$). At twelve hours a 68% elevation was observed ($p < .05$), which decreased to within control level by twenty-four hours (Table 2). A secondary elevation to 110% above control activity occurred on day three ($p < .05$), but returned to normal by day six. A third significant elevation occurred on day twelve which, as in the case of the serum LDH pattern, remained elevated at day fifteen. However, due to the small sample size of day fifteen, the elevation is not significantly different from the control (Fig. 2).
TABLE 1
SERUM LEVELS OF LACTATE DEHYDROGENASE AT VARIOUS INTERVALS AFTER EXPOSURE TO 2,000 RADS OF \(\gamma\)-RADIATION.
SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY \(t\)-TEST, USING A POOLED ESTIMATE OF VARIANCE

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity mU/ml</th>
<th>Significant Difference From Control</th>
<th>% Elevation From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>(1.31 \times 10^3)</td>
<td>+</td>
<td>139</td>
</tr>
<tr>
<td>6 Hour</td>
<td>6</td>
<td>(3.14 \times 10^3)</td>
<td>+</td>
<td>48</td>
</tr>
<tr>
<td>12 Hour</td>
<td>6</td>
<td>(1.94 \times 10^3)</td>
<td>-</td>
<td>21</td>
</tr>
<tr>
<td>24 Hour</td>
<td>5</td>
<td>(1.59 \times 10^3)</td>
<td>+</td>
<td>52</td>
</tr>
<tr>
<td>3 Day</td>
<td>4</td>
<td>(2.00 \times 10^3)</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6 Day</td>
<td>4</td>
<td>(1.27 \times 10^3)</td>
<td>-</td>
<td>(-) 3.3</td>
</tr>
<tr>
<td>9 Day</td>
<td>3</td>
<td>(1.36 \times 10^3)</td>
<td>-</td>
<td>3.0</td>
</tr>
<tr>
<td>12 Day</td>
<td>3</td>
<td>(2.35 \times 10^3)</td>
<td>+</td>
<td>79</td>
</tr>
<tr>
<td>15 Day</td>
<td>2</td>
<td>(1.89 \times 10^3)</td>
<td>-</td>
<td>44</td>
</tr>
</tbody>
</table>

Pooled Variance: 356561
Pooled Standard Deviation: 597
Figure 1. Serum levels of lactate dehydrogenase at various intervals after exposure to 2,000 rads of γ-radiation.
ACTIVITY IN (mU/ml serum) x 10^3
TABLE 2

SERUM LEVELS OF CREATINE KINASE AT VARIOUS INTERVALS AFTER EXPOSURE TO 2,000 RADS OF GAMMA RADIATION.
SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY t-TEST, USING A POOLED ESTIMATE OF VARIANCE

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity (mU/ml)</th>
<th>Significant Difference From Control @ p &lt; .05</th>
<th>% Elevation From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>208</td>
<td>+</td>
<td>131</td>
</tr>
<tr>
<td>6 Hour</td>
<td>5</td>
<td>481</td>
<td>+</td>
<td>68</td>
</tr>
<tr>
<td>12 Hour</td>
<td>6</td>
<td>350</td>
<td>-</td>
<td>44</td>
</tr>
<tr>
<td>24 Hour</td>
<td>6</td>
<td>299</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Day</td>
<td>4</td>
<td>437</td>
<td>+</td>
<td>110</td>
</tr>
<tr>
<td>6 Day</td>
<td>4</td>
<td>244</td>
<td>-</td>
<td>17</td>
</tr>
<tr>
<td>9 Day</td>
<td>3</td>
<td>272</td>
<td>-</td>
<td>31</td>
</tr>
<tr>
<td>12 Day</td>
<td>3</td>
<td>439</td>
<td>+</td>
<td>111</td>
</tr>
<tr>
<td>15 Day</td>
<td>2</td>
<td>365</td>
<td>-</td>
<td>76</td>
</tr>
</tbody>
</table>

Pooled Variance: 13812

Pooled Standard Deviation: 118
Figure 2. Serum levels of creatine kinase at various intervals after exposure to 2,000 rads of γ-radiation.
ACTIVITY (mU/ml serum)
Assays of serum glutamate oxaloacetate transaminase (GOT) activities revealed an increase of 54% at six hours post-irradiation \( (p < .05) \). Maximal elevation occurred after twelve hours, showing an activity increase of 66% \( (p < .05) \). After twenty-four hours serum GOT levels returned to normal (Table 3). An unexpected drop in activity to 59% of the control level on day six was not consistent with the directional hypothesis. However, since the probability value for a non-directional test showed significance at the 5% level, a possible rationale for this observed decrease will be presented elsewhere in this chapter. Serum GOT levels for days nine and twelve following exposure do not show significant deviations from control values (Fig. 3). However, a general trend of elevation does occur from day six to day fifteen, showing definitive increases in enzyme levels. Such secondary increases, as noted in all enzyme systems studied, may be indicative of the manifestation of major biochemical lesions induced by the ongoing metabolism of the injured tissue cells.

**Homogenate Assays**

Lactate dehydrogenase activity in heart muscle homogenates (Table 4) decreased 36% by twelve hours post-irradiation \( (p < .05) \). This decrease remained statistically significant after twenty-four hours, showing a 33% mean loss, returning to normal after three days. Activity levels from three to six days after exposure displayed a general decreasing trend, however, were not statistically different with respect to the control activity (Fig. 4). Day nine showed a secondary loss of
TABLE 3

SERUM LEVELS OF GLUTAMATE OXALOACETATE TRANSAMINASE AT VARIOUS INTERVALS AFTER EXPOSURE TO 2,000 RADS OF \( \gamma \)-RADIATION. SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY t-TEST, USING A POOLED ESTIMATE OF VARIANCE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity (mU/ml)</th>
<th>Significant Difference From Control @ ( p &lt; .05 )</th>
<th>% Elevation From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>49.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Hour</td>
<td>6</td>
<td>75.5</td>
<td>+</td>
<td>54</td>
</tr>
<tr>
<td>12 Hour</td>
<td>5</td>
<td>81.4</td>
<td>+</td>
<td>66</td>
</tr>
<tr>
<td>24 Hour</td>
<td>6</td>
<td>53.5</td>
<td>-</td>
<td>09</td>
</tr>
<tr>
<td>3 Day</td>
<td>4</td>
<td>47.9</td>
<td>-</td>
<td>(-) 2.6</td>
</tr>
<tr>
<td>6 Day</td>
<td>4</td>
<td>29.2</td>
<td>2P*</td>
<td>(-) 41</td>
</tr>
<tr>
<td>9 Day</td>
<td>3</td>
<td>36.3</td>
<td>-</td>
<td>(-) 26</td>
</tr>
<tr>
<td>12 Day</td>
<td>3</td>
<td>48.9</td>
<td>-</td>
<td>(-) 0.6</td>
</tr>
<tr>
<td>15 Day</td>
<td>3</td>
<td>60.1</td>
<td>-</td>
<td>+ 22</td>
</tr>
</tbody>
</table>

Pooled Variance: 105

Pooled Standard Deviation: 10.3

*Where activity levels differed significantly from control levels and did not agree with the directional hypothesis, a non-directional, two-tailed t-test was used to determine the level of significance.
Figure 3. Serum levels of glutamate oxaloacetate transaminase at various intervals after exposure to 2,000 rads of γ-radiation.
TABLE 4

HEART HOMOGENATE LEVELS OF LACTATE DEHYDROGENASE AT VARIOUS INTERVALS AFTER EXPOSURE TO 2,000 RADS OF $\gamma$-RADIATION. SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY $t$-TEST, USING A POOLED ESTIMATE OF VARIANCE

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity mU/mg Muscle</th>
<th>Significant Difference From Control $@ p &lt; .05$</th>
<th>% Decrease From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>457</td>
<td>+</td>
<td>20</td>
</tr>
<tr>
<td>6 Hour</td>
<td>3</td>
<td>365</td>
<td>+</td>
<td>20</td>
</tr>
<tr>
<td>12 Hour</td>
<td>3</td>
<td>291</td>
<td>+</td>
<td>36</td>
</tr>
<tr>
<td>24 Hour</td>
<td>3</td>
<td>305</td>
<td>+</td>
<td>33</td>
</tr>
<tr>
<td>3 Day</td>
<td>3</td>
<td>462</td>
<td>-</td>
<td>(−) 1.0</td>
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<tr>
<td>6 Day</td>
<td>3</td>
<td>426</td>
<td>-</td>
<td>6.9</td>
</tr>
<tr>
<td>9 Day</td>
<td>3</td>
<td>362</td>
<td>+</td>
<td>21</td>
</tr>
<tr>
<td>12 Day</td>
<td>3</td>
<td>470</td>
<td>-</td>
<td>3.0</td>
</tr>
<tr>
<td>15 Day</td>
<td>3</td>
<td>470</td>
<td>-</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Pooled Variance: 2414

Pooled Standard Deviation: 49.1
Figure 4. Heart homogenate levels of lactate dehydrogenase at various intervals after exposure to 2,000 rads of $\gamma$-radiation.
homogenate activity significantly different from the control at the 5% level. However, activities returned to normal by day twelve.

Creatine kinase levels in heart homogenates (Table 5) reflected similar changes with an observed loss of 21% from control level at twelve hours post-irradiation. Activity levels after twenty-four hours reflected a 25% mean loss which was not statistically different from the twelve hour measurement. Although enzyme levels returned to normal during day three, a secondary drop in homogenate activity, occurring during day six and day nine, showed statistically significant losses of 17% and 25% respectively (Fig. 5).

Glutamate oxaloacetate transaminase (GOT) loss from homogenate preparations shows a pattern similar to the previous system studied. The mean post-irradiation activity loss after twelve hours is 30% (p < .05), but returns to within control limits by twenty-four hours (p > .05). Homogenate GOT levels from day three to day fifteen fluctuate within control limits, and do not display statistically significant alterations with respect to control activities (Fig. 6).

**Mitochondrial Assays**

Loss of mitochondrial GOT activity, induced by an acute radiological dosage of 2,000 rads, is not significant at the 5% level of probability (Table 7). Although a mean activity loss of 36% occurred after six hours post-irradiation, the large sample variance limits the precision of measurement and may obscure the significance of such small alterations (Fig. 7).
TABLE 5

HEART HOMOGENATE LEVELS OF CREATINE KINASE AT VARIOUS INTERVALS AFTER EXPOSURE TO 2,000 RADS OF \( \gamma \)-RADIATION. SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY \( t \)-TEST, USING A POOLED ESTIMATE OF VARIANCE

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity ( \text{mU/g Muscle} )</th>
<th>Significant Difference From Control ( @ p &lt; .05 )</th>
<th>% Decrease From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>690</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>6 Hour</td>
<td>2</td>
<td>594</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>12 Hour</td>
<td>3</td>
<td>545</td>
<td>+</td>
<td>21</td>
</tr>
<tr>
<td>24 Hour</td>
<td>3</td>
<td>519</td>
<td>+</td>
<td>25</td>
</tr>
<tr>
<td>3 Day</td>
<td>3</td>
<td>616</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td>6 Day</td>
<td>3</td>
<td>573</td>
<td>+</td>
<td>17</td>
</tr>
<tr>
<td>9 Day</td>
<td>3</td>
<td>518</td>
<td>+</td>
<td>25</td>
</tr>
<tr>
<td>12 Day</td>
<td>3</td>
<td>629</td>
<td>-</td>
<td>8.8</td>
</tr>
<tr>
<td>15 Day</td>
<td>2</td>
<td>635</td>
<td>-</td>
<td>7.9</td>
</tr>
</tbody>
</table>

Pooled Variance: 2712

Pooled Standard Deviation: 52.1
Figure 5. Heart homogenate levels of creatine kinase at various intervals after exposure to 2,000 rads of Y-radiation.
ACTIVITY mU/mg muscle

