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Genesis of Epinephrine Arteriopathy

John F. Zaroslinski
Loyola University Chicago

GENESIS OF EPINEPHRINE ARTERIOPATHY

By

JOHN F. ZAROSLINSKI

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DEDICATION

This thesis is dedicated to the many faculty members of the Stritch School of Medicine without whose encouragement, understanding and stimulus this thesis would not be possible. Foremost among these is Y. Thomas Oester, Ph. D., M.D., who is a gentleman and a scholar in the truest sense. His patience, understanding and breadth of comprehension has been a model to be emulated by his many students.

Biography

John F. Zaroslinski was born in Chicago, Illinois on the 12th of September 1925. He attended and was graduated from Wells High School in June of 1943. Subsequently he enrolled at the University of Chicago. In the fall of 1943 he enlisted in the U.S. Army and served as a combat infantryman in Europe and in the Pacific. Upon honorable discharge from the army he resumed studies at the University of Chicago, majoring in physiology and was graduated with a Ph. B. degree in 1949.

His graduate studies at this institution were interrupted in 1951 when he was employed as a chemist by the Armour Pharmaceutical Company. In 1953 he was employed as a pharmacologist and later a senior pharmacologist by Baxter Laboratories in Morton Grove Illinois. While at Baxter he was engaged in studies of the mode of action of Piromen, a bacterial polysaccharide. In early 1958 he joined Armar-Stone Laboratories as Scientific Director, where he was in charge of research and scientific activities.

In June 1961, he began his graduate studies in pharmacology in the department of pharmacology of the Stritch School of Medicine. He has been a graduate assistant in this department since July of 1962.

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Chapter 1

INTRODUCTION

Progress in the study of arterial disease has undergone considerable evolution in the last fifty years. Advances in depth of comprehension of biological processes among all of the basic medical sciences has preceded and conditioned progress in the field of arteriosclerosis. Rapid strides in anatomy, biochemistry, physiology and pharmacology have not only given us better understanding of the function of normal living cells, but have also provided both routes and vehicles by which pathological processes might be better investigated. As a consequence of this progression it has become increasingly evident that arteriosclerosis is most likely the resultant of a multiplicity of factors. These many interrelationships require in our opinion a multidisciplinary approach which is problem rather than procedure oriented.

Development of this dissertation was conditioned by progress made by other investigators as well as by unanswered questions the resolution of which it was felt is vital to the appreciation of the etiology of arteriosclerosis. Epinephrine induced arteriopathy has gained greater stature as more recent studies have demonstrated the interrelationship of the medial and the adventitial layers of the arterial wall. Two divergent hypotheses have been advanced to explain the etiology of epinephrine arteriopathy; one suggests this condition is a consequence of hemodynamic effects, while a second hypothesis favors a metabolic etiology. The object of this dissertation was to determine whether the various drug interactions which intensify or conversely ameliorate epinephrine arteriopathy are better explained on a biochemical or a vasopressor basis or by an hypothesis combining both of

these factors.

Study of arteriosclerosis has evolved from early descriptive phases defining the morphology and epidemiology of the lesion to include the most sophisticated descriptive and quantitative methodology available. Early investigations emphasized the occurrence of atheromatous or "mush like" lesions in the arterial wall which presumably were responsible for the mortality associated with this condition. Observation of lipid in the human atheroma was followed by Anitschkow's discovery (1913) that feeding a diet high in cholesterol to the rabbit produced lesions which were similar in some respects to those described in humans.

Investigations which followed suggested that generation of the atheroma represented a distinct and separate form of arterial pathology limited in extent to the intimal or innermost layer of the arterial wall. Thus, the term atherosclerosis was coined to more clearly define and differentiate the atheromatous lesion from other forms of arterial pathology. Central to this line of inquiry was the tacit assumption that atherosclerosis was primarily a consequence of deranged lipid metabolism. The simplicity of this concept has had considerable appeal.

Discreditation of the unitarian theory of atherogenesis resulted from more precise quantitation and study of epidemiological data which has disclosed that deranged lipid metabolism is not a sine qua non of atherosclerosis. Ancel Keys (1963), perhaps the leading proponent of the hyperlipemic theory of atherogenesis, has recently stated "In the modern era of increasing sophistication, single cause theories of the etiology of atherosclerosis have given way to the general concept of a multiplicity of influences that promote the disease. The total atherogenic force is viewed

as the sum or resultant of a number of environmental factors operating on the basic genetic endowment".

Similarly, Stamler (1962), long a respected worker in this field, has stated "Atherosclerosis is a disease of multifactorial causation. A large number of factors, exogenous and endogenous, interact with diet to influence the development of the (atherosclerotic) lesion". In supporting this statement Stamler states that the Framingham study in which a large series of patients were studied in regard to a number of factors such as blood lipid hypertension, excessive smoking, obesity etc., seems to indicate that when two or more of the "defects" were found in any one individual the likelihood of atherosclerotic coronary disease occurring was considerably enhanced.

Dole et al. (1963) has recently attacked the lipid theory stating "clinicians have overemphasized the importance of plasma lipids. Changes in the composition of blood plasma are easy to detect but are unreliable as measures of vascular disease or as guides to treatment. Arteriosclerosis occurs without hyperlipemia and hyperlipemia without vascular lesions".

Changes in concept of the etiology of atherosclerosis have been accompanied by considerable modification in definition of the nature and extent of the lesion.

Early nomenclature characterized atherosclerosis as a degenerative condition associated with advanced age in which small pustule-like outpocketings containing stainable lipid formed on the intimal or innermost layer of arteries. In contrast, the term medial sclerosis was applied to changes located primarily in the media or middle layer of the arterial wall, characterized by degeneration of the muscularis, fatty degeneration, necrosis and finally calcium deposition.

More recently, the World Health Organization (1958) has defined atherosclerosis as "a variable combination of changes in the intima of arteries.....consisting of focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, and associated with medial changes".

Although many investigators have characterized arteriosclerosis as a disease of advancing age, more recently McGill et al. (1963), Taylor (1962) and others have indicated that this condition has its onset in childhood. This school of investigators has reported that the earliest lesions are believed to be fatty streaks occurring in the intima of children and adolescents. Thus according to McGill (1963), lipid deposition begins in the aorta in the first year of life, in the coronaries in the second decade and in the cerebral vessels in the third decade. These fatty streaks are seen grossly, in fresh or formalin fixed arterial tissue, as yellow opaque flecks or streaks on the intimal surface. Some of the fatty deposits become covered with a cap of fibrous connective tissue, forming what is referred to as the fibrous plaque. Other fatty streaks are believed to regress without formation of the fibrous plaque. According to McGill et al. (1963) it is the fibrous plaque which sets the stage for the clinically manifested form of arteriosclerosis, as a result of hemorrhage within the plaque and thrombosis over the plaque. Either of these complications may reduce the size of the lumen, resulting in angina, myocardial infarction, cerebral infarction or peripheral gangrene. According to Taylor (1962), the arteries of adults usually contain both fatty and fibrous plaques while in the older population fibrous lesions predominate. Progression of the lesion is associated with widening and thickening of the plaque and may be associated with necrosis and degeneration of the central

region of the lesion. Further progression of the lesion includes the accumulation of many small crystals of cholesterol as well as an extension of the lesion into the media. With increasing concentration of lipids within the foam cells many of these cells disintegrate and an acellular mass of lipid and necrotic debris forms. This latter accumulation of cholesterol and other lipids provides the semi-soft or mushy inclusion which characterizes the atheroma. Subsequent changes may include ulcerations of the surface of the atheroma possibly acting as focal point for thrombus formation, or hemorrhage may occur within the plaque. Calcium salts frequently deposit within the atherosclerotic plaque, forming flakes or plates that may be as large as 1 to 2 cm. in diameter. In some cases the arterial wall may become so severely calcified that the vessel will shatter when sectioning is attempted. Medial involvement is, according to Taylor, a common consequence of the progressive development of intimal atherosclerotic lesions and is frequently found in atherosclerotic aneurysms.

Hass (1962) has stated "it is likely that if calcific medial degeneration could be prevented, other factors which enhance progression of most undesirable atheroarteriosclerotic changes could be tolerated well by most people. If calcific medial degeneration could be prevented , the chain of reactions arising therefrom could best be minimized or interrupted by measures for keeping the blood cholesterol level below 180 milligrams per cent, for preventing unduly increased blood pressure and when indicated for reducing the clotting tendency of the blood". In further exposition of these points Hass states that, since lipids invariably accumulate in the intima of many parts of the arterial system in older segments of the population, the idea has arisen that some metabolic abnormality enhances the tendency for

these substances to accumulate through either a mural mechanism, a change in the composition of plasma lipids or an increase in their level. Hass (1962) further states that "there is little doubt that the level and composition of the blood lipids especially cholesterol and its esters are factors in the development of the disease, especially when the total blood cholesterol level is long sustained at high levels. But there is no proof that cholesterol or lipid levels, customary in man are in themselves responsible for the disease". The reasons which Hass (1962), gives to substantiate his position are as follows: 1) there is no consistent correspondence between the rate of progression or severity of the disease and the level of blood cholesterol or lipids. 2) The disease occurs eventually in almost everyone irrespective of the magnitude or the duration of these levels. 3) Patients with high cholesterol levels of long duration may have less disease than those with lower levels of similar duration.

Hass (1962) has attempted to resolve the discrepancies between theoretical considerations and empirical observations by formulating a concept which embodies the susceptibility of the arterial wall to various types of damage. For example, all vessels do not undergo medial degeneration and the degree of calcification accompanying this damage is not consistent, while in other cases coexistence of these two phenomena are closely related. Thus he feels that it is necessary to characterize the susceptibility of each part of the arterial system to deleterious factors which may be multiple and not necessarily identical in all systems or in all people. Similarly, since the deposition of lipid varies in both humans and animals as well as within each species Hass feels there is reason for assuming that the "lipoidosis potential" or susceptibility to lipid deposition of vessels from different

individuals may vary. Another facet of Hass' theory encompasses the "mesenchymal reaction potential". He indicates that in the course of medial degeneration the deteriorative changes may activate mesenchyme in the media, adjacent intima, and adventitia. The subsequent increase in collagen and activation of immature vascular mesenchyme assists in repair of the media. Similarly, activation of mesenchyme in the intima may occur resulting in phagocytosis of the lipid and formation of foam cells.

Arteriosclerosis in Animals

The very widespread occurrence of arteriosclerosis in its various forms in man obviously dictates the need for model systems in laboratory animals which simulate the various aspects of the pathologic processes which occur in humans. Research along these lines has taken two principal directions a) Study of naturally occurring lesions in various species of animals and b) Induction of arteriosclerosis in various species by chemical, physical, hormonal and dietary processes.

Study of naturally occurring lesions has been quite extensively pursued. Species varying from the aardvark to the wallaby have assiduously been dissected and degree of arteriosclerotic involvement has been correlated with what is known of the living habits of the particular group. From these studies recently reviewed by Lindsay and Chaikoff (1963) certain generalizations have come forth. It appears that most species of mammals studied exhibit naturally occurring arteriosclerotic lesions. This group includes: Marsupials such as kangaroos, bandicoots, dasyures, wallabies; Primates such as lemurs, macaques, baboons, chimpanzees, gorillas; Rodents such as rats, mice, squirrels, guinea pigs; Carnivores such as dogs, wolves, foxes, coyotes; Felidae such as domestic cats; Lagomorpha such as rabbits, hares,

etc. Among birds naturally occurring arteriosclerosis has been found in chickens, pigeons, ducks, geese, swans, etc.