0 400 600 800

CONTROL
HOUR INTERVALS
DAY INTERVALS

HOURS
6 12 24

DAYS
3 6 9 12 15
<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity (ml/mg Muscle)</th>
<th>Significant Difference From Control @ p &lt; .05</th>
<th>% Decrease From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>132</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6 Hour</td>
<td>3</td>
<td>106</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>12 Hour</td>
<td>3</td>
<td>92.3</td>
<td>+</td>
<td>30</td>
</tr>
<tr>
<td>24 Hour</td>
<td>3</td>
<td>114</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>3 Day</td>
<td>3</td>
<td>142</td>
<td>-</td>
<td>(-) 8.0</td>
</tr>
<tr>
<td>6 Day</td>
<td>3</td>
<td>156</td>
<td>-</td>
<td>(-) 18</td>
</tr>
<tr>
<td>9 Day</td>
<td>2</td>
<td>107</td>
<td>-</td>
<td>19</td>
</tr>
<tr>
<td>12 Day</td>
<td>3</td>
<td>134</td>
<td>-</td>
<td>(-) 2.0</td>
</tr>
<tr>
<td>15 Day</td>
<td>2</td>
<td>149</td>
<td>-</td>
<td>(-) 13</td>
</tr>
</tbody>
</table>

Pooled Variance: 414

Pooled Standard Deviation: 20.3
Figure 6. Heart homogenate levels of glutamate oxaloacetate transaminase at various intervals after exposure to 2,000 rads of γ-radiation.
TABLE 7

MITOCHONDRIAL LEVELS OF GLUTAMATE OXALOACETATE TRANSAMINASE AT VARIOUS INTERVALS AFTER EXPOSURE TO 2,000 RADS OF Y-RADIATION. SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY t-TEST, USING A POOLED ESTIMATE OF VARIANCE

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity mU/mg Biuret Protein</th>
<th>Significant Difference From Control @ p &lt; .05</th>
<th>% Decrease From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>1.31 x 10^3</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6 Hour</td>
<td>3</td>
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<td>-</td>
<td>36</td>
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<tr>
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<td>17</td>
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<tr>
<td>24 Hour</td>
<td>3</td>
<td>1.14 x 10^3</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>3 Day</td>
<td>3</td>
<td>1.64 x 10^3</td>
<td>-</td>
<td>(-) 25</td>
</tr>
<tr>
<td>6 Day</td>
<td>3</td>
<td>1.34 x 10^3</td>
<td>-</td>
<td>(-) 2.0</td>
</tr>
<tr>
<td>9 Day</td>
<td>3</td>
<td>1.49 x 10^3</td>
<td>-</td>
<td>(-) 13</td>
</tr>
<tr>
<td>12 Day</td>
<td>3</td>
<td>1.11 x 10^3</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>15 Day</td>
<td>3</td>
<td>1.30 x 10^3</td>
<td>-</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Pooled Variance: 133812

Pooled Standard Deviation: 366
Figure 7. Mitochondrial levels of glutamate oxaloacetate transaminase at various intervals after exposure to 2,000 rads of γ-radiation.
Although the heart has often been regarded as one of the most radioresistant organs of the body (Burch et al. 1968), the onset of radiation-induced heart disease has been recognized as an important manifestation of thoracic exposure (Stewart and Fajardo 1971). The present investigation documents biochemical evidence of initial cardiac injury in response to the acute administration of ionizing radiation to the thoracic area of rats.

One of the biochemical indicators in acute radiation injury is an elevation of serum lactate dehydrogenase activity following whole-body exposure (Hori et al. 1968). Lactate dehydrogenase, located in most organs, has an important function in carbohydrate metabolism, and under normal conditions remains at a fairly constant level. Glutamate oxaloacetate transaminase and creatine kinase are both found in relatively high concentrations in cardiac muscle. GOT, involved in the mediation of protein metabolism, and CK in the restoration of available energy for muscular contraction, play important roles as biochemical markers for the clinical detection of myocardial injury (Dreyfus et al. 1960; Schmidt et al. 1965) and were therefore selected as potential indicators for the study of radiation injury to cardiac muscle.

Serum levels of all three enzymes revealed transient elevations within twelve hours of thoracic irradiation, returning to within control levels by twenty-four hours. Similar response patterns have been documented in whole-body exposure (Becker et al. 1962; Almonte et al. 1965;
Takamori et al. 1969; Hori et al. 1970), indicating that such patterns appear to be representative of a general radiological response and are not related to the radiosensitivity of the system studied. Lactate dehydrogenase and creatine kinase serum levels displayed greater than two-fold initial elevations while GOT showed an increase of only 50% above normal. Although these elevations are significant, they are not remarkable. Because of the smallness of the changes and the sample variance within a population, clinical application of these systems as biochemical markers for radiation injury to the cardiac muscle does not appear likely unless one had pre-irradiation serum levels on the subject. In addition, studies by Brent and co-workers (1958) reveal that no apparent correlation exists between induced serum elevations and the likelihood of death.

All serum enzyme patterns indicate a trend toward normality after reaching initial maximal levels. Such a pattern suggests a possible re-instatement of membrane integrity following a transient alteration of membrane permeability. However, the effect might equally well be mediated by a lowering of the efflux rate of enzymatic material from the cell due to a decrease of the concentration gradient between cellular and extracellular levels caused by the depletion of the cellular constituents. Such factors, possibly operating in concert, combined with the rapid removal of the extracellular enzymes through active uptake in the extracellular fluid (Schmidt et al. 1965) may account for a "pseudo" transient nature of the initial elevations. Note that the restoration of
tissue enzyme levels to control values on day three (due possibly to enhanced synthesis of the enzymes) tends to restore the concentration gradient. This could subsequently lead to enhanced leakage and a secondary elevation of serum LDH and CK if the normal permeability of the cell membrane had not yet been restored. Although tissue homogenate GOT levels return to normal on day three, the serum level of GOT, unlike LDH and CK, did not increase significantly above normal at that time. This may indicate a significant restoration of membrane permeability with respect to GOT. Some overcompensatory repair mechanism for GOT permeability, whether at the cellular or hormonal level, or an enhanced inactivation of the serum enzyme could conceivably account for the noted transient reduction in serum GOT levels as shown in Figure 3.

Trends in the serum enzyme levels occurring after initial elevations suggest the possible manifestation of secondary radiation effects resulting from metabolic or biochemical lesions in the target organ. However, a strict interpretation of the serum elevations with respect to changes in the heart must be approached with extreme caution since radiation damage to peripheral tissues cannot be eliminated. In addition, physiological alterations mediated by hormonal action may further distort the relationship.

The significant decreases in the homogenate levels of the enzymes parallel the initial serum elevations and indicate that the observed serum elevations are not a result of enhanced tissue levels of the enzymes, but rather are a consequence of radiation-induced leakage of biologically
active material out of the injured cells. Similar alterations in the cellular levels of biologically active molecules in post-irradiated cells and organelles have been recorded (Okada and Peachey 1957; Roth and Eickel 1959; Kawasaki and Sakurai 1969). Secondary serum elevations, however, do not appear to be related to any significant secondary depletions of homogenate activities. It is unlikely that direct inactivation of the enzymes could account for the noted initial decreases in the homogenate activities since it has been demonstrated that most enzymes are relatively radioresistant (Schlenk et al. 1946; Ord and Stocken 1953; Winstead and Reece 1970), requiring substantially larger doses to initiate any significant inactivation.

It appears, at the biochemical level, that in vivo injury to the mitochondria with 2,000 rads of gamma radiation is not significant at the 5% confidence level. The apparent resistance of the mitochondrial membrane to damage, in comparison with the cell membrane, may result from the double membrane character of the mitochondria or physico-chemical differences in the structure of the membrane systems. It is, moreover, probable that the GOT concentration gradient between mitochondria and cytoplasm is not nearly as great as the cytoplasm-plasma gradient. Thus, the motive force and resultant leakage of the enzyme from the damaged mitochondria would not reflect as accurately the injury sustained from radiation damage to the organelle.
CHAPTER IV
RADIATION-INDUCED ENZYME EFFLUX FROM RAT HEART:
II EXERCISE-TRAINED ANIMALS

INTRODUCTION

Apart from various long-term performance studies (Jones et al. 1967; Belksy et al. 1972) surprisingly little is known about the effects of exercise on irradiated subjects. Since it is generally known that the ongoing metabolism of a cell is damaged by radiation, it is logical to propose that additional consequences of radiation exposure may become apparent under conditions of physiological stress. The determination of such consequences would allow an assessment of the irradiated animal's functional status and may ultimately prove important in the development of clinical therapeutic treatment programs.

The present study was, therefore, undertaken to evaluate the effect of exercise as an added metabolic stress in rats subjected to localized thoracic irradiation. The investigation involved an analysis of serum LDH, CK and GOT, with respect to alterations of these enzymes in the target organ, as indicators of radiation injury to the heart. The results compiled in this chapter were also statistically compared to the data compiled in the previous chapter in an effort to relate the extent of radiation injury between exercised and sedentary animals.
MATERIAL AND METHODS

Training Procedure

Many investigators have demonstrated that exercise stress of untrained subjects results in sharp elevations of plasma enzymes (Altland and Highman 1961; Halonen and Konttinen 1962; Fowler et al. 1962). However, such elevations are temporary and rapidly return to normal levels (Schlang 1961; Papadopoulos et al. 1968). Changes in serum enzyme levels after exercise of trained and untrained subjects studied by Fowler et al. (1962) revealed that serum enzyme elevations were considerably smaller in trained subjects than in untrained individuals. Similar observations have been made in man (Nuttall and Jones 1968) and other animals (Beaton 1966). Evidence compiled by Garbus and co-workers (1964) indicates that exercise training effectively protects against a rise in serum enzymes. Similar conclusions were also drawn by Highman and Altland (1963), who revealed that rats trained for six hours daily for seventeen to twenty days showed no significant changes in serum enzymes immediately following exercise stress.

In order to minimize serum elevations due to exercise itself, male Sprague-Dawley rats of an average weight of 65 gm and an average age of thirty days were placed on an exhaustive exercise training program. The age of the rats was chosen to correspond after thirty days of training to the average age of the animals utilized in the previous tests. After initial orientation the rats were exercised daily on a variable speed treadmill at speeds between 25 - 40 meters/minute for 30 - 50 minutes
until fatigue was evident. Following the last day of training, twenty-four hours prior to irradiation, the animals were placed under light ether anaesthesia and marked laterally across the top of the scapula to define the target area.

**Irradiation Procedure**

Exercised rats were irradiated in an identical manner as reported in Chapter III. Sham-irradiated, exercised control animals were treated accordingly. As soon as possible following irradiation all animals (including the sham-irradiated controls) were exercised in the normal manner and thereafter at twenty-four hour intervals approximately six to twelve hours previous to sacrifice.

Irradiated animals were sacrificed at intervals of six, twelve, and twenty-four hours and then at daily intervals of three, six, nine, and twelve days. The blood sampling procedure, preparation of heart homogenate, mitochondrial isolation, methods of enzyme analysis, mitochondrial protein determinations and methods of data analysis were all carried out in the manner previously reported.

**RESULTS**

**Effects of Y-Radiation on Exercised Rats**

**Serum Assays** The mean activity of LDH in serum increased 238% from control level six hours after exposure to 2,000 rads of gamma radiation. This elevation was significant beyond the .05 confidence level and remained so after twelve hours post-irradiation, showing a
mean increase of 166% (Table 1A). After twenty-four hours serum LDH activity returned to within normal limits. Although the mean activity was 90.5% higher than the control value, this elevation was not statistically different at the .05 level of confidence. No further significant deviations above the control value were recorded; however, there does occur a secondary elevation between sampling days three to twelve (Figure 1A).

Serum CK activity exhibited an initial increase to 182% above the mean control activity six hours following exposure (p < .05). After twelve hours the serum CK activity of irradiated animals remained significantly elevated (Table 2A), but levels returned to within control limits by twenty-four hours (Figure 2A). This initial response is strikingly comparable to the observed elevations of LDH, as was reported in Chapter III. Serum CK levels remained within control limits on days three and six, but did show a marginally significant elevation on day nine (p < .05). Enzyme levels did not show significant elevations on day twelve.

The mean GOT activity in the serum of irradiated, exercised rats exhibited a moderate increase of 50.6% at the six hour interval (p < .05). The enzyme activity remained significantly elevated twelve hours after exposure, showing a mean increase of 31.3%, but returned to control level after twenty-four hours (p > .05). Although serum GOT activity of rats sampled three and six days after irradiation demonstrated decreases of 31.3% and 25.5% respectively, these fluctuations were not significant at
TABLE 1A
SERUM LEVELS OF LACTATE DEHYDROGENASE IN EXERCISE-TRAINED RATS
AT VARIOUS INTERVALS AFTER EXPOSURE TO 2,000 RADS
OF γ-RADIATION. SIGNIFICANT DIFFERENCES
BETWEEN CONTROL AND TEST MEANS WERE
DETERMINED BY t-TEST, USING A
POOLED ESTIMATE OF VARIANCE

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity mU/ml</th>
<th>Significant Difference From Control @ p &lt; .05</th>
<th>% Increase From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>7.14 x 10^2</td>
<td>+</td>
<td>238</td>
</tr>
<tr>
<td>6 Hour</td>
<td>3</td>
<td>24.1 x 10^2</td>
<td>+</td>
<td>166</td>
</tr>
<tr>
<td>12 Hour</td>
<td>3</td>
<td>19.0 x 10^2</td>
<td>+</td>
<td>90.5</td>
</tr>
<tr>
<td>24 Hour</td>
<td>3</td>
<td>13.6 x 10^2</td>
<td>-</td>
<td>(-) 44.8</td>
</tr>
<tr>
<td>3 Day</td>
<td>2</td>
<td>3.97 x 10^2</td>
<td>-</td>
<td>14.6</td>
</tr>
<tr>
<td>6 Day</td>
<td>3</td>
<td>8.18 x 10^2</td>
<td>-</td>
<td>116</td>
</tr>
<tr>
<td>9 Day</td>
<td>3</td>
<td>15.4 x 10^2</td>
<td>-</td>
<td>58.3</td>
</tr>
<tr>
<td>12 Day</td>
<td>3</td>
<td>11.3 x 10^2</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

POOLED VARIANCE: 322717

POOLED STANDARD DEVIATION: 568
Figure 1A. Serum levels of lactate dehydrogenase in exercise-trained rats at various intervals after exposure to 2,000 rads of $\gamma$-radiation.
ACTIVITY IN (mU/ml serum) x 10^3

Day Intervals

Control

HOURS

6 12 24

DAYS

3 6 9 12
TABLE 2A
SERUM LEVELS OF CREATINE KINASE IN EXERCISE-TRAINED RATS
AT VARIOUS INTERVALS AFTER EXPOSURE TO 2,000 RADS
OF γ-RADIATION. SIGNIFICANT DIFFERENCES
BETWEEN CONTROL AND TEST MEANS WERE
DETERMINED BY t-TEST, USING A
POOLED ESTIMATE OF VARIANCE

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity mU/ml</th>
<th>Significant Difference From Control @ p &lt; .05</th>
<th>% Increase From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>152</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Hour</td>
<td>3</td>
<td>429</td>
<td>+</td>
<td>182</td>
</tr>
<tr>
<td>12 Hour</td>
<td>3</td>
<td>350</td>
<td>+</td>
<td>130</td>
</tr>
<tr>
<td>24 Hour</td>
<td>2</td>
<td>303</td>
<td>-</td>
<td>99.2</td>
</tr>
<tr>
<td>3 Day</td>
<td>3</td>
<td>105</td>
<td>-</td>
<td>(-) 30.9</td>
</tr>
<tr>
<td>6 Day</td>
<td>3</td>
<td>214</td>
<td>-</td>
<td>40.8</td>
</tr>
<tr>
<td>9 Day</td>
<td>3</td>
<td>320</td>
<td>+</td>
<td>111</td>
</tr>
<tr>
<td>12 Day</td>
<td>3</td>
<td>241</td>
<td>-</td>
<td>59.0</td>
</tr>
</tbody>
</table>

POOLED VARIANCE: 5284

POOLED STANDARD DEVIATION: 72.7
Figure 2A. Serum levels of creatine kinase in exercise-trained rats at various intervals after exposure to 2,000 rads of γ-radiation.
ACTIVITY IN mU/ml serum

HOURS

DAYS

0 100 200 300 400 500

HOUR INTERVALS

CONTROL

DAY INTERVALS
the .05 level of confidence (Table 3A). Serum GOT activities remained within control limits for the remaining sampling periods. However, there occurred a demonstrable elevating trend from day three to day twelve (Figure 3A).

**Homogenate Assays** LDH activity in prepared heart homogenates of irradiated animals showed a mean decrease of 32.3% at six hours following exposure (p < .05). Although LDH levels returned to normal at the twelve hour interval, a subsequent loss of 19.6% in activity occurred after twenty-four hours (Table 4A). Rats sacrificed three days following irradiation did not show significant alterations in the homogenate level of the enzymes. However, animals sampled on days six, nine and twelve showed respective losses of 29.6%, 24.3% and 15.9%, which were all found statistically significant at the 5% level (Figure 4A).

In contrast to the observed losses of cellular LDH, heart homogenate levels of CK in irradiated rats did not exhibit significant variations from sham-irradiated control animals within the first three days (Table 5A). Although a temporary loss of CK activity was observed on day six (p < .05), homogenate levels returned to normal on days nine and twelve (Figure 5A).

The mean GOT activities of post-irradiated rats showed a pattern strikingly similar to the homogenate CK pattern discussed above. No significant alterations in mean GOT levels, with respect to the control value, were found within the first three days (Table 6A). Despite a significant loss of 17.5% in enzyme activity on day six, GOT levels remained statistically identical to the mean control level for the remaining
TABLE 3A

SERUM LEVELS OF GLUTAMATE OXALOACETATE TRANSAMINASE IN EXERCISE-TRAINED RATS AT VARIOUS INTERVALS AFTER EXPOSURE TO 2,000 RADS OF γ-RADIATION.

SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY t-TEST, USING A POOLED ESTIMATE OF VARIANCE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity mU/ml</th>
<th>Significant Difference From Control @ p &lt; .05</th>
<th>% Increase From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>54.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Hour</td>
<td>3</td>
<td>82.2</td>
<td>+</td>
<td>50.6</td>
</tr>
<tr>
<td>12 Hour</td>
<td>3</td>
<td>71.7</td>
<td>+</td>
<td>31.3</td>
</tr>
<tr>
<td>24 Hour</td>
<td>3</td>
<td>54.1</td>
<td>-</td>
<td>(-) 0.9</td>
</tr>
<tr>
<td>3 Day</td>
<td>3</td>
<td>37.5</td>
<td>-</td>
<td>(-) 31.3</td>
</tr>
<tr>
<td>6 Day</td>
<td>3</td>
<td>40.7</td>
<td>-</td>
<td>(-) 25.5</td>
</tr>
<tr>
<td>9 Day</td>
<td>3</td>
<td>49.8</td>
<td>-</td>
<td>(-) 8.8</td>
</tr>
<tr>
<td>12 Day</td>
<td>3</td>
<td>61.5</td>
<td>-</td>
<td>12.6</td>
</tr>
</tbody>
</table>

POOLED VARIANCE: 83.7

POOLED STANDARD DEVIATION: 9.15
Figure 3A. Serum levels of glutamate oxaloacetate transaminase in exercise-trained rats at various intervals after exposure to 2,000 rads of Y-radiation.
ACTIVITY IN μU/ml serum

HOURS

DAYS

CONTROL
DAY INTERVALS
TABLE 4A

HEART HOMOGENATE LEVELS OF LACTATE DEHYDROGENASE IN EXERCISE-TRAINED RATS AT VARIOUS INTERVALS AFTER EXPOSURE TO 2,000 RADS OF Y-RADIATION. SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY t-TEST, USING A POOLED ESTIMATE OF VARIANCE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity</th>
<th>Significant Difference</th>
<th>% Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mU/mg Muscle</td>
<td>From Control @ p &lt; .05</td>
<td>From Control</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>622</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Hour</td>
<td>3</td>
<td>421</td>
<td>+</td>
<td>32.3</td>
</tr>
<tr>
<td>12 Hour</td>
<td>3</td>
<td>548</td>
<td>-</td>
<td>11.9</td>
</tr>
<tr>
<td>24 Hour</td>
<td>3</td>
<td>500</td>
<td>+</td>
<td>19.6</td>
</tr>
<tr>
<td>3 Day</td>
<td>3</td>
<td>548</td>
<td>-</td>
<td>11.9</td>
</tr>
<tr>
<td>6 Day</td>
<td>3</td>
<td>438</td>
<td>+</td>
<td>29.6</td>
</tr>
<tr>
<td>9 Day</td>
<td>3</td>
<td>471</td>
<td>+</td>
<td>24.3</td>
</tr>
<tr>
<td>12 Day</td>
<td>3</td>
<td>523</td>
<td>+</td>
<td>15.9</td>
</tr>
</tbody>
</table>

POOLED VARIANCE: 223.4

POOLED STANDARD DEVIATION: 47.3
Figure 4A. Heart homogenate levels of lactate dehydrogenase in exercise-trained rats at various intervals after exposure to 2,000 rads of γ-radiation.
ACTIVITY IN mU/mg muscle

HOURS
0  200  400  600  800  1000
CONTROL

DAY INTERVALS

DAYS
6  12  24  3  6  9  12
HEART HOMOGENATE LEVELS OF CREATINE KINASE IN EXERCISE-TRAINED RATS AT VARIOUS INTERVALS AFTER EXPOSURE TO 2,000 RADS OF Γ-RADIATION. SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY t-TEST, USING A POOLED ESTIMATE OF VARIANCE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity</th>
<th>Significant Difference</th>
<th>% Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mU/mg Muscle</td>
<td>From Control @ p &lt; .05</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>757</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6 Hour</td>
<td>3</td>
<td>773</td>
<td>-</td>
<td>(-) 2.11</td>
</tr>
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<td>12 Hour</td>
<td>3</td>
<td>723</td>
<td>-</td>
<td>4.5</td>
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<tr>
<td>24 Hour</td>
<td>3</td>
<td>734</td>
<td>-</td>
<td>3.0</td>
</tr>
<tr>
<td>3 Day</td>
<td>3</td>
<td>807</td>
<td>-</td>
<td>(-) 6.61</td>
</tr>
<tr>
<td>6 Day</td>
<td>3</td>
<td>646</td>
<td>+</td>
<td>14.7</td>
</tr>
<tr>
<td>9 Day</td>
<td>3</td>
<td>745</td>
<td>-</td>
<td>1.6</td>
</tr>
<tr>
<td>12 Day</td>
<td>3</td>
<td>754</td>
<td>-</td>
<td>0.4</td>
</tr>
</tbody>
</table>

POOLED VARIANCE: 3199

POOLED STANDARD DEVIATION: 56.6
Figure 5A. Heart homogenate levels of creatine kinase in exercise-trained rats at various intervals after exposure to 2,000 rads of \( \gamma \)-radiation.
TABLE 6A

HEART HOMOGENATE LEVELS OF GLUTAMATE OXALOACETATE TRANSAMINASE IN EXERCISE-TRAIN RATS AT VARIOUS INTERVALS AFTER EXPOSURE TO 2,000 RADS OF γ-RADIATION. SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY t-TEST, USING A POOLED ESTIMATE OF VARIANCE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity mU/mg Muscle</th>
<th>Significant Difference From Control @ p &lt; .05</th>
<th>% Decrease From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>160</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6 Hour</td>
<td>3</td>
<td>145</td>
<td>-</td>
<td>9.4</td>
</tr>
<tr>
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<tr>
<td>3 Day</td>
<td>3</td>
<td>166</td>
<td>-</td>
<td>(-) 3.8</td>
</tr>
<tr>
<td>6 Day</td>
<td>3</td>
<td>132</td>
<td>+</td>
<td>17.5</td>
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<tr>
<td>9 Day</td>
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<tr>
<td>12 Day</td>
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<td>160</td>
<td>-</td>
<td>0.0</td>
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</tbody>
</table>

POOLED VARIANCE: 111.1

POOLED STANDARD DEVIATION: 10.5
Figure 6A. Heart homogenate levels of glutamate oxaloacetate transaminase in exercise-trained rats at various intervals after exposure to 2,000 rads of Y-radiation.
ACTIVITY IN mU/mg muscle

DAYS

12 9 6 3 24 12 6

ACTIVITY IN mU/mg muscle

0

100

200

HOURS

DAY INTERVALS

HOUR INTERVALS

CONTROL
Mitochondrial Assays

GOT enzyme levels of heart mitochondria isolated at the stated intervals did not show significant alterations until twenty-four hours after irradiation (Table 7A). Samples isolated at twenty-four hours demonstrated a 36.8% mean loss with respect to the mean control value ($p < .05$). Mitochondrial enzyme levels returned to normal by day three and showed no further significant deviations (Figure 7A).