In most species of mammals and birds arteriosclerosis seems to be initiated by degeneration of certain components of the vascular wall. If lipids appear during the course of the development of the arteriosclerotic lesions, this appearance seems to be a secondary phenomenon (Lindsay & Chaikoff, 1963). The site of lesions varies with species studied. For example in rats and cows the lesions involve primarily the intima. In the dog and cat both intimal and medial involvement may occur together. According to Fox (1933), naturally occurring lesions in the rabbit are most prominent in the thoracic aorta where they appear as elevated intimal granulations or large flat plaques. Lindsay and Chaikoff (1963), are of the opinion that the naturally occurring lesions in the rabbit are similar to lesions in the human. Duff and Ritchie (1957) have described three types of lesions in aortas of rabbits. One consisted of focal accumulation of polymorphonuclear leucocytes in the intima and media. A second type involved medial degeneration, a process related to aging, which in advanced cases had converted the aorta to a wide calcific tube with irregular fusiform dilatations. The intima was thickened by fibroblastic proliferation and the elastic laminae and muscularis zone of the media were destroyed or disorganized by varying numbers of mononuclear cells that rarely contained Sudan IV staining droplets. The third was an uncommon, naturally occurring lesion consisting of clumps of mononuclear cells immediately under the endothelium. According to Lindsay and Chaikoff (1963), in most species of animals, demonstrable lipid material cannot be found in the early degenerative lesions and in early arteriosclerotic plaques. Moreover in most species in which lipid material is found in fibrous intimal

plaques, its presence is restricted mainly to larger and presumably older mature plaques, usually in the deepest segments adjacent to or in the media. These authors state "It must be emphasized that unlike the arteriosclerotic lesion in man, most later arteriosclerotic plaques in animals do not usually contain large amounts of lipid". They also state that in a number of species of animals medial disease overshadows intimal fibrosis and thickening. Extensive calcification of degenerating elastic tissue and the appearance of increased amounts of mucoid ground substance suggested to Lindsay and Chaikoff (1963), that the medial lesion may be basically similar to that of the intima. Bragdon (1952, 1954) has reported that fatty streaks found in arterial tissue of young children and adolescents are also present in the young rabbit, suggesting another similarity between the human and rabbit forms of the arteriosclerotic process.

Experimentally induced lesions in animals have been induced by dietary as well as chemical, physical and hormonal means. Anitschkow (1913) produced "atherosclerosis" in the rabbit by feeding a diet supplemented by addition of cholesterol. Among the advantages of this procedure is the fact that lesions are produced in a relatively short time and the ease of handling the rabbit in the laboratory. Hartroft and Thomas (1963) indicate that the pathology produced in the rabbit by this means differs from the lesions produced in man in that the foam cell is the most prominent component of the lesion, the ascending aorta is more severely involved than the abdominal, and the lesions almost never proceed to ulceration, calcification and thrombosis. Another disadvantage of the procedure according to these authors is the observation that the rabbit is by nature a strict vegetarian with a serum cholesterol level much lower than that of man, while the levels of cholesterol

associated with lesions in the rabbit are far in excess of those usually seen in man. Hass et al. (1960)have reported induction of arteriosclerosis in rabbits by feeding them excessive amounts or irradiated ergosterol. Lesions induced were characterized by bone resorption and by abnormal deposition of calcium salts in various tissues. Calcium appeared first in the inner media of the aortic arch. With time the calcific deposits spread in depth and proceeded along the abdominal aorta and its branches. The principal changes which these investigators reported, appeared in the internal elastic membrane and the media, while the fibroblastic intimal proliferation reactions and vascularized stromal resorption of the media were the principal manifestations of repair.

Tsaltas (1962) has reported induction of arteriosclerosis in the rabbit by injection of papain. These lesions were reported to include the development of raised white circumscribed plaques in the arch and descending portions of the aorta and its major branches. The lesions according to Tsaltas usually involved the subintimal portions of the media, but some extended through the entire wall. Elastica was fragmented and the lesions contained an abundant deposition of PAS (Periodic Acid Schiff) positive material and calcium. Connective tissue proliferation and cartilagenous and osseous metaplasia were also observed.

Katz and Pick (1961) have produced atherosclerosis in the chicken by addition of cholesterol to their regular diet. These lesions reportedly on rare occasion may ulcerate or calcify, but according to Hartroft and Thomas (1963), differ from human lesions in that calcification and ulceration occurs far less often than in human lesions.

The rat has also been employed in the study of arteriosclerotic lesions. Wissler et al. (1954) and Fillios et al. (1956) have reported aortic lesions following addition of cholesterol, bile salts and thiouracil to a semi-synthetic diet. Wexler et al. (1960) produced aortic lesions in the rat by repeated administration of large amounts of ACTH. This group has also reported occurrence of aortic lesions in females which had been repeatedly bred. Cholesterol supplementation and thiouracil added to the normal diet has been reported by Steiner and Kendall (1946) to produce atherosclerosis in dogs. Cholesterol feeding in pigeons (Lofland 1961) also results in atheroma formation.

Epinephrine Induced Medial Arteriopathy One of the key findings in this area was the report by Mikulcich and Oester (1950) that medial arterial sclerosis, originally reported by Josué (1903) to follow injection of large intravenous doses of epinephrine, could be greatly augmented in both severity and incidence by concomitant subcutaneous administration of thyroxine. Subsequent studies from this same laboratory [Oester (1959); Friedman, Oester and Davis (1955); Oester, Davis and Friedman (1955); and Roszkowski and Oester (1956)] have described several drugs which appear to be of protective value in the epinephrine-thyroxine regimen.

Friedman, Oester and Davis (1955) reported that although both epinephrine and norepinephrine were capable of producing medial sclerosis when administered alone or in combination with thyroxine the extent of damage produced by epinephrine and epinephrine-thyroxine regimen produced not only a greater incidence but also a greater severity of the lesions. Oester, Davis and Friedman (1955) reported that when intravenous administration of a cholesterol suspension was added to the epinephrine-thyroxine regimen,

intimal sclerosis associated with proliferation and foam cell formation resulted; medial damage on the other hand was similar to that observed when animals were subjected to only an epinephrine-thyroxine regimen. Davis, Oester and Friedman (1955) reported that ATP (subcutaneous) was able to decrease the severity and incidence of epinephrine-thyroxine induced medial damage. More recently O'Sullivan (1962) has reported that phentolamine (Regitine) administered prophylactically on a subacute basis was able to reduce both the severity and incidence of medial damage; phenoxybenzamine (Dibenzyline) under similar conditions appeared relatively ineffective. Iproniazid (Marsalid) at low doses, was found to significantly decrease the incidence and severity of medial damage produced by injections of epinephrine not accompanied by thyroxine administration. Higher doses of iproniazid on the other hand appeared to intensify incidence and severity of these lesions.

Conclusions drawn from O'Sullivan's study were that 1) the protection afforded by iproniazid was a consequence of its hypotensive action, 2) the "well defined and clear cut" protective action of phentolamine was a consequence of its hypotensive and adrenergic blocking action, and 3) the rather poor protection afforded by phenoxybenzamine (Dibenzyline) was ascribed to the lack of marked hypotensive action apart from the inhibition of the epinephrine induced pressor effect. O'Sullivan has ascribed the effect of thyroxine in increasing severity of incidence and severity of epinephrine induced medial damage to an increase in resting blood pressure as well as to a potentiation of the pressor response following intravenous epinephrine. Similarly, O'Sullivan (1962) has ascribed the protective effect of ATP to the hypotensive action of this drug and to a reduction of cardiac rate following epinephrine. His statement "that the primary influence in the development

of epinephrine induced sclerosis of the rabbit aorta is the increase in intraluminal pressure and stroke volume consequent to the administration of epinephrine" supports the contention that the medial sclerotic effects of epinephrine and drugs either augmenting or protecting against medial sclerosis are a consequence of their cardiovascular and pressor effects.

Waters (1954) and Waters and de Suto-Nagy (1950) as well as Taylor (1954) have also supported the contention that one of the principal factors in production of atherosclerosis may be the injury of the arterial wall by hypertention or acute rises in arterial intraluminal pressure.

A relationship between medial arteriosclerosis and atherosclerosis has been suggested by Constantinides et al. (1958). They found in a chance observation that the aortas of some rabbits that developed accidental renal damage while being fed a high cholesterol diet exhibited patchy medial necrosis associated with an unusually severe degree of intimal atherosclerosis. Subsequently Constantinides et al. subjected rabbits to a thyroxine-epinephrine regimen similar to that used by Oester et al. (1955). Rabbits were given epinephrine intravenously at a dose of 50 /Kg. for a period of ten days. During the last five days of the treatment period thyroxine at a dose of 1 mg./kg. was administered subcutaneously. After a four day period these animals were given a 1% cholesterol, 5% cottonseed oil diet for three weeks and then sacrificed. On autopsy it was found that cholesterol fed controls which had not been subjected to the epinephrine-thyroxine regimen did not show any evidence of atherosclerosis, while the incidence of atherosclerosis was 89% in the epinephrine-thyroxine animals. Aortic cholesterol concentration in the epinephrine-thyroxine pretreated animals was six times higher than in cholesterol fed controls. Microscopic

examination of the aortae disclosed that for the most part atheromatous plaques were found overlying areas of medial necrosis. Although the period of cholesterol feeding was only three weeks, the thickness of many of the atheromas was of a magnitude observed only in animals subjected to at least an eight week cholesterol feeding. Constantinides et al. (1960) have concluded that prior medial injury greatly increases the atherogenic response of the aortic intima toward hypercholesterolemia and atheroma formation.

Constantinides and his associates emphasize that these findings are of great significance since they describe a mechanism whereby atherosclerosis can develop in the absence of conspicuous or prolonged blood lipid elevation. They also help explain the pathogenesis of some lipid-poor atheromatous plaques in humans which have been described as "underlaid by foci of medial elastic destruction."

These findings gain a particular importance in that numerous agents such as ACTH (adrenocorticotropic hormone), unsaturated fatty acid deficiency, infection, etc., have been shown to produce medial necrosis in mammalian arteries. Thus Constantinides and his associates view medial injury as a "final common pathway through which a multitude of stresses might sensitize the intima to blood lipids and thus promote atherogenesis even in the presence of only slight and transitory hypercholesterolemia."

Workers in the field have been aware of the possibility of medial damage contributing to atheroma formation. However, the absence of medial damage underlying intimal atheromas has in many instances tended to negate such a conclusion. Constantinides et al. (1960) feel that one explanation of these findings may be in a greater potential for medial repair to occur as compared with other aortic strata. Another significant factor according to

this group is that in some of their experiments they found that in later stages medial lesions can break down completely, fusing with the overlying intimal plaques. In this way subatheromatous calcium deposits may become "intra-atheromatous". Thus a lesion which began as an uncalcified atheroma resting on partly necrotic calcified media may end as a calcified atheroma resting on an apparently normal media. Perhaps of importance is the observation that some of the lesions produced by Constantinides, which were predominently fibrous and lipid-poor, are more similar to the human atheromatous lesions than the tightly packed plaques characteristically produced by high lipid diets in the absence of medial injury.

Submicroscopic Aspects The use of electronmicroscopy as well as density gradient centrifugation procedures has contributed considerably to our understanding of the relationship of cellular ultrastructure and function. Siekevitz (1958) has attempted to draw together some of the information which has been made available in recent years to formulate a "working concept" of cellular organization which although hypothetical in some respects provides a profile which assists in the unification of various isolated concepts of cellular ultrastructure. According to Siekevitz one of the structures whose functional relationship to the cell is not too well understood is the endoplasmic reticulum which is believed to consist of a system of lumina limited by a membrane approximately 75 Å thick. These lumina take the form of canals, vesicles and cisternae within the cell. These spaces appear to be interconnected and form a "continuum" which permeates the cytoplasm. of nearly all cells. The endoplasmic reticulum fragments on homogenization forming the so called microsomal fraction. According to Siekevitz a common finding in electron photomicrographs is a generous concentration of pinocytotic vesicles which appear in some instances to travel toward the endoplasmic reticulum

with which they coalesce on contact. Thus Siekevitz views the pinocytotic vesicle as a possible means by which extracellular matter may be transported to the endoplasmic reticulum.

The nucleus appears to be bounded by two concentric membranes. On close examination the inner one of the two membranes curves back to merge with the outer membrane at the edges of the pores which have been described in the nucleus. The outer membrane's origin according to Siekevitz is in the endoplasmic reticulum. In a further exposition of these concepts he postulates the occurrence of enzymes such as hexokinase along the wall of the endoplasmic reticulum.

Rhodin (1962) has indicated that rough-surfaced endoplasmic reticulum is extremely scarce in the intestinal smooth muscle but quite abundant in vascular muscle cells. He further states "the function of the rough surfaced endoplasmic reticulum and the ribosomes is related to protein synthesis within a cell. In cells with a high rate of protein synthesis these cell constituents are quite abundant."

Subsequent to the characterization of the mitochondria as the principal site of oxidative phosphorylation, considerable research effort has been expended to elucidate the morphology of this structure and to relate structure to function. It is generally accepted that mitochondrial particles are bounded by two membranes, the outer of which forms the outer shell, while the inner evaginates forming protrusions into the lumen called crista, (Lehninger 1961 a).