The Effect of Exercise Training on Sham-irradiated Control Rats

In order to determine the effect of the exercise training procedure on the enzyme levels in serum, heart homogenate and heart mitochondria of sham-irradiated control animals, the mean enzyme levels of the exercised samples were statistically compared to the levels of sedentary controls. To obtain a pooled variance between the exercised and sedentary groups, variances previously pooled for each enzyme system were subjected to a test of homogeniety, using the $t$ ratio. Degrees of freedom were determined using the total population utilized in calculating the original pooled variances in each system. The ratios obtained clearly showed that the variances within exercised and sedentary groups were homogeneous and were therefore pooled to obtain a single variance factor. Mean enzyme levels were subjected to a two-tailed $t$-test for a non-directional hypothesis and significance limits were set at the 5% level of confidence.

Serum LDH and CK levels demonstrated slight reductions as a consequence of exercise training. However, the decreased activities were not
TABLE 7A

MITOCHONDRIAL LEVELS OF GLUTAMATE OXALOACETATE TRANSAMINASE IN EXERCISE-TRAINED RATS AT VARIOUS INTERVALS AFTER EXPOSURE TO 2,000 RADS OF \( \gamma \)-RADIATION.

SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY \( t \)-TEST, USING A POOLED ESTIMATE OF VARIANCE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity</th>
<th>Significant Difference From Control @ ( p &lt; .05 )</th>
<th>% Decrease From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td>1.08 x 10^3</td>
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<td>1.9</td>
</tr>
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<td>1.06 x 10^3</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>12 Hour</td>
<td>3</td>
<td>0.94 x 10^3</td>
<td>+</td>
<td>36.8</td>
</tr>
<tr>
<td>24 Hour</td>
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<td>0.68 x 10^3</td>
<td>-</td>
<td>(-) 11.1</td>
</tr>
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<td>1.20 x 10^3</td>
<td>-</td>
<td>(-) 22.2</td>
</tr>
<tr>
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<td>3</td>
<td>1.32 x 10^3</td>
<td>-</td>
<td>(-) 9.3</td>
</tr>
<tr>
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<td>1.18 x 10^3</td>
<td>-</td>
<td>(-) 15.7</td>
</tr>
<tr>
<td>12 Day</td>
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<td>1.25 x 10^3</td>
<td>-</td>
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</tr>
</tbody>
</table>

POOLED VARIANCE: 56836

POOLED STANDARD DEVIATION: 238
Figure 7A. Mitochondrial levels of glutamate oxaloacetate transaminase in exercise-trained rats at various intervals after exposure to 2,000 rads of Y-radiation.
ACTIVITY IN (mU/mg biuret protein) x 10^3

HOURS
0 0.5 1.0 1.5 2.0
       DAY INTERVALS

DAYS
6 12 24 3 6 9 12

CONTROL

[Graph showing activity levels over time and intervals]
significantly different from sedentary values at the .05 level of confidence (Table 8A). Serum GOT, similarly, did not exhibit significant changes between the groups (2p > .05). Although heart homogenate levels of GOT and CK did not demonstrate any significant alterations, homogenate LDH of the exercised animals did exhibit a 36.1% mean increase in activity (2p < .05). The mean activity of mitochondrial GOT in exercised animals was not significantly different from that of sedentary animals.

Comparison of Radiation-Induced Alterations in Enzyme Concentrations in Serum, Heart Homogenate and Heart Mitochondria in Exercised and Sedentary Rats.

In order to obtain confidence intervals for the percentage changes in enzyme activities previously reported, the pooled standard deviations for the previous data were divided by the known control activities in each table and multiplied by 100. The resultant factors represented the standard deviation (s) for the recorded percentage changes. The resulting variances (s²) were subjected to an f ratio test and were found to be homogeneous. The variances of the exercised and sedentary groups were therefore pooled to obtain a single pooled variance. Percentage increases were subjected to a non-directional, two-tailed t-test set at the 5% limit of confidence.

**Serum Enzyme Elevations.** Significant increases in the percentage elevation of serum LDH in exercised, irradiated rats occurred at six and twelve hours following exposure in comparison with irradiated, sedentary animals (Table 9A). No significant differences in LDH elevations between
TABLE 8A
ENZYME LEVELS OF SHAM-IRRADIATED, EXERCISED AND SEDENTARY CONTROLS. A COMPARISON OF EXERCISE-INDUCED CHANGES IN ENZYME ACTIVITIES OF SERUM (S), HEART HOMOGENATE (H), AND HEART MITOCHONDRIAL (M) FRACTIONS.

<table>
<thead>
<tr>
<th>Assay System</th>
<th>Sedentary Level</th>
<th>Exercised Level</th>
<th>Pooled S</th>
<th>Significant Difference @ 2p &lt; .05</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-LDH</td>
<td>1,310 mU/ml</td>
<td>714 mU/ml</td>
<td>586</td>
<td>-</td>
</tr>
<tr>
<td>S-CK</td>
<td>208 mU/ml</td>
<td>152 mU/ml</td>
<td>104</td>
<td>-</td>
</tr>
<tr>
<td>S-GOT</td>
<td>49.2 mU/ml</td>
<td>54.6 mU/ml</td>
<td>9.08</td>
<td>-</td>
</tr>
<tr>
<td>H-LDH</td>
<td>457 mU/mg</td>
<td>622 mU/mg</td>
<td>48.3</td>
<td>+</td>
</tr>
<tr>
<td>H-CK</td>
<td>690 mU/mg</td>
<td>757 mU/mg</td>
<td>54.4</td>
<td>-</td>
</tr>
<tr>
<td>H-GOT</td>
<td>132 mU/mg</td>
<td>160 mU/mg</td>
<td>16.3</td>
<td>-</td>
</tr>
<tr>
<td>M-GOT</td>
<td>1,310 mU/mg</td>
<td>1,080 mU/mg</td>
<td>313</td>
<td>-</td>
</tr>
</tbody>
</table>

Mean enzyme levels were subjected to a two-tailed t-test for a non-directional hypothesis. Significance limits were set at the .05 level of confidence.
TABLE 9A

COMPARISON OF ALTERATIONS IN SERUM ENZYME LEVELS OF POST-IRRADIATED SEDENTARY AND EXERCISED RATS.

<table>
<thead>
<tr>
<th>Post-Irradiation Time</th>
<th>Enzyme System</th>
<th>% Activity in Sedentary Rats</th>
<th>% Activity in Exercised Rats</th>
<th>Significant Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Hours</td>
<td>LDH</td>
<td>239 (6)</td>
<td>338 (3)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>231 (5)</td>
<td>282 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>GOT</td>
<td>154 (6)</td>
<td>151 (3)</td>
<td>-</td>
</tr>
<tr>
<td>12 Hours</td>
<td>LDH</td>
<td>148 (6)</td>
<td>266 (3)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>168 (6)</td>
<td>230 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>GOT</td>
<td>166 (5)</td>
<td>131 (3)</td>
<td>-</td>
</tr>
<tr>
<td>24 Hours</td>
<td>LDH</td>
<td>121 (5)</td>
<td>191 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>144 (6)</td>
<td>199 (2)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>GOT</td>
<td>109 (6)</td>
<td>101 (3)</td>
<td>-</td>
</tr>
<tr>
<td>3 Days</td>
<td>LDH</td>
<td>152 (4)</td>
<td>55.6 (2)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>210 (4)</td>
<td>69.1 (3)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>GOT</td>
<td>97.4 (4)</td>
<td>68.7 (3)</td>
<td>-</td>
</tr>
<tr>
<td>6 Days</td>
<td>LDH</td>
<td>96.7 (4)</td>
<td>115 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>117 (4)</td>
<td>141 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>GOT</td>
<td>59.0 (4)</td>
<td>74.5 (3)</td>
<td>-</td>
</tr>
<tr>
<td>9 Days</td>
<td>LDH</td>
<td>103 (3)</td>
<td>216 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>131 (3)</td>
<td>111 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>GOT</td>
<td>74.0 (3)</td>
<td>91.2 (3)</td>
<td>-</td>
</tr>
<tr>
<td>12 Days</td>
<td>LDH</td>
<td>179 (3)</td>
<td>158 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>211 (3)</td>
<td>159 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>GOT</td>
<td>99.4 (3)</td>
<td>113 (3)</td>
<td>-</td>
</tr>
</tbody>
</table>

LDH POOLED STANDARD DEVIATION: 58.4  
CK POOLED STANDARD DEVIATION: 53.5  
GOT POOLED STANDARD DEVIATION: 19.5

Figures in parenthesis represent the number of rats sampled.

Values are expressed as percent activity with respect to control values of 100%.
exercised and sedentary animals occurred during the remaining sampling intervals.

Although no statistically significant differences in serum CK elevations between exercised and sedentary groups occurred during the first twenty-four hours, a temporary decrease was found in exercised animals sacrificed after three days ($2p < .05$). No further differences in serum elevations were found during the remaining sampling times.

Post-irradiation serum GOT elevations showed no significant differences between sedentary and exercised animals at the 5% level of confidence.

**Heart Homogenate Enzyme Levels** Although the percentage decrease of LDH enzyme activity in the six hour post-exposure samples did not differ significantly between exercised and sedentary animals, there was an increased percentage activity in exercised rats at twelve hours ($2p < .05$), suggesting a more rapid recovery process to the initial enzyme loss. Except for a temporary decrease in the LDH activity of the exercised samples on day six, no further significant differences are apparent (Table 10A).

Homogenate CK activities in the exercised rats generally remained significantly higher in most post-irradiation samples and, in contrast to the transient leakage demonstrated in irradiated sedentary rats, did not show an initial loss of activity (Tables 5 & 5A).

The mean activity of GOT in heart homogenates was generally greater in exercised rats during the initial twenty-four hour post-irradiation
TABLE 10A

COMPARISON OF ALTERATIONS IN HOMOGENATE ENZYME LEVELS OF POST-IRRADIATED SEDENTARY AND EXERCISED RATS.

<table>
<thead>
<tr>
<th>Post-Irradiation Time</th>
<th>Enzyme System</th>
<th>% Activity in Sedentary Rats</th>
<th>% Activity in Exercised Rats</th>
<th>Significant Difference @ 2p &lt; .05</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Hours</td>
<td>LDH</td>
<td>79.9 (3)</td>
<td>67.7 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>86.1 (2)</td>
<td>102 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>GOT</td>
<td>80.4 (3)</td>
<td>90.6 (3)</td>
<td>-</td>
</tr>
<tr>
<td>12 Hours</td>
<td>LDH</td>
<td>63.7 (3)</td>
<td>88.1 (3)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>79.1 (3)</td>
<td>95.5 (3)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>GOT</td>
<td>70.0 (3)</td>
<td>95.6 (3)</td>
<td>+</td>
</tr>
<tr>
<td>24 Hours</td>
<td>LDH</td>
<td>66.8 (3)</td>
<td>80.4 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>75.3 (3)</td>
<td>97.0 (2)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>GOT</td>
<td>86.8 (3)</td>
<td>92.5 (3)</td>
<td>-</td>
</tr>
<tr>
<td>3 Days</td>
<td>LDH</td>
<td>101 (3)</td>
<td>88.1 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>89.3 (3)</td>
<td>107 (3)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>GOT</td>
<td>108 (3)</td>
<td>104 (3)</td>
<td>-</td>
</tr>
<tr>
<td>6 Days</td>
<td>LDH</td>
<td>93.1 (3)</td>
<td>70.4 (3)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>83.1 (3)</td>
<td>85.3 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>GOT</td>
<td>118 (3)</td>
<td>82.5 (3)</td>
<td>+</td>
</tr>
<tr>
<td>9 Days</td>
<td>LDH</td>
<td>79.2 (3)</td>
<td>75.7 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>75.1 (3)</td>
<td>98.4 (3)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>GOT</td>
<td>81.2 (2)</td>
<td>95.0 (3)</td>
<td>-</td>
</tr>
<tr>
<td>12 Days</td>
<td>LDH</td>
<td>103 (3)</td>
<td>84.1 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>91.2 (3)</td>
<td>99.6 (3)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>GOT</td>
<td>102 (3)</td>
<td>100 (3)</td>
<td>-</td>
</tr>
</tbody>
</table>

LDH POOLED STANDARD DEVIATION: 9.28
CK POOLED STANDARD DEVIATION: 7.55
GOT POOLED STANDARD DEVIATION: 11.85

Figures in parenthesis represent the number of rats sampled.

Values are expressed as percent activity with respect to control values of 100%.
**TABLE 11A**

**COMPARISON OF ALTERATIONS IN MITOCHONDRIAL G6PD LEVELS OF POST-IRRADIATED SEDENTARY AND EXERCISED RATS.**

<table>
<thead>
<tr>
<th>Post-Irradiation Time</th>
<th>% Activity in Sedentary Rats</th>
<th>% Activity in Exercised Rats</th>
<th>Significant Difference @ 2p &lt; .05</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Hours</td>
<td>63.7 (3)</td>
<td>98.1 (3)</td>
<td>-</td>
</tr>
<tr>
<td>12 Hours</td>
<td>82.6 (3)</td>
<td>87.0 (3)</td>
<td>-</td>
</tr>
<tr>
<td>24 Hours</td>
<td>86.9 (3)</td>
<td>63.2 (3)</td>
<td>-</td>
</tr>
<tr>
<td>3 Days</td>
<td>125 (3)</td>
<td>111 (3)</td>
<td>-</td>
</tr>
<tr>
<td>6 Days</td>
<td>102 (3)</td>
<td>122 (3)</td>
<td>-</td>
</tr>
<tr>
<td>9 Days</td>
<td>113 (3)</td>
<td>90.7 (3)</td>
<td>-</td>
</tr>
<tr>
<td>12 Days</td>
<td>84.4 (3)</td>
<td>84.3 (3)</td>
<td>-</td>
</tr>
</tbody>
</table>

**POOLED STANDARD DEVIATION:** 25.1

Values are expressed as percent activity with respect to control values of 100%.

Figures in parenthesis represent the number of rats sampled.
interval, although the deviations are statistically significant only at the twelve hour interval. Unlike the temporary decrease in sedentary animals noted after 12 hours (Table 6, Chapter III), there was no significant loss of homogenate GOT activity in irradiated, exercised rats (Table 6A). Except for a temporary decrease in activity on day six, the homogenate GOT activity of exercised rats did not differ significantly from the post-exposure values of sedentary animals for the remaining sampling intervals.

Heart Mitochondrial Enzyme Levels  Mean GOT enzyme levels in mitochondria isolated from the hearts of exercised and sedentary rats exposed to radiation showed no significant differences for all sampling periods involved (Table 11A).

DISCUSSION

The large but transient serum enzyme elevations exhibited by LDH and CK revealed a significant post-irradiation response which was maximal six hours following radiation exposure, returning to control levels by twenty-four hours. Such a pattern suggests a rapid but temporary leakage of biological material out of the damaged target tissues. Similar biological responses have been reported in studies involving whole-body exposure Takemori et al. 1969; Hori et al. 1970), and in the previous study conducted in this laboratory involving thoracic irradiation of sedentary male rats (MacWilliam and Bhakthan, submitted for publication). Although serum LDH showed no further statistically significant elevations during the
remaining sampling periods, both LDH and CK were, in general, elevated beyond day three (Figure 3, Chapter III and Figure 3A), indicating the possible development of secondary injury processes, as was suggested in the previous work. The small increase in the post-exposure serum GOT activities of exercised rats is also similar to the response noted previously in sedentary animals, and indicates enhanced leakage of the enzyme from the damaged tissues. The resultant return to normal in serum GOT levels after twenty-four hours indicates the possible re-instatement of membrane integrity. Although no further significant serum GOT elevations occurred during the remaining sampling intervals, an elevating trend similar to the LDH and CK patterns occurred from day three to day twelve. This evidence adds further support to the suggested development of secondary radiation effects, which may include the development of major biochemical lesions or metabolic alterations.

The pattern of temporary reductions in LDH activity of heart homogenates within the twenty-four hour period following radiation exposure indicates an initial leakage of enzyme material from the target organ, which inversely parallels the observed transient serum elevations. Such a relationship has been previously observed in sedentary animals and lends further support to the suggestion that radiation induces changes in membrane permeability, resulting in an efflux of biological material out of the injured cell. Although samples taken twelve hours and three days following exposure do not demonstrate any marked reduction in LDH enzyme activity, all remaining sampling times exhibit significant loss of the
enzyme. The lack of normalization after twelve days following exposure suggests that the permeability of the damaged membrane with respect to LDH has not been restored. An alternate, but less likely, suggestion is that radiation exposure may have reduced or altered the normal metabolic requirements for the enzyme, resulting in a decreased cellular level. Similar losses of biologically active material from irradiated systems have been independently observed (Okada and Peachey 1957; Kawasaki and Sakurai 1969). In contrast to the LDH effects, homogenate levels of CK and GOT in irradiated, exercised rats did not exhibit significant decreases in activity within the first twenty-four hours. These observations contrast with the results obtained with sedentary rats (Chapter III), in which demonstrable losses in homogenate activities occurred. The data suggest that exposure of exercised rats to 2,000 rads of gamma radiation does not cause significant alterations in the permeability of these enzymes in the target organ. It may be that exercise stress protects against the loss of biological material from the damaged cell. This proposal is, however, in disagreement with the observed leakage of homogenate LDH and, as well, the noted enhancement in serum enzyme levels of CK and GOT. A second and more plausible suggestion is that exercise, as a physiological stress, may induce specific metabolic adjustments such as increased synthesis of proteins. Recent evidence of stimulated synthesis of nuclear base proteins following exposure of isolated rabbit lymphocytes to 1,000 R of X-radiation has been reported (Ghosh 1971).

The observed temporary loss of mitochondrial GOT from samples isolated
twenty-four hours after *in vivo* exposure suggests that radiation may induce transient modifications in the membrane permeability of heart mitochondria in exercise-stressed rats, resulting in a leakage of the enzyme out of the subcellular organelle and into the cytoplasm. Evidence of secondary permeability changes could not be found.

Sham-irradiated exercised rats, sacrificed after thirty days of exhaustive training, do not exhibit significant differences in serum levels of the three enzymes with respect to the sedentary sham-irradiated animals. The results indicate that the animals were well adapted, physiologically, to the training procedure and suggest that there were no false serum enzyme elevations in the irradiated animals due to exercise. Similar results indicating the protective effect of exercise training against a rise in serum enzymes have been reported (Garbus et al. 1964). Although exercise-induced modifications in heart homogenate levels of CK and GOT were negligible, the significantly increased activity of tissue LDH in the trained animals indicates a metabolic adaptation to the imposed physiological stress.

In comparing the radiation response of exercised and sedentary rats, significant increases in the percentage elevation of serum LDH in exercised animals were noted within the first twenty-four hours following exposure. The data suggests that exercise, as an added post-irradiation stress, further modifies the altered permeability of the damaged target cells, resulting in an enhanced efflux in response to the initial injury. Such differences may, however, be partially attributable to an increase in the cell-serum enzyme gradient caused by the noted enhancement of the cellular
enzyme level as a result of the training procedure. In contrast, the data for serum CK and GOT reveal that no significant enhancement in post-exposure serum elevation occurred during the entire sampling period, suggesting that the metabolic stress of exhaustive exercise in irradiated animals did not compound the initial radiological injury noted in sedentary rats with respect to those enzymes. In addition, it is interesting to note that unlike LDH, homogenate levels of CK and GOT did not increase significantly as a result of the training procedure, and would not, therefore, contribute to any significant increase in the enzyme concentration gradient. Hence, it appears that although there is evidence of enhanced enzyme efflux due to post-irradiation exercise stress, this response is not a general characteristic of all enzymes studied and may, instead, reflect increased cell-serum enzyme gradients of specific components as a result of the exercise training itself.

The significant difference noted at the twelve hour sampling interval between homogenate LDH levels of exercised and sedentary subjects indicates an enhanced recovery process to the initial enzyme loss may occur in the exercise-trained animals. This proposal is, however, very tenuous, based on the available data, and is meant primarily as a suggestion for further investigation. Unlike homogenate LDH, the cellular loss of CK generally remains significantly retarded in exercised subjects with respect to the sedentary irradiated rats. The data indicate that post-exposure exercise of trained animals markedly inhibits the loss of CK from the heart observed in sedentary groups. This occurrence does not
necessitate the preservation of membrane integrity, which would be contrary to the observed serum elevations, but indicates, instead, that exercise may stimulate protein synthesis as a possible result of altered metabolic priorities due to the stress condition. Recent evidence of GOT homogenate levels in exercised subjects also displays a similar retardation of initial enzyme loss from the damaged target organ. Although a significant decrease in the enzyme activity of exercised animals occurred on day six, this transient decrease could not be corroborated in later samples.

Mitochondrial levels of GOT, although showing a temporary reduction in exercised animals twenty-four hours after exposure, do not differ significantly between exercised and sedentary animals. It can be concluded that exercise, as a metabolic stress in animals receiving 2,000 rads of gamma radiation to the thoracic region, does not cause any remarkable alterations in the permeability of the mitochondrial membrane with respect to the enzyme system in study.
CHAPTER V
RADIATION-INDUCED ENZYME EFFLUX FROM RAT HEART:
III. EFFECT OF DIETARY SUPPLEMENTATION WITH
VITAMIN E ON LIPID PEROXIDE FORMATION

INTRODUCTION

In studies with tissue homogenates and isolated systems it has been observed that exposure to ionizing radiation can induce the formation of peroxides, known to cause extensive destruction of lipids (Hauggaard 1968). However, the ability of living organisms to destroy peroxides has made the search for radiation-catalyzed lipid oxidation difficult. Evidence of in vitro lipid peroxide formation as a result of radiation exposure has been documented (Wills and Wilkinson 1967; Wills 1970). In addition, in vivo studies by Bacq and co-workers (1951) detected peroxides in extracts of mouse and rat depot fat following irradiation. However, the extremely small values obtained did not furnish conclusive evidence that radiation exposure in living systems initiates in vivo peroxidation of lipids.