Lehninger (1961 b) has indicated that the enzymes of respiration and coupled phosphorylation are more or less firmly imbedded in or on the mitochondrial membranes, and that the cristae are the site of these enzymatic

activities. His group has fragmented mitochondria and found that sizing of the particles by differential centrifugation produces particles which appear to be relatively constant in regard to content per milligram of protein nitrogen, of cytochrome oxidase, beta hydroxy butyric dehydrogenase, succin-oxidase and ATP-ase regardless of particle size. These findings suggest to Lehninger that the membranes are made up of large numbers of recurring structural units each of which may contain a complete assembly of respiratory carriers present in a fixed ratio. He further indicates that an individual liver mitochondrion may contain between five and ten thousand of these respiratory assemblies.

Although a complete appreciation of the various functions of mitochondria has not been attained, Lehninger indicates that these particles possess three rather conspicuous properties namely 1) catalysis of respiration and energy coupling 2) occurrence of reversible swelling and contraction leading to water movements which are geared to respiration and 3) ion transport also geared to the respiratory chain.

Recent evidence accumulated in a number of laboratories suggests that the swelling and contraction of the mitochondria may be an expression of respiratory modulation of these particles. This swelling and contraction in turn may be a reflection of mechano-chemical changes of the respiratory and coupling enzymes, analogous to the mechano-chemical activities of the actomyosin complex.

Perhaps one of the most useful pharmacological tools available for the study of mitochondrial function is thyroxine. It has been well known for many years that thyroxine pretreatment of animals increased respiration of tissue slices from various organs. Hoch and Lipman (1954) reported that a

consistent decrease in the P/O ratio could be obtained in isolated liver mitochondria following thyroxine addition if the mitochondria were preincubated with thyroxine prior to addition of substrate. Aebi and Abelin (1953) reported that liver mitochondria from thyrotoxic rats exhibited an increased tendency to spontaneous swelling in vitro. Subsequently Klemperer (1955) found an increase in water content in thyroxine treated mitochondria. Tapley et al. (1955) reported that thyroxine added in vitro enhances the swelling of KCl suspended normal rat liver mitochondria.

From the preteeding discussion it is apparent that previously held opinions regarding the cause of arteriosclerosis are undergoing considerable revision. In the past, many authorities in this field have held that atherosclerosis is the consequence of a single particular predisposing factor. More recently Keys (1963) and Stamler (1962) have suggested that atherosclerosis is due to a multiplicity of causes. Atherosclerosis has been looked upon primarily as a lesion of the intimal layer of the arterial wall. As a consequence of the studies of Hass (1960, 1962), Constantinides (1958) and others it is becoming increasingly evident that lesions of the medial layer of arteries may play an important role in the formation of intimal lesions. Epinephrine induced arteriopathy, in the rabbit, appears to be an excellent model system in which lesions of the media may be studied (Oester 1959). Because of the possible relevance of epinephrine induced medial lesions to human atherosclerosis it is important that the mechanism or mechanisms by which these lesions are produced be more fully understood.

Chapter II

Statement of Problem

As a consequence of the increasing appreciation of the role of medial damage as a predisposing factor in arteriosclerosis, considerable speculation has centered around factors which may precipitate or cause this type of pathology. O'Sullivan (1962) and Lorenzen (1959, 1961 a, b) have interpreted medial arteriopathy induced by epinephrine in the rabbit as a resultant of the pressor effects of this drug. This position has been challenged by Milch and Loxterman (1964) who have suggested that "epinephrine induces a defect in the biochemical architecture of the arterial wall which renders it susceptible to degenerative change."

Examination of literature in this field suggested that a better appreciation of the importance of possible etiological factors might be gained in studies which simulated the experimental conditions under which medial arteriopathy is produced in the rabbit. In this regard it was felt that central to the study of possible etiological factors was a study of the pressor effects of epinephrine, norepinephrine, and sclerosis antagonists in both the normal and thyroxine pretreated rabbit where blood pressure was monitored in an unanesthetized animal. Consideration of the various drug interactions suggested that results of blood pressure determinations in the unanesthetized rabbit would give some indication as to whether results previously reported by other investigators might best be explained on the basis of pressor effects of epinephrine or whether the nature of drug interactions is such as to suggest that observed results might be explained on the basis of a biochemical theory. In the event that pressor data did not support either of these hypotheses a third possibility might be likely;

namely, that epinephrine arteriopathy is a consequence of a biochemical defect induced in the arterial wall which exerts a permissive action on the induction of lesions by purely hydrostatic forces.

Because of the paucity of literature data on subacute biochemical effects of epinephrine and thyroxine in the rabbit aorta it was felt that examination of some of the tissue constituents would provide information pertinent to the present study. Therefore, a subsequent phase of this investigation included study of subacute effects of epinephrine on various parameters such as aortic lactic acid, glycogen, glucose lactic dehydrogenase, in animals subjected to the two week thyroxine-epinephrine regimen described by Oester et al. (1954, 1955).

Limitations of these biochemical studies suggested that experiments in which the effects of both epinephrine and thyroxine on carbohydrate metabolism and protein synthesis in the isolated aorta might provide more meaningful information.

The very rapid growth of radioisotope methodology in recent years suggested that this discipline could be used to particular advantage to elucidate and characterize both normal metabolism in the rabbit aorta as well as to provide additional clarification regarding cellular effects of the sclerogenic regimen.

In summary this problem entailed an evaluation of pressor effects of epinephrine in conjunction with various sclerogenic regimens as well as with agents reported to protect against epinephrine induced sclerosis. A second phase of this problem included a survey of several possible biochemical effects which might contribute evidence supporting a biochemical basis of epinephrine induced medial arteriopathy.

Chapter III

Materials and Methods

Phase I

New Zealand white rabbits, obtained from a local source, weighing two to four kilograms were used throughout. Since previous studies by Oester et al. (1955) failed to establish a sex dependent relationship, animals of both sexes were used in accordance with the procedures already described (o.c.). In all acute experiments animals were weighed previous to any experimental procedure. In sub-acute experiments, during which animals were treated for periods of fourteen days, daily record of body weight was kept and dosage adjusted accordingly. Animals were housed in steel cages and fed a diet of Purina Rabbit Chow. The quarters were well ventilated and room temperature was kept at a relatively constant level throughout the experimental period.

In acute experiments designed to determine effects of epinephrine and its antagonists on blood pressure, the rabbits were weighed, anesthetized with chloroform, and immobilized on an animal restraining board. Subsequently an incision was made in the inguinal region and the femoral artery bared by careful blunt dissection. A polyethylene cannula (PE 90) filled with heparin dissolved in normal saline was inserted approximately six inches into the vessel so that the open lumen was located in the midthoracic region of the aorta. Pilot experiments disclosed that in the weight range used, this procedure invariably placed the open end of the cannula in the mid thoracic aorta. After insertion the external end of the cannula was plugged with a small piece of wire and then heat-sealed to provide double protection against leakage. The incision site was then closed with cotton sutures, the catheter

taped to the inner aspect of the thigh, and the animal was allowed to recover from anesthesia. Righting reflex usually reappeared within fifteen minutes.

In early experiments blockade of the cannula within 24 hours after initial cannulation caused loss of many preparations. Since no detectable differences in response to epinephrine were observed in animals prepared the previous day, as compared with animals used two hours after surgery, all subsequent blood pressure experiments were carried out in animals approximately two hours after cannulation.

Subsequent to recovery from anesthesia animals were placed on the animal restraining board and the cannula connected to a blunted 19 gauge needle attached to a Statham P23A pressure transducer. A Model 5 Grass Polygraph was employed to record blood pressure. Prior to use the Grass Polygraph was balanced and calibrated in accordance with manufacturers operation manual.

Intravenous administration of epinephrine and norepinephrine was accomplished by means of a constant infusion pump calibrated to deliver a constant volume of 0.8 cc per 3 minutes. Adjustment of total dose to 50γ/Kg. was made by varying the concentration of drug. Thus while the volume remained constant (0.8 ml.) in all cases the epinephrine or norepinephrine was administered at a rate of 50γ/Kg./3 minutes. All intravenous injections were made via the marginal ear vein. Both the route and period of administration conformed to that previously used by Oester (1955) and O'Sullivan (1962).

Preparation of Drugs Unless otherwise specified all drugs were administered in a vehicle of sterile-non-pyrogenic saline.

Epinephrine: was prepared from crystalline l-epinephrine base (Carnegies Limited, Lot #3387). Stock solution contained 1 mg./ml. of saline, solubilised by addition of one minim of concentrated HCl, reagent grade, per twenty mls. of solution. One milligram of sodium bisulfite per ml. was added as an anti-oxidant. The stock solution was stored in a freezer at 0°F and thawed prior to final 1:10 dilution. In several experiments USP reference Standard Epinephrine Bitartrate was adjusted to above concentration. Pressor responses were identical to those elicited by the epinephrine base obtained from Carnegies Limited.

Thyroxine: L-thyroxine (Synthroid, Baxter Laboratories, Lot #61254) was used throughout. Stock solutions were prepared in sterile saline. Solubilisation of thyroxine was accomplished by dropwise addition of 0.4N NaOH. The solution, 0.25 mgs./ml, was stored in a freezer at 0°F.

Phentolamine methanesulfate U.S.P. (Regitine® crystals, Ciba, Lot #A-0836) were dissolved (10 mg./ml.) in sterile saline immediately before use.

Phenoxybenzamine hydrochloride (Dibenzyline®, Smith, Kline and French, Lot #11-DZ) was dissolved at a concentration of 2 mgs./ml. in sterile saline prior to use.

Iproniazid (Marsilid®, crystals, Hoffman La Roche, Lot #32) was dissolved in sterile saline at a concentration of 5 mgs./ml. The pH was adjusted to approximately 5.0 with 4N NaOH just prior to use.

Adenosine Triphosphate Sodium Salt was obtained from Nutritional Biochemicals Corp. (Lot #6419). A concentration of 100 mgs./ml. (in sterile distilled water) was prepared immediately prior to use.

Norepinephrine Bitartrate (Winthrop Chemicals Corp. Lot #N2570B). The powder was dissolved at a concentration of 100 mcg. (base)/ml. in sterile

saline prior to use.

The injection protocol included control administration of epinephrine or norepinephrine during a three minute period followed by a fifteen minute period during which return toward normal was allowed to occur unless otherwise specified. When ATP, phenoxybenzamine or phentolamine were administered prophylactically, the subcutaneous administration of these drugs was performed 30 minutes before challenge with epinephrine. In the case of both phentolamine and phenoxybenzamine a fraction of the total dose was also administered intravenously (marginal ear vein) five minutes before the epinephrine challenge. Thyroxine pretreatment consisted of subcutaneous injection of 0.1 mg./Kg. of thyroxine daily in accordance with the procedure employed by O'Sullivan.

The following doses of drugs employed were used: Epinephrine, 50γ/kg. (as base); Norepinephrine, 50γ/kg. (as base); Thyroxine, 0.1 mg./kg.; ATP, 200 mgs./kg.; Phentolamine (Regitine), 5 mg./kg. s.c. and 5 mg./kg. i.v.; Phenoxybenzamine (Dibenzyline), 2 mg./kg. s.c. and 1 mg./kg. i.v.; Iproniazid, 30 mg./kg. s.c. 24 hrs. before experiment and then s.c. again 30 min. before epinephrine challenge, five minutes prior to epinephrine challenge an additional 15 mg./kg. was administered intravenously.

Phase II

Drugs employed were prepared and administered as described in Phase I. At least eight animals were employed per group unless otherwise stated. The following determinations on aortic tissue were performed;

1) Tissue glycogen using the method of Seifter et al. (1950).

2) Tissue lactic acid using the method of Barker and Summerson (1941).

3) Tissue inorganic phosphorus using the method of Fiske and Subbarow (1929).

4) Glucose using the glucose oxidase method (Glucostat reagent, Worthington Biochemicals Corp., Freehold, N.J.).

(See appendix for description of details of the procedures.)

Normal unanesthetized animals received 50γ/Kg. of epinephrine and were sacrificed by separation of the cervical vertebrae at two, three, four, five, six and eight hours following administration of the drug. The chest cavity was opened and the aorta rapidly removed. After washing and removal of the adventitia by stripping, two samples of aortic tissue were excised and weighed on a Mettler balance. One of the two samples was placed in 10% KOH and heated in boiling water for a period of 30 minutes in preparation for the glycogen analysis. The second piece was placed in cold TCA and homogenized. The latter tissue supernatant was employed for phosphate and lactic acid determinations.

Phase IIb

New Zealand rabbits as previously described in Phase I were given daily treatment for a two week period as follows:

Group 1. Normal controls (1 cc/kg. i.v. saline).

2. Thyroxine controls, 0.1 mg./kg. s.c.

3. Epinephrine 50γ/kg. of epinephrine (expressed as base) i.v. as in Phase I.

4. Epinephrine 50γ/kg. (ct. Phase I) and thyroxine 0.1 mg./kg. s.c.

On termination of the two week treatment period the animals were sacrificed as in Phase IIa and the aorta divided into three sections. The first two were processed as described in Phase IIa while the third section was homogenized in 5 cc ice cold distilled water and resulting supernatant

employed for glucose, lactic dehydrogenase and protein determinations. Lactic dehydrogenase was determined in accordance with the procedure of Sobel et al. (1957) described in detail in the appendix. Protein was determined in accordance with the procedure of Gornall (1948; see appendix). For purposes of comparison, samples of thigh muscle were also removed and treated as described above.

Phase III

Rabbits were decapitated and the chest cavity rapidly opened. The aorta was removed by rapid but careful dissection, care being taken not to exert any significant traction as the tissue was being removed. The aorta was then transferred to a petri dish containing either Krebs-Ringer phosphate or Krebs-Ringer bicarbonate buffer at room temperature. During the period of subsequent dissection performed so as to minimize trauma, the tissue was continuously oxygenated using either 100% oxygen or 95% oxygen - 5% CO_2 mixture. After the bulk of the adventitia and adherent fat was removed the aorta was sliced using a sharp scalpel into twelve sections. The first six successive sections of the first half were combined with corresponding successive sections of the second portion so that each sample contained one piece from both the high and low thoracic aorta. Pilot experiments indicated that reproducibility from experiment to experiment was greater if room temperature buffer was used in preference to ice-cold buffer which tended to produce erratic results. The paired sections were placed into a second dish which also was oxygenated. The samples were then removed to filter paper moistened with buffer and any visibly remaining bits of adventitia were removed. The tissue was then rapidly weighed on a torsion balance and returned to the second petri dish awaiting completion of final cleaning and

weighing of the entire series of tissues. Identity of tissues was maintained by using a compartmentalized tissue holder similar to those used in processing tissues for histological fixation.

Aortic tissue pairs were then transferred to disposable metabolism flasks (see appendix) and gassed for a period of 10 minutes with shaking st 37°C. A mixture of 95% O_2 - 5% CO_2 was used in the case of Krebs-Ringer bicarbonate buffer, while the Krebs-Ringer phosphate buffer was gassed with 100% oxygen.

Subsequently they were recapped with serum stoppers; small plastic beakers containing fluted filter paper wet with 4 N KOH were suspended from the stopper. In all experiments radioactive substrate was added before tissue addition to flask. In drug experiments, drugs were also added before tissue gassing. Concentrations used were as follows: adenosine triphosphate 2 mg/ml phentolamine 0.1 mg/ml and phenoxybenzamine 0.03 mg/ml. After gassing was completed epinephrine was added to appropriate samples. The flasks containing tissues were incubated for varying periods of time in a metabolic shaker at 80 strokes per minute using a 1½ inch stroke. Temperature was maintained at 37°C. At conclusion of incubation period, experiments in which collection of $C^{14}O_2$ was desired, incubation was terminated by the addition of 0.5 ml. of 4 N H_2SO_4. The flasks were then shaken for an additional 30 minute period, at low speed in an Eberbach shaker to effect complete trapping of CO_2. At the end of this period the exterior of the KOH beaker was washed with distilled water, (washings being returned to incubation flask) and the serum cap was then placed atop a counting vial containing 0.5 ml. of Hyamine 10 X (Packard Instrument Co.). Once the serum cap was firmly sealed in place 0.4 ml. of 4 N_2H SO_4 was added to the hanging plastic beaker to effect

release of the CO_2 trapped by the KOH. The counting vial was subsequently shaken for an additional period of twenty minutes to effect transfer of CO_2 to the Hyamine in the bottom of the vial. When the second shaking was completed the serum cap was removed and 10 ml. of Bray's solution (1960; see appendix) added. A plastic cap was then affixed and the sample counted in a Packard 314X liquid scintillation counter. The aortic sample remaining in the original incubation flask was transferred to a sample vial containing 10 ml. of a 2:1 chloroform-methanol mixture and shaken for a period of six hours to remove moisture and fat. Subsequently, the chloroform-methanol mixture was decanted and the procedure repeated with shaking for an additional 2 hours. The aortic samples were then allowed to "dry" at room temperature for an additional 2 hours and then weighed to determine dried defatted weight. It was originally hoped to determine protein via the method of Gornall; however, the addition of acid to release CO_2 caused sufficient denaturation to make use of this procedure for estimation of protein nitrogen not feasible. The medium containing flasks were capped and stored at 0°F. until subjected to further analytical process.

Procedures involved in study of incorporation of uniformly labelled lysine C^{14} into protein were carried out in a manner similar to that described above, except that no attempt was made to collect CO_2 and incubation was terminated by removal from medium and freezing of tissue rather than by addition of acid.

The tissue was prepared for counting by the procedure described by Farrese and Reddy (1963). In brief, the aortic sections were added to an ice cold homogenizer and homogenized in 5.0 mls of ice cold saline. During the entire period of homogenization the tissue grinder was kept in an ice water

bath. The tissue debris was then removed by centrifugation and the supernatant used for preparation of protein by adding 1 ml. of cold 20% TCA to a 1 ml. aliquot of the protein. The resultant precipitate was then washed 3 times with cold 5% TCA, extracted twice with 5% TCA for 15 minutes at 90°C to remove nucleic acids, and washed again with cold TCA. Subsequently the residue was washed twice with ethyl acetate to remove lipids. The latter procedure appeared more desirable than the usual chloroform extraction since it minimized flotation of protein particles. Finally, the protein residue was dried in a counting vial, 0.5 ml. of Hyamine 10X added, and the sample incubated for 18-20 hours at 55°C to effect digestion of the protein. Under these conditions digestion of protein appeared complete and no discernable yellowing of the Hyamine was evident. Upon completion of the digestion 10 mls. of Bray's solution was added to the vial and, the sample counted for a minimum of ten minutes in a Packard 314X liquid scintillation counter.

In all cases sample counts were adjusted for instrument efficiency based on counting of a sealed C^{14} reference standard. Background averaged approximately 80 CPM and sample counts in each experiment were corrected for background. Correction for quenching was performed in accordance with the channels ratio method described by Baillie (1963) and Bush (1963). Briefly, the method involved preparation of several triplicate samples containing varying concentrations of Hyamine 10X a strongly quenching substance. Counts recorded in the "A" channel were divided by counts recorded in the "B" channel; thds ratio was plotted against counting efficiency corrected for instrument efficiency. This linear plot was then used to correct samples for loss of counts due to quenching.

Thus all radioisotope data was converted from CPM (counts per minute) to DPM (disintegrations per minute).

Chapter IV

Results

Systolic Blood Pressure Effects of pretreatment by various drugs on the pressor response to epinephrine in the unanesthetized rabbit are illustrated in Figures 1 - 9. Specific data obtained in these experiments are listed in Tables 1 - 3. Figure 1 illustrates the pre-infusion control level of systolic pressure and peak systolic response following epinephrine. Peak systolic level attained following norepinephrine is denoted by horizontally hatched bars. The peak systolic level (Fig. 1) attained following epinephrine, was not significantly different from that following norepinephrine, ($p>0.05$). Similarly there was no significant difference between net increase in systolic pressure produced by these two drugs (Fig. 2). The percentage increase in systolic pressure (Fig. 3) following norepinephrine was smaller than that following epinephrine ($p = 0.05$). This difference was in part a reflection of the higher control systolic level of the norepinephrine group.

Pretreatment of rabbits for two weeks with thyroxine produced a rise in the pre-infusion level of systolic pressure (Fig. 1); however, both the net and percentage increase in systolic pressure following epinephrine were significantly lower in magnitude than in saline treated controls [p values 0.05 and 0.001 respectively (see Figs. 1 - 3 and Tables 1a and 1b)]. The peak systolic level in this group following epinephrine was not significantly different than that observed in saline controls ($p>0.05$).

Iproniazid, at a dose reported by O'Sullivan (1962) to augment epinephrine arteriopathy lowered the peak pressor level attained following epinephrine administration. The net systolic pressure increase in iproniazid pretreated animals was not significantly different from that observed in

saline pretreated controls (Fig. 2), while the percentage increase was lower (Fig. 3).

Administration of ATP caused a decrease in control systolic pressure to approximately 55% of that observed in saline pretreated controls (Fig. 1). The peak systolic level following epinephrine was significantly lower than that observed in controls; however, the net increase did not differ significantly from that in controls. The percentage increase increase in systolic pressure was significantly higher than that observed in controls and is in part a reflection of lower control levels of systolic pressure in the ATP pretreated animals. Administration of ATP to animals pretreated for two weeks with thyroxine (Table 1) did decrease the control systolic pressure; however, the peak systolic pressure level following epinephrine was almost identical to that observed in thyroxine pretreated controls. Both the net and percentage increase in systolic pressure following epinephrine were higher in animals which were pretreated with both ATP and thyroxine as compared with animals receiving thyroxine alone (Table 1).

According to O'Sullivan (1962) phentolamine (Regitine) is more potent in inhibiting epinephrine arteriopathy than phenoxybenzamine (Dibenzyline). In the present studies phentolamine pretreatment resulted in a lower preinfusion systolic level than did pretreatment with phenoxybenzamine (Fig. 1). Similarly the peak systolic level following epinephrine infusion was lower in the phentolamine pretreated animals than in the phenoxybenzamine pretreated animals [p 0.05 (Table 1a, b)]. The net and percentage increase in systolic pressure response to epinephrine, although higher in the phentolamine pretreated group was not statistically different from that obtained in phenoxybenzamine pretreated animals.

Diastolic Blood Pressure changes following pretreatment by various drugs and following infusions of epinephrine and norepinephrine are silustrated in Figs. 4 - 6. Data corresponding to these values is listed in Table 2a and b. Friedman et al. (1955a and b) reported that norepinephrine administered on a subacute basis produced an incidence and severity of medial arteriopathy significantly lower than that produced by epinephrine administered under identical conditions. In the present study the peak diastolic pressure level (Fig. 4) was not significantly different following infusion of these two drugs. Similarly both the net and percentage increase in diastolic pressure following infusion of these two drugs was quite similar with no statistically significant difference being observed (Figs. 5, 6 and Tables 2a, b). Subacute thyroxine pretreatment produced an increase in preinfusion level of diastolic pressure and also increased the rise in diastolic pressure following epinephrine infusion as compared with saline pretreated controls (Fig. 4).

Iproniazid at a dose reported by O'Sullivan (1962) to augment epinephrine arteriopathy lowered the preinfusion level of the diastolic pressure. However, neither the peak diastolic level attained after epinephrine infusion nor the net increase differed significantly with that observed in saline treated controls (Figs. 4, 5 and Tables 2a, b). The percentage increase in diastolic pressure following epinephrine was higher than that observed in saline controls (Fig. 6) and was significant at the 5% level (Tables 2a, b).

ATP pretreatment decreased the preinfusion diastolic pressure more than pretreatment with any of the drugs studied (Fig. 4). The net increase with epinephrine was almost identical to that observed in saline controls. The percentage increase in diastolic pressure following epinephrine exceeded

that produced by any of the drugs studied. This observation is in part a reflection of the rather low pre-infusion diastolic pressure level. Administration of ATP to animals pretreated for two weeks with thyroxine significantly decreased the pre-infusion diastolic level [p 0.001 (Tables 2a, b)] as compared with animals pretreated with thyroxine alone. The net and percentage increase in diastolic pressure following epinephrine infusion did not however significantly differ from that observed in the thyroxine controls (Figs. 5, 6 and Tables 2a, b).

Phentolamine decreased the pre-infusion diastolic pressure to a greater extent than did phenoxybenzamine (Fig. 4). However, following epinephrine infusion the peak diastolic response net increase as well as percentage increase in diastolic pressure did not differ significantly between the two groups (Tables 2a, b).

Mean Blood Pressure changes in general paralleled effects observed in analysis of systolic and diastolic pressure changes. As in the case of diastolic and systolic pressure the peak mean pressure and net increase in mean pressure was not significantly different following epinephrine or norepinephrine infusions (Fig. 7 and 8). As in the case of diastolic pressure the percentage increase in mean blood pressure did not differ significantly when these two drugs were compared (Fig. 9 and Tables 3a, b).