Recent evidence by Wills (1970) suggests that vitamin E may act as an effective inhibitor against peroxidation in irradiated rat-liver microsomes. It is expected that vitamin E may act as a chain-breaking lipid antioxidant, inhibiting free-radical peroxidation via hydrogen abstraction or through the formation of a lipid-tocopherol complex (Tappel 1972). Although many in vitro studies support the antioxidant hypothesis, the lack of conclusive evidence of either peroxide formation in vivo, or presence of peroxides in tissues (Schwarz 1972) has severely
hampered the study of the antioxidant activity of vitamin E and its relationship to free-radical induced peroxide formation in the living organism.

The present investigation attempted to furnish evidence of in vivo peroxide formation in irradiated rat heart and relate such damage to the established leakage of biologically active material out of the damaged target organ. In addition, the study attempted to relate the degree of post-irradiation cardiac damage to the dietary level of α-tocopherol administered prior to the irradiation procedure.

MATERIAL AND METHODS

Male Sprague-Dawley rats of an average weight of 300 gm were selected at random and divided into two groups. The first group (normal diet) was fed standard Purina lab chow, known to contain 66 mg of α-tocopherol (1 IU = 1 mg) per kgm of diet. The second group (fortified diet) was fed powdered lab chow fortified with 13.2 gm of D-L α-tocopherol acetate (250 IU = 1 gm) per kg of diet. The vitamin was purchased from Nutritional Biochemicals Corporation, Ohio. This gave a tocopherol concentration factor of approximately 50 times the endogenous level, known to cause significant variations in the rate of in vitro lipid peroxide formation in liver homogenates of irradiated mice (Dawes and Wills 1972). Both groups were fed and watered ad libitum for two weeks.

1D. Shelton, Ralston-Purina Co., St. Louis; through personal communication.
prior to irradiation.

Irradiation Procedure

All animals were prepared for irradiation as described previously (Chapter III). The rats were irradiated in groups of four using an Eldorado 8 gamma unit (Atomic Energy of Canada). The dose rate was calculated to be 266 rads/minute at the surface and exposure time was corrected for an absorbance of 15.7% to give an estimated dosage of 2,000 rads to the heart. Sham-irradiated controls for each group were treated in an identical manner to the experimental animals, excluding actual exposure. At pre-determined intervals of six, twelve and twenty-four hours following exposure, rats were selected and placed under light ether anaesthesia for sacrifice.

Heart Muscle Preparation for Thiobarbiturate Analysis

Immediately after extracting the blood, hearts were excised, trimmed of fatty and connective tissue and rinsed in ice-cold 0.15 M KCl. After quickly blotting dry, the tissue was plunged into liquid nitrogen until frozen, weighed, wrapped in tin foil and stored on dry ice. Samples were analyzed within three to five days.

Thiobarbiturate Analysis for Lipid Peroxides

Heart tissue was thawed at room temperature, cut into a fine mince and placed into 2.0 ml of 0.15 KCl per gm of tissue. KCl replaced the usual sucrose medium, since it has been determined that carbohydrates interfere with this analysis (Wills 1967). The tissue was homogenized
in a manual ground-glass blender and to the resulting homogenate was added 1.0 ml of 10% trichloroacetic acid per ml of suspension. After agitating, the mixture was centrifuged for five minutes and the resultant supernatant was carefully extracted and filtered through Whatman GF/A glass paper to remove residual material. To 4.0 ml of the filtrate was added 1.0 ml of 1% thiobarbiturate and the solution was heated at 100°C for ten minutes (Tappel and Zalkin 1959). The resultant colour was measured at 530 nm on a Beckman DB-GT spectrophotometer against a solution blank containing 1.0 ml of water per 4 ml of supernatant. Peroxide values (TBA values) are expressed as mmoles of malonaldehyde per litre of supernatant, using the molar extinction coefficient of 1.56 x 10⁵ M⁻¹ cm⁻¹ determined by Sinnhuber et al. (1958).

RESULTS

Serum Enzyme Analysis

Serum LDH levels of rats fed the normal diet did not reveal a significant elevation until twelve hours after exposure (Table 1B). The twelve hour interval, however, exhibited a mean activity increase of 73% (p < .05). Serum LDH levels returned to normal after twenty-four hours (Figure 1B). Although the animals maintained on the tocopherol-fortified diet exhibited the same general post-irradiation pattern (Figure 2B), at no time was the mean serum elevation statistically distinct from the sham-irradiated controls of that group (Table 2B). Furthermore, the mean percentage elevations (Table 3B) comparing the irradiation response
TABLE 1B

POST-IRRADIATION SERUM LACTATE DEHYDROGENASE LEVELS IN RATS MAINTAINED ON STANDARD PURINA LAB CHOW. SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY t-TEST, USING A POOLD ESTIMATE OF VARIANCE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity mU/ml Serum</th>
<th>Significant Difference From Control @ p &lt; .05</th>
<th>% Increase From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>$1.35 \times 10^3$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Hour</td>
<td>5</td>
<td>$1.47 \times 10^3$</td>
<td>-</td>
<td>9.0</td>
</tr>
<tr>
<td>12 Hour</td>
<td>4</td>
<td>$2.34 \times 10^3$</td>
<td>+</td>
<td>73.0</td>
</tr>
<tr>
<td>24 Hour</td>
<td>4</td>
<td>$1.13 \times 10^3$</td>
<td>-</td>
<td>(-) 16.3</td>
</tr>
</tbody>
</table>

POOLED VARIANCE: 300022

POOLED STANDARD DEVIATION: 548
Figure 1B. Post-irradiation serum lactate dehydrogenase levels in rats maintained on standard Purina lab chow.
ACTIVITY IN (mU/ml serum) x 10^3

HOURS

0 1.0 2.0 3.0

6 12 24

CONTROL HOUR INTERVALS
TABLE 2B

POST-IRRADIATION SERUM LACTATE DEHYDROGENASE LEVELS IN RATS MAINTAINED ON VITAMIN E-FORTIFIED LAB CHOW FOR TWO WEEKS PRIOR TO IRRADIATION. SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY t-TEST, USING A POOLED ESTIMATE OF VARIANCE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Activity mU/ml Serum</th>
<th>Significant Difference From Control @ p &lt; .05</th>
<th>% Increase From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>611</td>
<td>-</td>
<td>21.0</td>
</tr>
<tr>
<td>6 Hour</td>
<td>5</td>
<td>736</td>
<td>-</td>
<td>41.0</td>
</tr>
<tr>
<td>12 Hour</td>
<td>4</td>
<td>860</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>24 Hour</td>
<td>4</td>
<td>528</td>
<td>-</td>
<td>(-) 13.6</td>
</tr>
</tbody>
</table>

POOLED VARIANCE: 94564

POOLED STANDARD DEVIATION: 308
Figure 2B. Post-irradiation serum lactate dehydrogenase levels in rats maintained on vitamin E-fortified lab chow for two weeks prior to irradiation.
ACTIVITY IN mU/ml serum

HOURS

0 250 500 750 1000

HOUR INTERVALS

CONTROL

24
TABLE 3B

COMPARISON OF ALTERATIONS IN SERUM LACTATE DEHYDROGENASE LEVELS BETWEEN RATS FED STANDARD LAB CHOW AND TOCOPHEROL-FORTIFIED DIETS.

<table>
<thead>
<tr>
<th>Post-Irradiation Time</th>
<th>% Activity Normal Diet</th>
<th>% Activity Tocopherol-Fortified Diet</th>
<th>Significant Difference @ ( p &lt; .05 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Hours</td>
<td>109 (5)</td>
<td>121 (5)</td>
<td>-</td>
</tr>
<tr>
<td>12 Hours</td>
<td>173 (4)</td>
<td>141 (4)</td>
<td>-</td>
</tr>
<tr>
<td>24 Hours</td>
<td>83.7 (4)</td>
<td>86.4 (4)</td>
<td>-</td>
</tr>
</tbody>
</table>

POOLED VARIANCE: 2098

POOLED STANDARD DEVIATION: 45.8

Figures in parenthesis represent the number of rats sampled.

Standard deviations, representing confidence limits of the percentage elevations, were calculated by dividing the pooled standard deviations of the serum activities by the control activity and multiplying this factor by 100. The resultant variances \( (S^2) \) were subjected to an \( f \) ratio and were found to be homogeneous. The variances of the normal diet and vitamin E-fortified diet were, therefore, pooled to obtain a single variance factor.
between the two groups were not significantly different \((p > .05)\) and indicate that the dietary tocopherol fortification did not significantly reduce the post-irradiation leakage of enzyme into the serum.

A comparison of serum LDH levels between sham-irradiated rats fed normal and fortified diets (Table 4B) did, however, reveal a significant reduction in the mean enzyme level of the tocopherol-fortified group \((2p < .05)\). The mean LDH serum activity of the control diet group was, in fact, 121% above the mean level found in the tocopherol-fortified animals.

**Lipid Peroxide Determination**

Malonaldehyde, a major degradation product of lipid peroxides, allows a sensitive estimation of the extent of peroxidation in living tissues by the formation of a coloured complex with thiobarbiturate. Although the validity of this determination has been questioned (Philpot 1963), Wills and Rotblat (1964) and Wills (1966) found that the thiobarbiturate (TBA) method correlated well with other contemporary estimations of peroxide determination.

The mean peroxide values (TBA values, expressed as malonaldehyde concentration) in the heart homogenate preparations of irradiated, normal diet rats did not differ significantly from the TBA values of the sham-irradiated controls (Table 5B). Although a 17.6% increase did occur after twenty-four hours (Figure 3B), this elevation was not statistically significant. Similarly, irradiated rats maintained on the tocopherol-fortified diet (Table 6B) did not exhibit any evidence of increased tissue levels of
TABLE 4B
COMPARISON OF LACTATE DEHYDROGENASE SERUM ACTIVITY BETWEEN SHAM-IRRADIATED CONTROL RATS FED STANDARD LAB CHOW AND TOCOPHEROL-FORTIFIED DIETS.

<table>
<thead>
<tr>
<th>Serum Activity Normal Diet (mU/ml Serum)</th>
<th>Serum Activity Tocopherol-Fortified Diet (mU/ml Serum)</th>
<th>Pooled Significant Difference</th>
<th>@ 2p &lt; .05</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.5 x 10^2 (6)</td>
<td>6.11 x 10^2 (6)</td>
<td>±445</td>
<td>+</td>
</tr>
</tbody>
</table>

Figures in parenthesis represent the number of rats sampled.

Variances previously calculated for each system were subjected to a test of homogeneity using the f ratio. The ratio indicated that the variances were homogeneous and were, therefore, pooled together to obtain a single variance factor.
TABLE 5B

CONCENTRATIONS OF TBA REACTANTS, EXPRESSED AS NMOLAR VALUES, IN THE FINAL SUPERNATANT OF HEART HOMOGENATES FROM RATS FED STANDARD PURINA LAB CHOW BEFORE IRRADIATION. SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY t-TEST, USING A POOLED ESTIMATE OF VARIANCE

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Concentration of Malonaldehyde (nmoles/l)</th>
<th>Significant Difference From Control @ p &lt; .05</th>
<th>% Increase From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>551</td>
<td>-</td>
<td>3.99</td>
</tr>
<tr>
<td>6 Hour</td>
<td>4</td>
<td>573</td>
<td>-</td>
<td>0.54</td>
</tr>
<tr>
<td>12 Hour</td>
<td>6</td>
<td>554</td>
<td>-</td>
<td>17.6</td>
</tr>
<tr>
<td>24 Hour</td>
<td>6</td>
<td>648</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

POOLED VARIANCE: 6525

POOLED STANDARD DEVIATION: 80.8
Figure 3B. Concentration of TBA reactants, expressed as nmolar malonaldehyde, in the final supernatant of heart homogenates from rats fed standard lab chow and tocopherol-fortified diets before irradiation.
CONTROL
DIII1':SHAM - IRRADIATED
AM
TOCOPHEROL FORTIFIED DIET: SHAM - IRRADIATED
CONTROL
DIE:T:
IRRADIATED
TOCOPHEROL FORTIFIED DIET: IRRADIATED

CONCENTRATION OF TBA REACTANTS
(nmolar malonaldehyde)

600
800
1000
1200
1400
1600
2000

0 6 12 18 24 HOURS
TABLE 6B

CONCENTRATION OF TBA REACTANTS, EXPRESSED AS NMOLAR VALUES, IN THE FINAL SUPERNATANT OF HEART HOMOGENATES FROM RATS FED TOCOPHEROL-FORTIFIED DIET. SIGNIFICANT DIFFERENCES BETWEEN CONTROL AND TEST MEANS WERE DETERMINED BY t-TEST, USING A POOLED ESTIMATE OF VARIANCE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Samples</th>
<th>Mean Concentration of Malonaldehyde (nmole/1)</th>
<th>Significant Difference From Control @ p &lt; .05</th>
<th>% Increase From Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>381</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Hour</td>
<td>5</td>
<td>382</td>
<td>-</td>
<td>0.26</td>
</tr>
<tr>
<td>12 Hour</td>
<td>6</td>
<td>322</td>
<td>-</td>
<td>(-) 15.5</td>
</tr>
<tr>
<td>24 Hour</td>
<td>5</td>
<td>328</td>
<td>-</td>
<td>(-) 13.9</td>
</tr>
</tbody>
</table>

POOLED VARIANCE: 7181

POOLED STANDARD DEVIATION: 84.7
**TABLE 7B**

COMPARISON OF MALONALDEHYDE CONCENTRATIONS IN THE FINAL SUPERNATANT OF HEART HOMOGENATES FROM SHAM-IRRADIATED CONTROL RATS FED NORMAL DIET AND VITAMIN E-FORTIFIED DIET.

<table>
<thead>
<tr>
<th>Mean Concentration Normal Diet (nmoles/l)</th>
<th>Mean Concentration Vitamin E-Fortified Diet (nmoles/l)</th>
<th>Pooled Difference</th>
<th>Significant Difference @ p &lt; .05</th>
</tr>
</thead>
<tbody>
<tr>
<td>551 (8)</td>
<td>381 (6)</td>
<td>±82.4</td>
<td>+</td>
</tr>
</tbody>
</table>

Figures in parenthesis represent the number of rats samples.

Variances determined were found to be homogeneous and were, therefore, pooled to obtain a single variance factor.
malonaldehyde (Figure 3B).

Comparison of heart homogenate TBA values between sham-irradiated rats fed normal and fortified diets (Table 7B) revealed a notable reduction of 31% in the malonaldehyde tissue level of those animals maintained on the vitamin E-enriched diet ($p < .05$) and indicates that dietary supplementation of vitamin E may serve to decrease \textit{in vivo} peroxide formation.

**DISCUSSION**

The initial radiation response of the rats maintained on the standard lab chow diet is noticeably different from previous results obtained in both exercised and sedentary animals. In contrast to the maximal six hour responses noted previously, the six hour response does not exhibit any significant enzyme elevation. In addition, the maximal response at twelve hours is noticeably decreased with respect to the maximal elevations previously documented. Such differences in serum LDH elevations may lie in the lower radiological dose rate used in the present work. However, a more likely reason may be the age differences between the animals used in the studies. In order to obtain sufficient heart tissue for the TBA analysis, rats employed in the present work weighed an average of 300 gms and were necessarily older than the 150 - 200 gm rats used previously. Such a finding indicates the chronological age of the animal may significantly affect the radiation response observed.

Although irradiated animals previously maintained on the tocopherol-
enriched diet did not exhibit any significant serum enzyme elevations (as was observed in the normal diet animals), the comparison of percentage elevations between groups did not reveal any significant protective action of vitamin E on the post-exposure response. Such evidence indicates that excessive quantities of vitamin E does not serve as a radioprotective agent in vivo. However, it may be that differences would have been noted between vitamin E deficient rats and rats maintained on a normal diet. Furthermore, it must be noted that the elevation of serum LDH as a radio-biological marker may not, as a result of large variance and relatively low response, serve as a sensitive enough indicator of damage.

The significant decrease in serum LDH levels in the sham-irradiated rats maintained on vitamin E indicates that the dietary administration of tocopherol decreases the normal serum level of the enzyme. Although the observed decrease may result from stimulated inactivation of the serum protein, a more plausible explanation involves a decreased rate of enzyme efflux from the tissues as a result of tocopherol-induced stabilization of membrane permeability. This supports the proposal of Lucy and Dingle (1964), Lucy (1972) and Diplock and Lucy (1973) that vitamin E acts as a stereochemical stabilizer of cellular membranes, without which membranes may demonstrate abnormally high permeability. In addition, the elevation of serum and plasma enzymes in clinically-induced tocopherol depletion has proved to be a useful means of observing the development of vitamin E deficiency (Oksanen 1970; Tollerud 1970; Hyldgaard-Jensen 1973). Studies by Boyd (1968), using lambs with experimental nutritional myopathy, showed
that administration of α-tocopherol completely stopped the abnormal leakage of enzymes observed under such conditions and provide further supporting evidence to the proposal of membrane stabilization.

The failure to secure evidence of radiation-induced lipid peroxide formation does not rule out the possibility that ionizing radiation may initiate autocatalytic peroxide formation in vivo. It has been reported that the amounts of peroxides formed in vivo as a result of radiation injury are very small (Bacq et al. 1951), which suggests that the accumulation of peroxide products within twenty-four hours of exposure may have been below the limits of experimental sensitivity. In addition, the in vivo condition allows for the ongoing metabolism and destruction of peroxides and by-products, so that malonaldehyde analysis of such systems may not reflect the true accumulation of peroxides in the damaged tissues. Dawes and Wills (1972) have reported marked increases in the rate of lipid peroxidation in incubated liver, kidney and heart homogenates of rats exposed to 500 - 2,000 rads of whole-body irradiation. In comparison to the results presently reported, it becomes evident that the in vivo analysis as compared to in vitro incubation of homogenates may yield vastly different results. Such is supported in a recent study on irradiated rats by Ichii and co-workers (1968). It was reported that, in all tissues analyzed, the amount of TBA chromagen formed in vivo was negligible at all periods tested unless homogenates were incubated in vitro. It is, therefore, difficult to support the assumption that analysis of TBA products in vivo is a realistic representation of the true situation of peroxide
formation. Due to the fact that radiation-induced peroxide formation could not be detected in either dietary group of rats, the protective function of vitamin E as a lipid antioxidant against radiation injury could not be evaluated. However, in a similar study using an identical tocopherol concentration factor between control and fortified diets as used in this study (Dawes and Wills 1972), it was reported that the tissue level of vitamin E clearly plays an important role in determining the rate of in vitro peroxidation of irradiated rat tissues. Such conflicting evidence, with respect to peroxidation, stresses the importance in selection of the method of analysis. It therefore appears essential to determine whether the in vivo tissue analysis or the in vitro incubation method is most representative of the true estimation of peroxide formation in the intact organism.

The relatively high levels of TBA reactants, expressed as nmole of malonaldehyde, do not represent the absolute levels of lipid peroxide products (Zalkin and Tappel 1960). Although the TBA reaction primarily measures malonaldehyde formed in peroxidation, animal tissues are also known to contain other TBA reactants (Sinnhuber et al. 1958) including aromatic aldehydes and carbohydrate condensation products which give rise to a significant background error. However, the 31% relative decrease in the concentration of TBA reactants in the heart homogenates of tocopherol-fortified, sham-irradiated control animals does indicate a notable decrease in the tissue level of peroxide products. It is apparent that dietary supplementation with vitamin E effectively decreases the
heart-tissue level of these accumulated TBA reactants. Although this could be mediated via stimulated metabolic degradation of the products, a more plausible explanation would be that supplementation of normal dietary levels of α-tocopherol effectively reduces the rate of ongoing lipid peroxide formation, leading to observable reductions in the tissue levels of these TBA chromatic reactants. Similar comparisons in the tissue levels of TBA reactants in normal and tocopherol-deficient rats have been previously reported (Zalkin and Tappel 1960; Kitabchi and Williams 1968).
CHAPTER VI
GENERAL CONCLUSIONS

IRRADIATION EFFECTS IN SEDENTARY ANIMALS

Irradiation of cardiac muscle induces a transient efflux of biologically active material out of the target organ which correlates with an increase of this material in the serum. It is, therefore, proposed that enhanced serum enzyme levels following radiation exposure result from leakage of the material across the damaged membrane and are not a consequence of increased levels within the heart muscle. Subsequent stabilization of both serum and homogenate enzyme levels indicate a possible reinstatement of cellular integrity following the observed initial alterations. Secondary elevations of serum enzyme levels are not accompanied by cytoplasmic losses and implicate some biochemical lesion or metabolic alteration within the target organ.

Although the possibility of cellular death and disruption cannot be discounted, the doses normally required to produce such effects via cytoplasmic injury are much higher than dosages used at the therapeutic level (Altman et al. 1970; Fajardo 1973). In addition, the similarity of serum and homogenate patterns for LDH and CK, in contrast to GOT, suggests that leakage from the myocardium is not due to massive cell disruption, since such a catastrophe would cause simultaneous loss of all biologically active material from the ruptured cell and thereby result in essentially identical leakage patterns for the enzymes studied. It appears, instead, that the efflux of these enzymes from the damaged cells may be regulated
by certain "limiting" factors such as concentration gradients, molecular size and geometry or electrostatic charge. Whereas LDH and CK appear to be controlled by similar factors, GOT leakage may be governed by some other limiting determinants influencing its membrane permeability as is suggested by the work of Henley et al. (1959).

IRRADIATION EFFECTS IN EXERCISED ANIMALS

Significant serum elevations of LDH, CK and GOT following thoracic irradiation of exercise-trained rats add further support to the contention that radiation injury causes alterations in cell membrane permeability, leading to a leakage of material out of the target organ. Similar patterns revealing rapid, transient elevation of serum enzymes have been previously observed (Takamori et al. 1969; Hori et al. 1970). Subsequent elevating patterns in later sampling intervals indicate the possible development of secondary radiation injury.

In contrast to the initial decrease in heart homogenate levels of CK and GOT observed in Chapter III, post-irradiation loss of the enzymes in hearts excised from exercised animals was significantly reduced. Although it may be suggested that exercise stress in post-irradiated animals protects against loss of material from the target organ, the observed loss of homogenate LDH and increased serum enzyme levels do not implicate a preservation of membrane integrity. A more plausible explanation may be that exercise induces specific metabolic adjustments which counteract the efflux of material from the damaged organ.
Enzyme levels of GOT in isolated rat heart mitochondria suggest that post-irradiation exercise did not cause significant alterations in membrane integrity with respect to irradiated sedentary animals.

**IRRADIATION EFFECTS IN VITAMIN E-FORTIFIED ANIMALS**

Comparison of percentage serum LDH elevation between rats fed standard Purina lab chow and tocopherol-fortified diets did not reveal any significant radioprotective action of the vitamin. Although such evidence suggests that vitamin E does not serve as a useful radioprotective agent, it is felt that the elevation of serum LDH may not be a sensitive enough radiobiological marker.

Significant reduction of serum LDH levels in sham-irradiated rats maintained on vitamin E indicates that the dietary administration of tocopherol decreases the serum enzyme level found under normal dietary conditions. It is known that extracellular enzymes exist in a "steady-state" situation, their concentration in the blood dependent upon the rate of efflux from tissue cells and their rate of inactivation. Significant reductions in normal serum levels must, therefore, occur through the alteration of either the leakage rate or the rate of inactivation. Although dietary supplementation with α-tocopherol may indirectly alter the rate of enzyme inactivation, a more plausible explanation involves a decrease in the rate of cellular efflux as a result of induced stabilization of cellular membrane permeabilities.