Thyroxine pretreatment increased both the pre-infusion mean blood pressure (Fig. 7) and the peak mean blood pressure following epinephrine infusion as compared with saline controls, while the net and percentage increase in mean blood pressure following epinephrine was not significantly different from that observed in saline controls (Figs. 8, 9 and Tables 3a, b). This parallels results obtained in analysis of diastolic pressure changes.

Iproniazid pretreatment employed a total dose of 75 mg./Kg. which approximates that reported by Spector et al. (1960) to produce almost total inhibition of monamine oxidase in the rabbit. This dose of iproniazid reduced pre-infusion mean blood pressure and decreased somewhat the peak mean pressure following epinephrine infusion. The latter difference was not statistically significant (Tables 3a, b). Both net and percentage increases in mean blood pressure following epinephrine were not significantly different from values obtained in saline controls.

ATP, as in the case of systolic and diastolic pressure, lowered mean arterial blood pressure. The rise following epinephrine infusion achieved a peak level lower than that in saline controls; however, the net increase in mean pressure was almost identical to that observed in controls, while the percentage increase was significantly higher (Figs. 8, 9 and Tables 3a, b).

Biochemical Studies In several pilot experiments the time course of effects of epinephrine on blood constituents such as glucose, lactic acid and inorganic phosphate were studied. In general, results were in accordance with data available on other species in that blood glucose and lactic acid rose in comparison with controls. These data suggested that changes in blood constituents plateaued approximately two to three hours following intravenous epinephrine administration. The rabbit aorta contributes only about 1 gram to the total body mass in a 2 kilo rabbit. Thus possible metabolic changes in aortic tissue would not be expected to be revealed by a study of blood constituents. Therefore tissue analysis of the aorta itself was undertaken. Pilot experiments were conducted in which aortic levels of glycogen, lactic acid and phosphate were determined following intravenous infusion of epinephrine. Slight decreases in aortic phosphate and lactic acid levels, reaching minima at 2 and 4

hours respectively were observed. These changes were not however, statistically significant. Epinephrine did cause a decrease in both aortic and skeletal muscle glycogen (Fig. 15 and Table 4). In the aorta, glycogen levels decreased approximately 40%, as compared with controls [p 0.05% (Table 4)]. In skeletal muscle obtained from the inner aspect of the thigh, glycogen fell approximately 66% as compared with controls (p 0.001%). This depletion was maximal at four hours after epinephrine administration.

Since thyroxine has a known glycogen depleting effect it was felt that determination of the effect of the sclerogenic regimen with appropriate controls might suggest further routes of exploration. Thus four groups of eight animals each were treated daily for fourteen days as follows:

Group 1. Saline controls

2. Thyroxine 0.1 mg./kg. s.c.

3. Epinephrine 50γ/kg. i.v.

4. Epinephrine 50γ/kg. i.v. + Thyroxine 0.1 mg./kg. s.c.

The results obtained are illustrated in Figs. 10 - 14.

Subacute administration of thyroxine or epinephrine alone did not significantly decrease aortic glycogen; however when administration of both drugs was combined a significantly lower level of aortic glycogen was observed (Fig. 10). Glycogen in skeletal muscle was somewhat higher in the thyroxine treated rabbits and lower in thyroxine + epinephrine treated animals as compared with controls. These differences were not statistically significant (Table 5).

Aortic glucose levels (Fig. 11) were elevated in rabbits which received either epinephrine or epinephrine plus thyroxine (p = 0.01%). Animals receiving thyroxine alone had aortic glucose levels not significantly

different from those found in saline treated controls. Although skeletal muscle glucose was elevated in all three experimental groups this increase was significant only in the group receiving epinephrine alone (Table 6).

Aortic lactic acid, in the thyroxine and epinephrine treated groups (Fig. 12), was similar in magnitude to that found in saline controls. The elevation in aortic lactic acid seen in the epinephrine plus thyroxine treated group was not statistically significant (Table 7). No significant changes in skeletal muscle lactic acid were observed in any of the experimental groups. Similarly no significant changes in lactic dehydrogenase levels were observed either in aorta or skeletal muscle (Table 8). Aortic phosphate was lower in thyroxine and thyroxine plus epinephrine groups, while in animals receiving epinephrine alone levels were almost identical to those observed in saline controls (Table 9).

Phase III

At this phase of the investigations it was felt that use of isotopically labelled substrates might provide evidence of a more direct nature as to the effects of both thyroxine and epinephrine on aortic tissue. A number of pilot experiments were conducted in an effort to achieve satisfactory consistency of results. Data obtained on $C^{14}O_2$ production and standard error were consistent with those reported by Mulcahy and Winegrad (1963). Kinetic studies were conducted on both normal and two week thyroxine pretreated animals. These data are illustrated in Figs. 16 and 17. In all radioisotope experiments counts per minute were corrected to disintegrations per minute (DPM) by use of the channels ratio method and extrapolated from a quench curve.

In aortic samples incubated for a period of two hours in Krebs-Ringer phosphate buffer (KRP) epinephrine appeared to increase $C^{14}O_2$ production by approximately thirty percent (Figs. 18, 21 and Table 10). When either ATP or phentolamine were added to the aortic samples containing epinephrine a decrease in $C^{14}O_2$ below control levels was noted. When phenoxybenzamine was added however, $C^{14}O_2$ production did not differ significantly from control values.

If on the other hand aortic samples were incubated in a Krebs-Ringer bicarbonate buffer containing 0.5μM/ml. of unlabelled glucose, a much more significant rise in $C^{14}O_2$ production was noted in the epinephrine treated aorta (Figs. 19, 22 and Table 11). Norepinephrine under equivalent conditions failed to produce a significant increase in $C^{14}O_2$ production (Table 13). ATP, although antagonizing the stimulant effect of epinephrine, did not appear to cause as great an inhibition as either phentolamine or phenoxybenzamine. When aortic samples were incubated with glucose 1-C^{14} some increase was observed over that with the label in the 6 position (Figs. 19 and 22). Epinephrine did not however appear to cause as great an effect on $C^{14}O_2$ production with the label in the #1 position as compared with C-6 labelled glucose.

When aortic samples from animals pretreated s.c. with 0.1 mg./kg. of thyroxine for two weeks, were incubated in KRB buffer as described above an almost two fold increase in $C^{14}O_2$ production was noted (Figs. 20, 23 and Table 12). Addition of epinephrine caused an additional two-fold increase in $C^{14}O_2$ production. This stimulation of $C^{14}O_2$ production was not blocked by ATP, but was inhibited by both phentolamine and phenoxybenzamine the former producing a greater inhibition. Incubation of aortic tissue from thyroxine pretreated animals with glucose labelled in the #1 position resulted in an

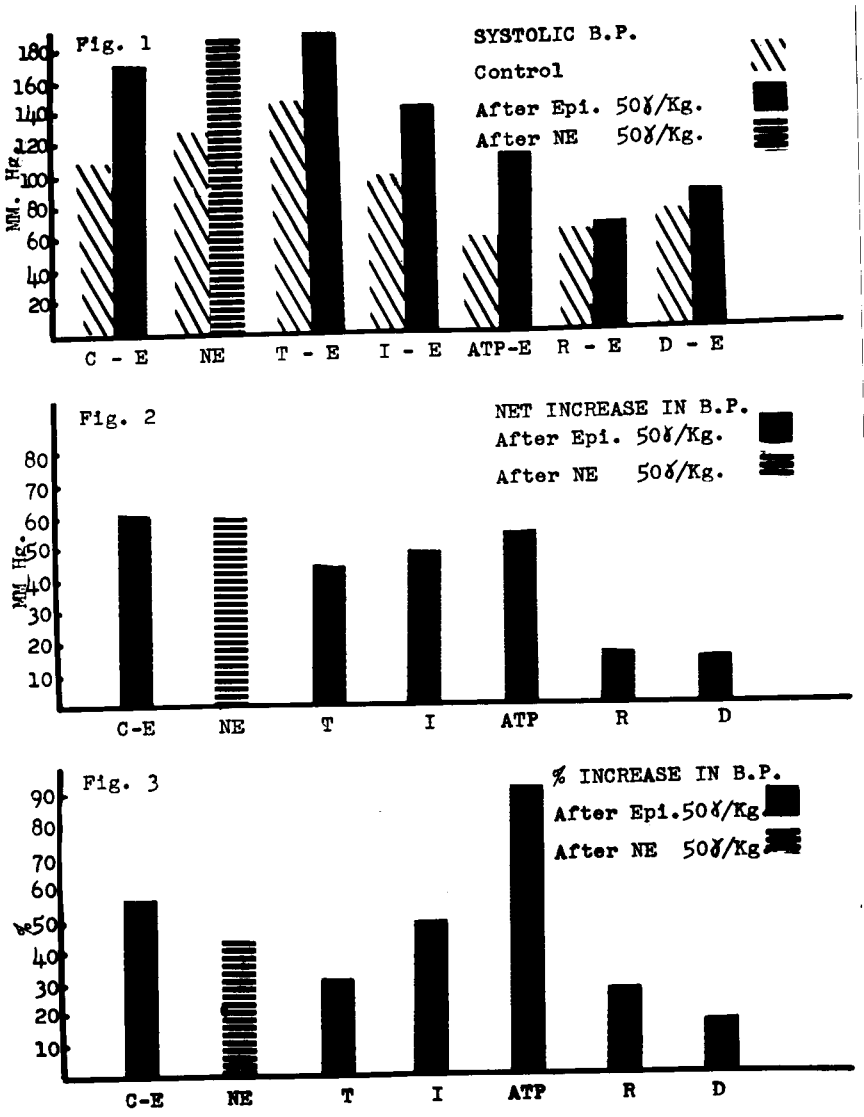
almost sixfold increase in $C^{14}O_2$ production as compared with only about a two fold increase observed in normal animals (Figs. 20, 23). Addition of epinephrine to aortic samples from thyroid animals incubated with glucose 1-C^{14} evoked little increase in $C^{14}O_2$ production (Figs. 20 and 23). Neither ATP nor phenoxybenzamine appeared to significantly affect $C^{14}O_2$ production from glucose 1-C^{14} while some inhibition was noted in samples treated with phentolamine.

Thus in all three series of experiments epinephrine produced a significant increase in $C^{14}O_2$ production when compared with control samples also incubated with glucose 6-C^{14}. Both phentolamine and phenoxybenzamine were found to inhibit $C^{14}O_2$ production, phentolamine being the more active of the two drugs in this respect. ATP appeared to effect some inhibition of the stimulant effect of epinephrine in the Krebs-Ringer phosphate (KRP) and Krebs-Ringer bicarbonate (KRB) buffers but only weakly so in the KRB + glucose series. When ATP was added to aortic samples from Thyroxine pretreated animals little or no effect was noted.

Incubation of aortic tissue with lysine U.L. C^{14} resulted in incorporation of this amino acid into protein. Epinephrine inhibited this incorporation (Figs. 24, 25). For example at one hour the rate of incorporation of labelled lysine into protein in tissue treated with epinephrine was only about a third of that observed in untreated controls (Fig. 24 and Table 14). Aortic tissue from animals pretreated with thyroxine for two weeks exhibited an incorporation rate less than forty percent of that observed in normal controls. As in the case of the normal animals, thyroid pretreated aortas appeared sensitive to the inhibitory effect of epinephrine on lysine incorporation into protein. ATP appeared to antagonize this inhibition, bringing the incorporation rate up to almost the control level in the thyroid

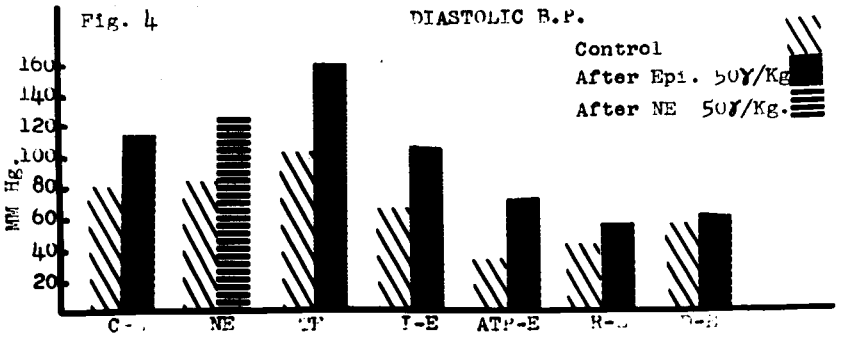
pretreated group. Neither phentolamine nor phenoxybenzamine appeared to effect any significant reversal of epinephrine inhibition of lysine incorporation into protein (Fig. 25 and Table 14).

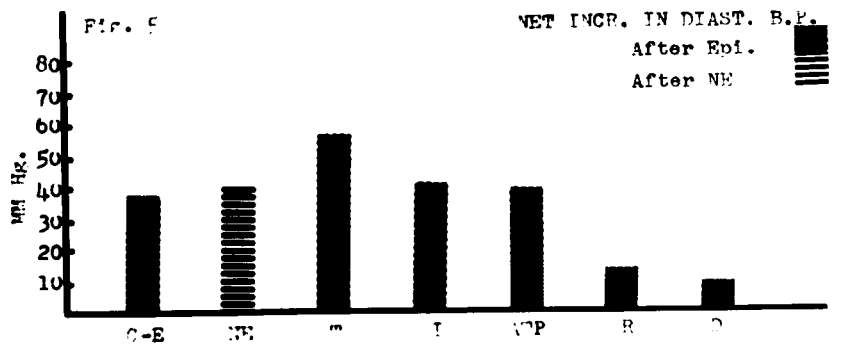
Acute Effects of Epinephrine (E) or Norepinephrine (NE) on Systolic Blood Pressure in the Unanesthetized Rabbit Following Pretreatment by Various Drugs.

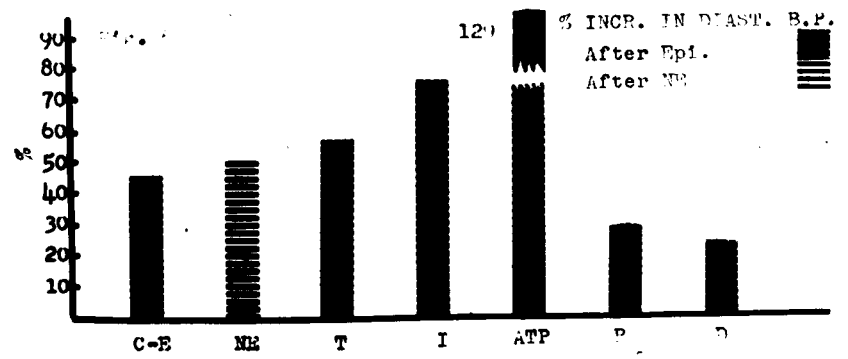


The following doses were employed: (E) epinephrine 50 /kg.; (NE) norepinephrine 50 /kg.; (T) thyroxine 0.1 mg./kg./14 days s.c.; (I) iproniazid 30mg/kg s.c. 24 hrs. before and again 30 minutes before epinephrine followed by 15 mg./kg. i.v.; (ATP) adenosine triphosphate 200 mg./kg. s.c.; (R) phentolamine Regitine 5 mg./kg. s.c. and 5 mg./kg.i.v.; (D) phenoxybenzamine (Dibenzyline) 2 mg./kg. i.v. and 1 mg./kg. i.v. All subcutaneous (s.c.) injections were administered 30 minutes prior to epinephrine challenge unless otherwise stated. All intravenous (i.v.) drug pretreatment was administered 5 minutes before epinephrine challenge. Eight animals used per group except norepinephrine group consisted of seven animals and thyroxine group consisted of nine animals. "Control" refers to systolic blood pressure subsequent to drug pretreatment but prior to E or NE.

Acute Effects of Epinephrine (E) or Norepinephrine (NE) on Diastolic Blood Pressure in the Unanesthetized Rabbit Following Pretreatment by Various Drugs.

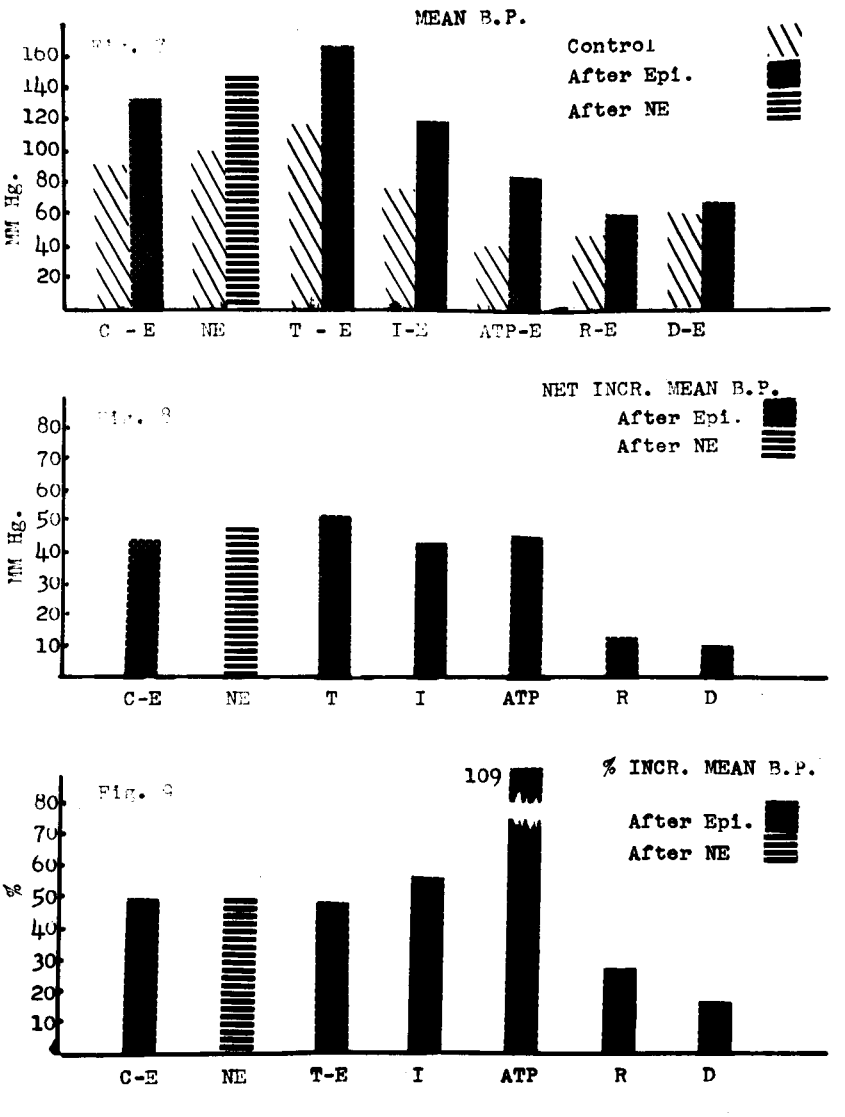






The following doses were employed: (E) epinephrine 50 /kg.: (NE) norepinephrine 50 /kg.; (T) thyroxine 0.1 mg./kg./14 days s. c.; (I) iproniazid 30 mg./kg. s.c. 24 hrs. before and again 30 minutes before epinephrine followed by 15mg./kg. i.v.; (ATP) adenosine triphosphate 200 mg./kg. s.c.; (R) phentolamine (Regitine) 5 mg./kg. s.c. and 5 mg./kg. i.v.; (D) phenoxybenzamine (Dibenzyline) 2 mg./kg. i.v. and 1 mg./kg. i.v. All Subcutaneous (s.c.) injections were administered 30 minutes prior to epinephrine challenge unless otherwise stated. All intravenous (i.v.) drug pretreatment was administered 5 minutes before epinephrine challenge. Eight animals used per group except norepinephrine group consisted of seven animals and thyroxine group consisted of nine animals. "Control" refers to diastolic blood pressure following drug pretreatment but prior to E or NE infusion.

Acute Effects of Epinephrine (E) or Norepinephrine (NE) on Mean Blood pressure in the Unanesthetized Rabbit Following Pretreatment by Various Drugs.



The following doses were employed: (E) epinephrine 50 /kg.; (NE) norepinephrine 50 /kg.; (T) thyroxine 0.1 mg./kg./14 days s.c.; (I) pronizazid 30 mg./kg. s.c. 24 hrs. before and again 30 minutes before epinephrine followed by 15 mg./kg.i.v.; (ATP) adenosine triphosphate 200 mg./kg.s.c.; (R) phentolamine (Regitine) 5 mg./kg.s.c. and 5 mg./kg.i.v.; (D) phenoxybenzamine (Dibenzyline) 2 mg./kg. i.v. and 1 mg./kg.i.v. All subcutaneous (s.c.) injections were administered 30 minutes prior to epinephrine challenge unless otherwise stated. All intravenous (i.v.) drug pretreatment was administered 5 minutes before epinephrine challenge. Eight animals used per group except norepinephrine group consisted of seven animals; thyroxine group consisted of nine animals. "Control" refers to mean blood pressure subsequent to drug pretreatment but prior to E or NE.

Effects of Subacute Administration of Thyroxine (T) and Epinephrine (E) on Various Tissue Constituents in the Rabbit.

Fig. 10

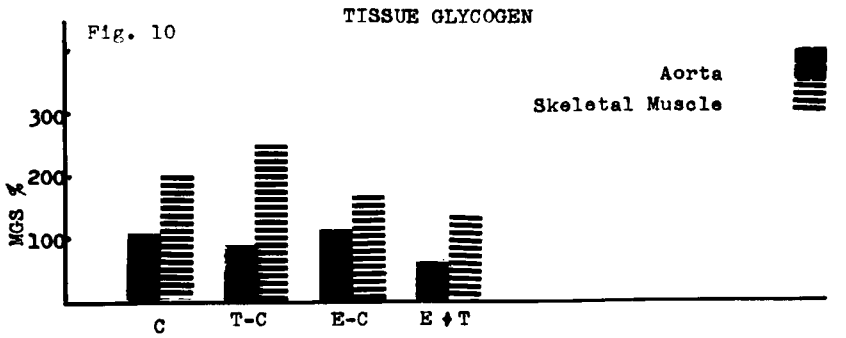


Fig. 11

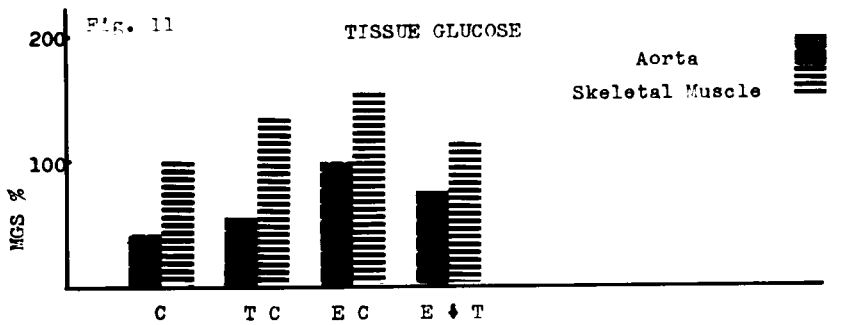
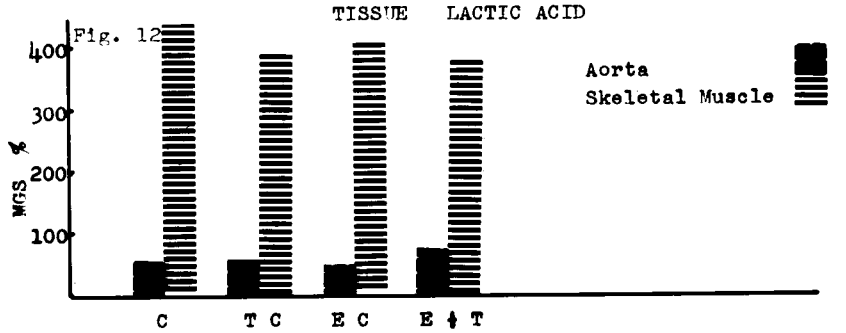
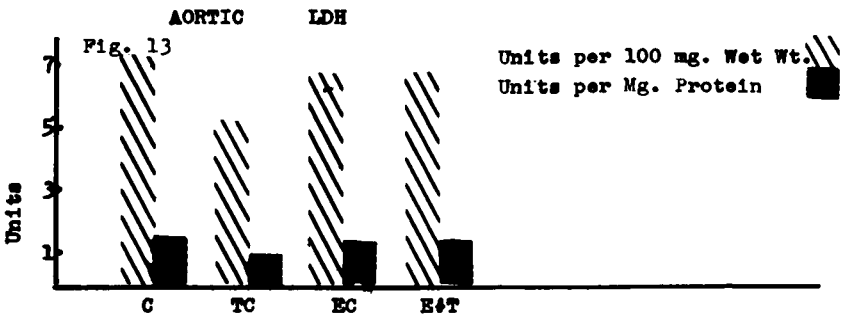


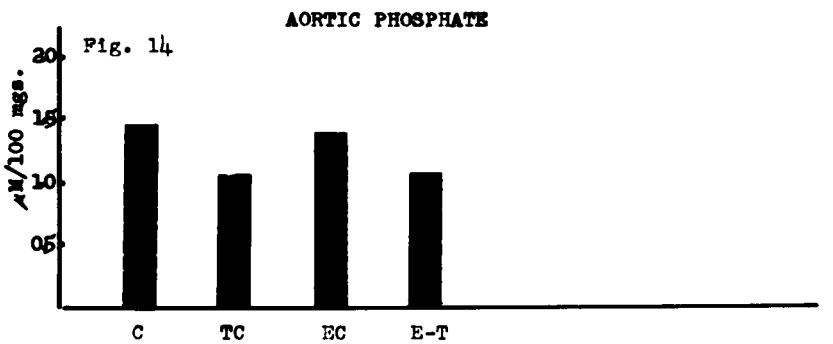
Fig. 12



Animals were treated as follows: (C) controls 1 ml./kg. saline i.v.; (EC) epinephrine 50 /kg. i.v.; (TC) thyroxine 0.1 mg./kg. s.c.; (E+T) epinephrine and thyroxine as above. Period of drug administration was 2 weeks.