Due to the fact that autocatalytic peroxide formation *in vivo* was
not detected, the radioprotective function of vitamin E as a lipid anti-
oxidant could not be evaluated. However, the failure to secure evidence
of peroxide formation does not rule out the possibility that ionizing
radiation may initiate peroxidation of membrane lipids. It is quite
conceivable that, because of minute quantities or ongoing metabolic
elimination, the amount formed was below the threshold of experimental
sensitivity. It was, however, shown that dietary supplementation with
vitamin E effectively reduces the normal heart-tissue level of peroxi-
dation products. It is proposed that dietary supplementation with large
amounts of α-tocopherol reduces the rate of ongoing peroxide formation
in the intact animal, leading to an observable reduction in the tissue
level of these compounds.
BIBLIOGRAPHY


SINNHUBER, R.O., T.C. YU and TO CHANG YU. Characterization of the red pigment formed in the 2-thiobarbituric acid determination of oxidative rancidity, Food Res. 23: 626 - 634, 1958.


### APPENDIXES

<table>
<thead>
<tr>
<th>APPENDIX</th>
<th>PAGE</th>
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<tbody>
<tr>
<td>1</td>
<td>145</td>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>153</td>
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<tr>
<td>4</td>
<td>155</td>
</tr>
<tr>
<td>5</td>
<td>157</td>
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</table>

1. Computer program for enzyme reaction order analysis and calculation of reaction rates
2. Computer programs for converting enzyme reaction rates for serum, homogenate and mitochondrial assays. Rates are converted from micromolar values into milli-unit values
3. Computer program for calculating various statistical parameters, including mean, variance and standard deviation
4. Computer program for calculation of a pooled estimate of variance
5. Computer program for t-test, using a pooled estimate of variance
Appendix 1. Computer program for enzyme reaction order analysis and calculation of reaction rates.
### C-34 FOCAL 91969

#### 11.01 C INITIALISE

**10.10 D 311E**

**10.12 C 11.60**

#### 11.01 C CONTROL SEQUENCE

**11.03 S \#1 =**

**11.04 A \"!!!\, EXPT, \#0 \?\" LAB.
\FACEX "B," TODAY'S DATE "D,\"**

**11.05 A \"TYPE INVESTIGATIONS INITIALS "D,\"**

**11.06 A \"EFFECT OF \"D,\" ON \"\, ABSOLUTELY \"A,\"!!!\"**

**11.10 D 1211 (D) 11.411 (A) D 11.30, 11.35, 11.20**

**11.20 D 13.30 D 13.55 WP = WP+1**


**11.40 C 11.10**

**11.42 S WP = WP-1 IF \#1 = 0 WP = WP 0 \#1 = A(N) S \#1 = A(N) S \#1 = 0**

**11.44 D 12.75D 14.D 15.5 WP = WP 11C 11.34**

**11.50 S B = 12.32X11 \#01-FIEN(D-FLOC(10)) D 11.52IT " OK \"YR**

**11.52 T \"ICH BLEIB ETW DEXOFF \ICH DRUCK LV 19" FLOC(10), \" NEXT TIME BUY\"**

**11.60 A \"IF \#1 DAILY PLAY SAFE \"PRESS 1\"!!!**

**11.62 A \"AV ORDER CALC GAITED ON \#00 0 Or 1 \"R\"\"**

**11.64 A \"FUNCTION \#1 \#2 0 Or 1 \"F\" \"\"**

**11.66 A \"FAEATUER \#1 \#2 0 Or 1 \"K\" \"K\"**

**11.68 A \"DATA A.R. TRANSMIT, \#1, 0 Or 1 \"D,\"\"**

**11.70 A \"TRANSMIT AT \#1, 0 Or 1 \"D,\"\"**

**11.72 A \"WEIGHTS PROJ. TO Y MAGNITUDE ON INITIAL \#0 Or 1 \"Y,\"\"**

**11.94 A \"LIVE CONSTRAINTS 1U NO ORIGIN \#1 FREE 0 Or 1 \"L,\"\"**

**11.96 A \"PREDICTED ORDER OF W\#1 1, Or 2 \"O,\"\"**

**11.98 A \"GRAPH GAITED \#1 \#2 0 Or 1 \"CH,\" 11.03**

#### 12.01 C INPUT OUTPUT

**12.02 I (DIG, 11.60) 12.66, 12.10**

**12.10 D 11.53A \"!!1\" \#1 = 0 \"B,\" (D) 12.59; \#1 = A \"\" VALUE OF FACTOR VARIED**

**12.12 T \#4 0 \"EXPT NO \"T,\" \" \"CALC \#4 0 \"XV,\" ENTER DATA\"**

**12.44 A \"FINAL VAL. OF Y \"Y,\" INITIAL VAL. OF Y \"Y,\" \#1 = \#1 S WP = 1**

**12.16 T \"Y \"Y VALUE \#1 \"Y \"Y VALUE\" = 73,0**

**12.18 T \#36P, \#4 2.5A \"Y = Y(Y+2.5) 12.32D Y(Y+2.5) \#1 = \#1 Y(Y+2.5)**

**12.50 S WP = WP+11C 12.18**

**12.92 A \"I\" \#1 \"\" \"CONCENTR \#1 \"\" \"\"**

**12.24 S \#1 = \#1 12.32S \#1 = \#1 \#1 \#1 S WP = WP-1 \"\" YINIT \"YR**

**12.34 S \# = FLOC(FY) 2.32X11 \#**

**12.50 I \#0 = 12.11.59**

**12.52 A \"\"\" \"\" \"PRESS RETURN BEFORE & AFTER PUNCHOUT\" D0IF \#1 = 1,157 T "0"**

**12.54 T \"Y \"YF, \"Y,\" \#1 = \#1 WP = WP-1 \"\" \"\"**

**12.56 T \"J \"J = 1,157 T "J"**

**12.58 A D0IF**
15.31 C GRAPHIC SUBROUTINE
15.35 I (C) 15.10 R
15.10 S Yv=Z(1); Yv=Z(5); F q=1; Yv=S Xd=X(V); S Yd=Y(V); S X(V)=k(V); I 152
15.11 G 15.20
15.12 S Y(V) =2(V); B Y(V) =X(V); Z(V) =YD; I (YV-Y(V)) 15.14 I (YV-Y(V)) 15.16 R
15.14 I (YV-Y(V)) 15.20
15.16 S Yv=Y(V); R
15.20 S Xv=X(V); S Xv=X(V)
15.22 S Xd=(Xv-XV)/33; S Yd=(Yv-YV)/33; I T!!
15.30 F N=1, BL T "--"
15.34 T #; S CO=1; F CV=1, VP ID 15.40
15.36 D 15.30; F N=1, VP; S Y(V) =Z(V); S X(V) =k(V)
15.38 T !!!! R
15.40 I (CO*XD-(X(CV)-XV))15.59; D 15.69; T "E", Y(CV), #; D 15.62; T "C", #; R
15.50 T "!!", !; S CO=CO+1; G 15.40
15.60 F N=YV, YD, Y(CV); T ""
15.62 T X(CV); #; F N=YV, YD, B*X(CV)+A#; T ""

31.01 S Z=FCO*(1139, 3664)
31.02 S Z=FCO*(1139, 2409)
31.03 S Z=FCO*(3074, 2369)
31.04 S Z=FCO*(3074, 3969)
*


Appendix 2. Computer programs for converting enzyme reaction rates for serum, homogenate and mitochondrial assays. Rates are converted from micromolar values into milli-unit values.
v SERUM

[1] 'THIS PROGRAM GIVES YOU ACTIVITY IN MILLI-UNITS PER ML. SERUM'

[2] ''

[3] 'PLEASE ENTER THE RATE IN MICROMOLS/MIN. LEAVE AT LEAST ONE'

[4] 'SPACE BETWEEN EACH SAMPLE VALUE'

[5] R=[]

[6] 'PLEASE ENTER THE TOTAL ASSAY VOLUME, AGAIN LEAVING A SPACE'

[7] A=[]

[8] 'NOW ENTER THE SERUM VOLUMES'

[9] H=[]

[10] 'THE ACTIVITY IN MILLI-UNITS/ML. SERUM IS'


[12] ACT;'MILLI-UNITS/ML.'
HOMOGEN

[1] 'THIS PROGRAM GIVES YOU ACTIVITY IN MILLI-UNITS/GRAM MUSCLE'
[2] ''
[3] 'TYPE IN RATE IN MICROMOLAR/MIN.'
[4] RT<-[]
[5] 'TYPE IN TOTAL ASSAY VOLUME'
[6] TA<-[]
[7] 'TYPE IN TOTAL HOMOGENATE VOLUME'
[8] HV<-[]
[9] 'TYPE IN PERCENT DILUTION'
[10] DIL<-[]
[11] 'ACTIVITY IN MILLI-UNITS PER GRAM MUSCLE'
[12] ACT+RT×TA×100%/HV×DIL
[13] ACT; 'MILLI-UNITS/GRAM MUSCLE'
\"
MITOCHON

[1] 'THIS PROGRAM GIVES YOU ACTIVITY IN MILLI-UNITS/GM PROTEIN'
[2] ''
[3] 'TYPE IN THE RATE'
[4] RATE<-()
[5] 'TYPE IN THE ASSAY VOLUME'
[6] ASV<-()
[7] 'NOW TYPE IN THE MITOCHONDRIAL VOLUME'
[8] MV<-()
[9] 'ENTER THE DILUTION FACTOR(PERCENT)'
[10] DIL<-()
[12] WP<-()
[13] ACTIV<-RATE×ASV×100÷(MV×DIL×WP)
[14] ACTIV ; 'MILLI-UNITS/MG. PROTEIN'

\v
Appendix 3. Computer program for calculating various statistical parameters, including mean, variance and standard deviation.
DSTAT X;MEAN;VAR;V;W;N;M
[1] 'SAMPLE SIZE
   ;N+D X+X[N\Delta X]
[2] 'MAXIMUM
   ;X[N]
[3] 'MINIMUM
   ;X[1]
[4] 'RANGE
   ;X[N]-X[1]
[5] 'MEAN
   ;MEAN+(/X)/N
[6] 'VARIANCE
   ;VAR+((X-MEAN)^2)/N-1
[7] 'STANDARD DEVIATION
   ;VAR*0.5
[8] 'MEAN DEVIATION
   ;((X-MEAN)/N)
[9] 'MEDIAN
   ;0.5X[N/2]+X[1+1/N]
[10] ((N>0)/0=pM)\&0<0&M+X[(W=(W\&V-1\&W)/V+(X\&1\&X)/N))/12
[12] 'MODE
   ;M
Appendix 4. Computer program for calculation of a pooled estimate of variance.
1] TYPE IN THE NUMBERS OF SAMPLES IN EACH GROUP N=A,B,C,...

2] TYPE IN THE STANDARD DEVIATION IN EACH GROUP S+X,Y,Z,...

\[ 3 \] VAR+S*2
\[ 4 \] N1+N-1
\[ 5 \] VN1+VAR*N1
\[ 6 \] SVN1+/-VN1
\[ 7 \] DEN+(+/-)*N
\[ 8 \] S2P+SVN1/DEN
\[ 9 \] S2P
\[ 10 \] S2P*.5

\[ \]
Appendix 5. Computer program for t-test, using a pooled estimate of variance.
\[ V \textsc{tpool} \]

[1] 'type mean x'

[1.5] \( Mx \leftarrow \)

[2] 'type mean y'

[2.5] \( My \leftarrow \)

[3] 'type pooled variance'

[3.5] \( P\text{var} \leftarrow \)

[4] 'type number samples in x'

[4.5] \( Nx \leftarrow \)

[5] 'type number samples in y'

[5.5] \( Ny \leftarrow \)

[6] \( \text{num} \leftarrow Mx - My \)

[7] \( \text{den} \leftarrow (\text{Pvar} \times ((\div Nx) + (\div Ny))) \times 0.5 \)

[8] \( T \leftarrow \text{num} \div \text{den} \)

[9] \( T \)

\[ V \]