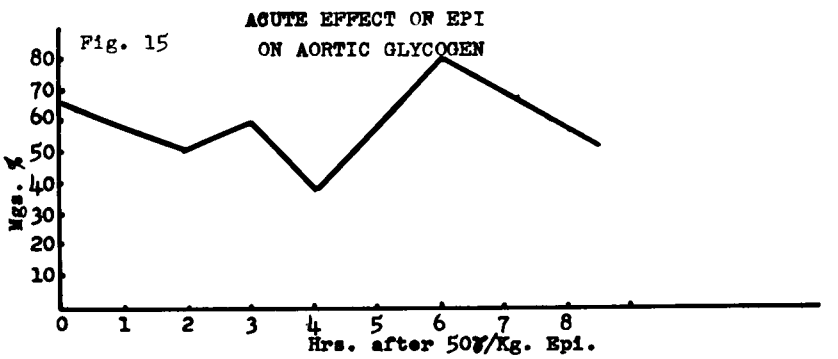
Effects of Subacute Administration of Thyroxine (T) and Epinephrine (E) on Various Tissue Constituents in the Rabbit.





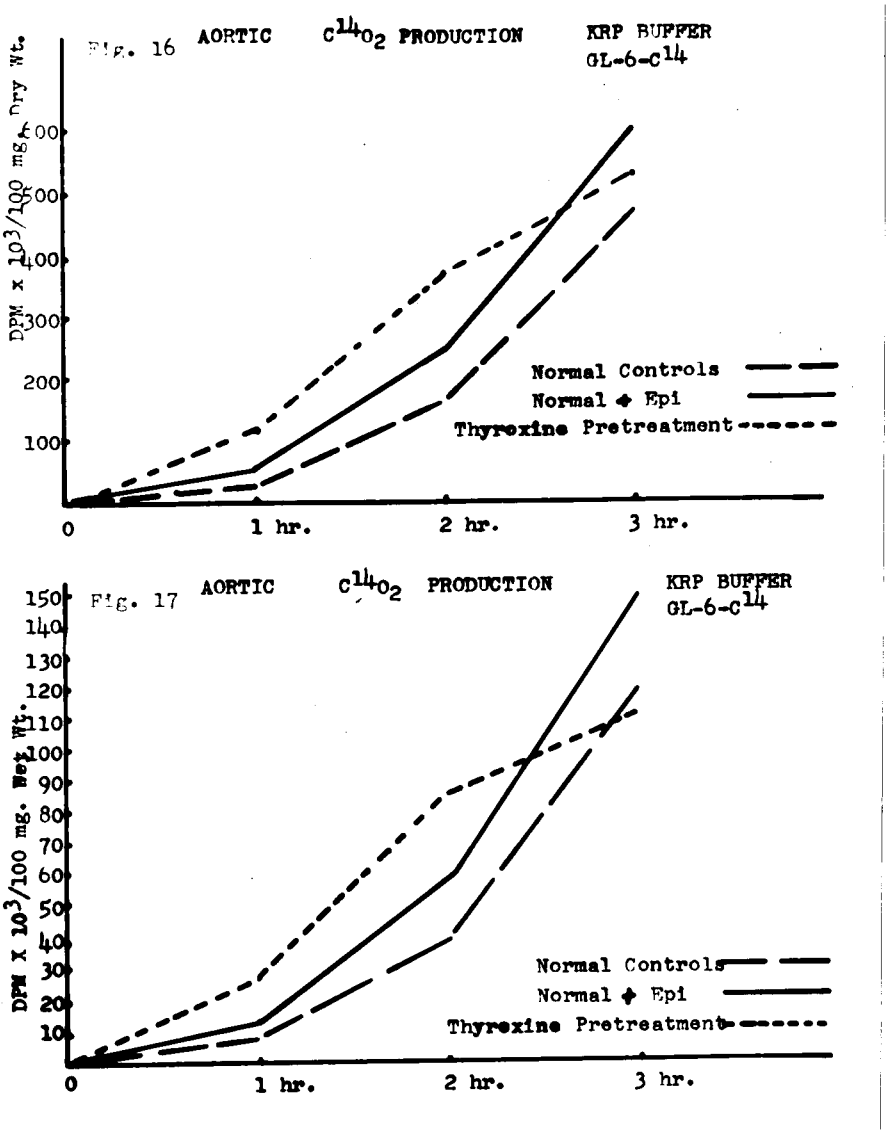
Animals were treated as follows: (C) controls 1 ml./kg. saline i.v.; (EC) epinephrine 50%/kg. i.v.; (TC) thyroxine 0.1 mg./kg. s.c.; (E + T) epinephrine and thyroxine as above. Period of drug administration was 2 weeks.

Acute Effects of Epinephrine on Aorta Glycogan



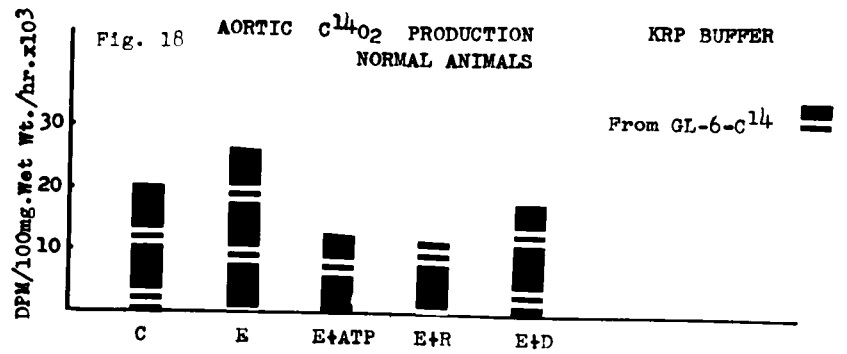
Rabbits were fasted 24 hrs. prior to administration of 50 /kg. epinephrine intravenously.

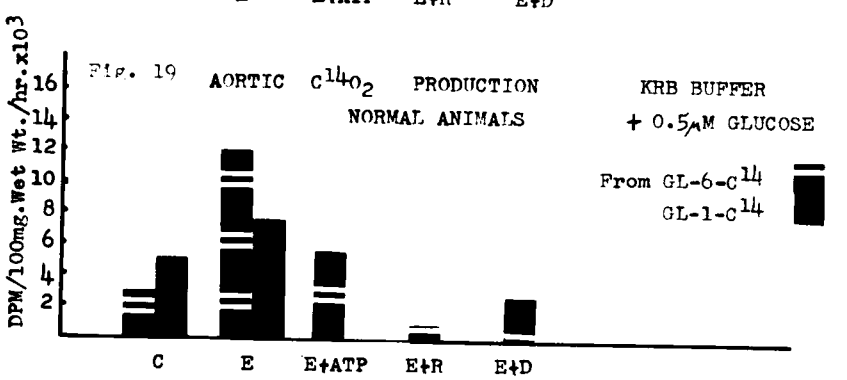
Effect of Incubation Time on In Vitro $C^{14}O_2$ Production From Glucose-6-C^{14} by Rabbit Aorta

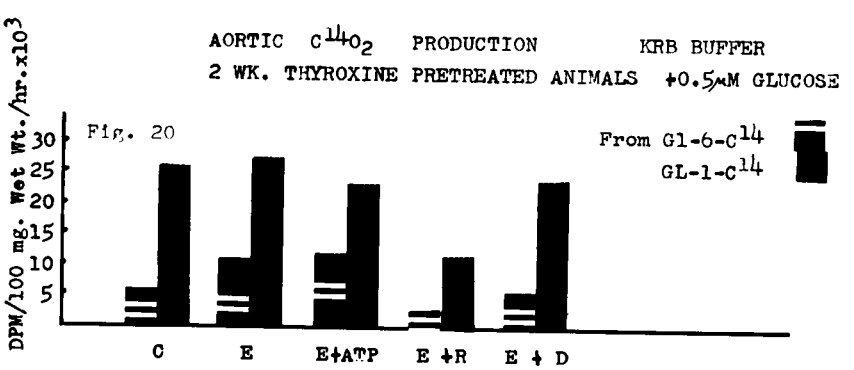


Incubation medium consisted of KRP - Krebs-Ringer Phosphate Buffer. Each tissue sample was incubated with 0.5 microcuries of glucose-6-C^{14}. Samples were shaken in a metabolic shaker at 80 strokes/minute. Temperature 37°C. Incubation of aortic samples was stopped at 1, 2 and 3 hours by addition of 1N sulfuric acid. Thyroxine pretreated animals received 0.1 mg./kg. of this drug subcutaneously for 14 days. (Epi) epinephrine 0.5γ/ml. of medium added at beginning of incubation.

In Vitro Incubation of Rabbit Aorta

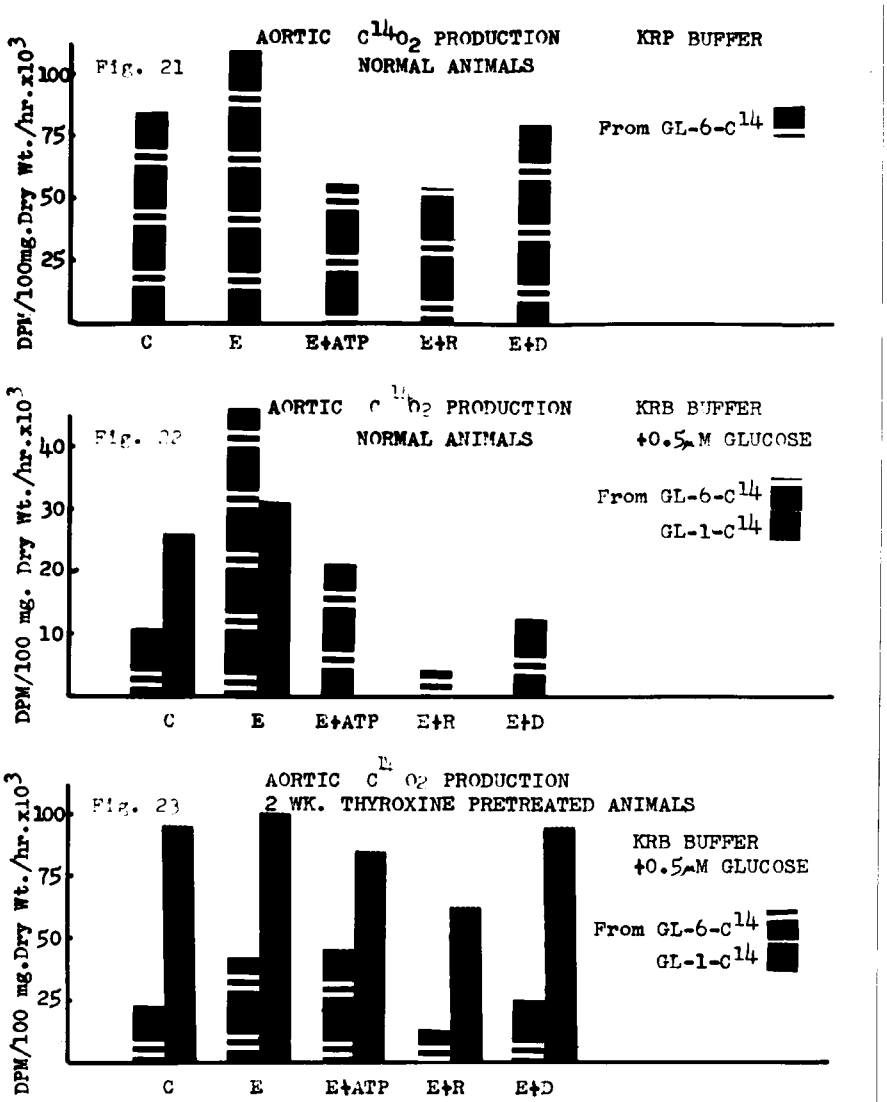






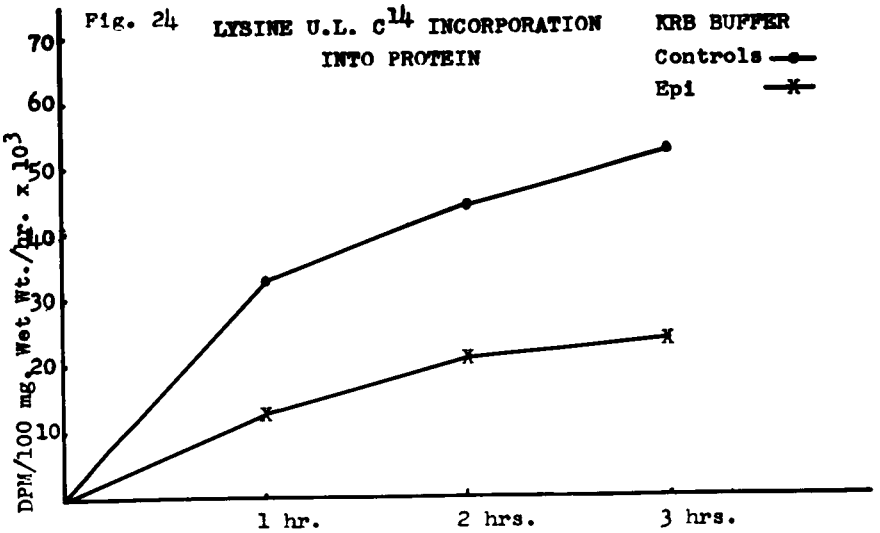
Incubation medium consisted of KRP - Krebs-Ringer Phosphate or KRB - Krebs-Ringer Bicarbonate buffers. Drug concentrations used: (E) epinephrine 0.5 /ml.; (ATP) adenosine triphosphate 2 mg./ml.; (R) phentolamine (Regitine) 0.1 mg./ml.; (D) phenoxybenzamine (Dibenzyline) 0.03 ml./ml.; 0.5 microcurie of C^{14} labelled glucose was added to each tissue sample. Incubation time was 2 hours at 37°C., 80 strokes per minute. Thyroxine pretreatment 0.1 mg./kg. subcutaneously for 14 days.

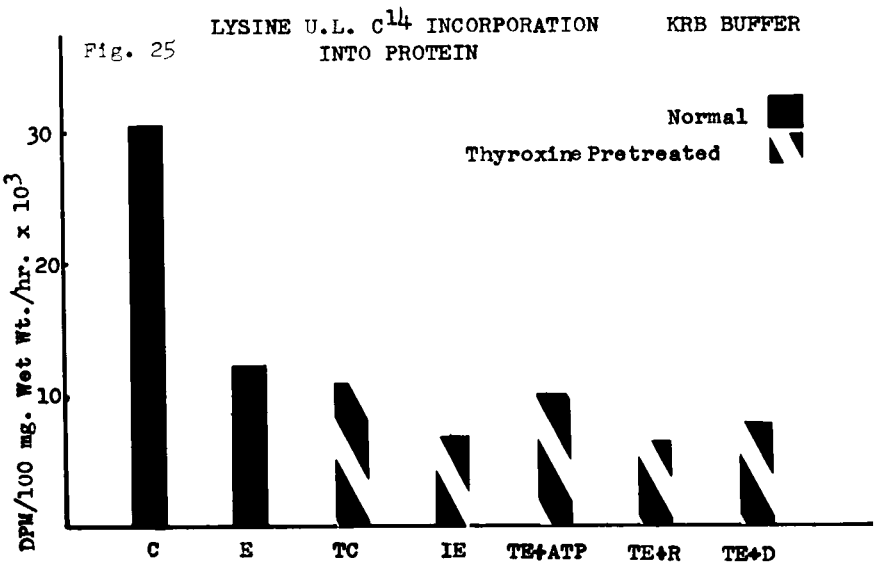
In Vitro Incubation of Rabbit Aorta



Incubation medium consisted of KRP - Krebs-Ringer Phosphate or KRB - Krebs-Ringer Bicarbonate buffers. Drug concentrations used: (E) epinephrine 0.5γ/ml.; (ATP) adenosine triphosphate 2 mg./ml.; (R) phentolamine [Regitine] 0.1 mg./ml.; (D) phenoxybenzamine [Dibenzyline] 0.03 ml./ml.; 0.5 microcurie of C^{14} labelled glucose was added to each tissue sample. Incubation time was 2 hours at 37°C., 80 strokes per minute. Thyroxine pretreatment 0.1 mg./kg. subcutaneously for 14 days.

In Vitro Incorporation of Uniformly Labelled (u.l.) Lysine C^{14} into Protein by Aortic Tissue.





Incubation medium consisted of KRB - Krebs-Ringer Bicarbonate buffer. Each tissue sample was incubated with 0.5 microcurie of u.l. lysine C^{14}. Drug concentrations used: (E) epinephrine 0.5 /ml.; (ATP) adenosine triphosphate 2 mg./ml.; (R) phentolamine (Regitine) 0.1 mg./ml.; (D) phenoxybenzamine (Dibenzyline) 0.03 mg./ml.; Incubation time was 2 hours at 37°C., 80 strokes per minute. Thyroxine pretreatment 0.1 mg./kg. subcutaneously for 14 days.

Table 1a

Effect of Intravenous Infusion of Epinephrine (EPI) or Norepinephrine (NE) Following Pretreatment by Various Drugs on Systolic Blood Pressure in the Unanesthetized Rabbit

Group	Pretreatment	No. of Exps.	Control Level mm.Hg.	Pressor Drug	Pressor Response mm.Hg.	Net Increase mm.Hg.	% Increase
1	Saline 1 ml/Kg s.c.	8	109 ±4.83	Epi 50γ/Kg.	172 ±8.72	62.5 ±5.68	57 ±4.62
2	Saline 1 ml/Kg s.c.	8	129 ±5.3	NE 50γ/Kg.	189 ±4.46	60 ±4.4	43 ±1.36
3	Thyroxine* 0.1 mg/Kg (s.c.)	9	146 ±4.6	Epi 50γ/Kg.	191 ±2.8	45 ±5.3	27 ±2.72
4	ATP 200 mg/Kg (s.c.)	8	60 ±2.35	Epi 50γ/Kg.	114 ±6.4	54 ±6.1	92 ±11.8
5	ATP 200 mg/Kg(s.c.) +Thyroxine* 0.1 mg/Kg (s.c.)	8	130 ±5.2	Epi 50γ/Kg.	190 ±10.4	60 ±8.58	47 ±7.38
6	Iproniazid 30 mg/Kg (s.c.)	7	96 ±4.0	Epi 50γ/Kg.	145 ±5.8	58 ±6.5	56 ±5.1
7	Phentolamine(Regitine) 5 mg/Kg (s.c.)+5 mg/Kg i.v.	8	63 ±5.12	Epi 50γ/Kg.	72 ±3.7	16 ±2.51	27 ±3.3
8	Phenoxybenzamine (Dibenzyline) 2 mg/Kg (s.c.)+1 mg/Kg i.v.	8	75 ±3.53	Epi 50γ/Kg	88 ±6.54	13 ±2.16	17 ±4.13

* Thyroxine was administered daily for a period of 2 weeks prior to experiments.
All other subcutaneous (s.c.) administrations were made 30 minutes prior to epinephrine administration.
Iproniazid (s.c.) was in addition administered 24 hrs. prior to group #6 experiments.
Intravenous (i.v.) drug pretreatment was administered 5 minutes prior to epinephrine infusions.

Statistical Analysis of Systolic Blood Pressure Changes in Table 1a

Group	Pressor Level	Net Increase	% Increase
1 vs 2	N.S.	N.S.	0.05
1 vs 3	N.S.	0.05	0.001
1 vs 4	<0.001	N.S.	0.05
1 vs 6	0.05	N.S.	N.S.
1 vs 7	<0.001	<0.001	<0.001
1 vs 8	<0.001	<0.001	<0.001
3 vs 5	N.S.	N.S.	0.01
7 vs 8	0.05	N.S.	N.S.

N.S. = Not Significant - probability level greater than 0.05 (5%)

Table 2a

Effect of Intravenous Infusion of Epinephrine (EPI) or Norepinephrine (NE) Following Pretreatment by Various Drugs on Diastolic Blood Pressure in the Unanesthetized Rabbit

Group	Pretreatment	No. of Exps.	Control Level mm.Hg.	Pressor Drug	Pressor Response mm.Hg.	Net Increase mm.Hg.	% Increase
1	Saline 1 ml/Kg s.c.	8	82 ±4.85	Epi 50 γ/Kg.	117 ±12.3	38 ±7.5	46 ±8.2
2	Saline 1 ml/Kg s.c.	9	84 ±1.53	NE 50 γ/Kg.	126 ±4.2	42 ±3.8	49 ±4.46
3	Thyroxine* 0.1 mg/Kg (s.c.)	9	102 ±3.3	Epi 50 γ/Kg.	158 ±2.56	57 ±3.43	57 ±5.25
4	ATP 200 mg/Kg (s.c.)	8	31 ±1.4	Epi 50 γ/Kg.	70 ±5.2	39 ±4.64	129 ±14.1
5	ATP 200 mg/Kg (s.c.) +Thyroxine* 0.1 mg/Kg (s.c.)	8	75 ±10.6	Epi 50 γ/Kg.	135 ±3.39	60 ±10.5	52 ±9.5
6	Iproniazid 30 mg/Kg (s.c.) +15 mg./Kg. (i.v.)	7	65 ±3.56	Epi 50 γ/Kg.	106 ±5.7	49 ±4.91	75 ±10.2
7	Phentolamine (Regitine) 5 mg/Kg (s.c.)+5 mg/Kg i.v.	8	45 ±1.78	Epi 50 γ/Kg.	59 ±3.47	13 ±2.54	29 ±4.8
8	Phenoxybenzamine (Dibenzyline) 2 mg/Kg (s.c.)+1 mg/Kg i.v.	8	54 ±2.5	Epi 50 γ/Kg.	59 ±4.9	8 ±3.29	23 ±3.4

* Thyroxine was administered daily for a period of 2 weeks prior to experiments.
All other subcutaneous (s.c.) administrations were made 30 minutes prior to epinephrine administration.
Iproniazid (s.c.) was in addition administered 24 hrs. prior to group #6 experiments.
Intravenous (i.v.) drug pretreatment was administered 5 minutes prior to epinephrine infusions.

Statistical Analysis of Diastolic Blood Pressure Changes in Table 2a

Group	Pressor Level	Net Increase	% Increase
1 vs 2	N.S.	N.S.	N.S.
1 vs 3	0.01	0.05	N.S.
1 vs 4	0.01	N.S.	<0.001
1 vs 6	N.S.	N.S.	0.05
1 vs 7	<0.001	0.01	N.S.
1 vs 8	<0.001	0.01	0.05
3 vs 5	<0.001	N.S.	N.S.
7 vs 8	N.S.	N.S.	N.S.

N.S. = Not Significant - probability level greater than 0.05 (5%).

Table 3a

Effect of Intravenous Infusion of Epinephrine (EPI) or Norepinephrine (NE) Following Pretreatment by Various Drugs on Mean Blood Pressure in the Unanesthetized Rabbit

Group	Pretreatment	No. of Exps.	Control Level mm.Hg.	Pressor Drug	Pressor Response mm.Hg.	Net Increase mm.Hg.	% Increase
1	Saline 1 ml/Kg s.c.	8	91 ±5.5	Epi 50γ/Kg.	135 ±10.2	44 ±6.6	49 ±6.57
2	Saline 1 ml/Kg s.c.	9	99 ±2.45	NE 50γ/Kg.	147 ±4.06	48 ±2.74	49 ±2.83
3	Thyroxine* 0.1 mg/Kg (s.c.)	9	117 ±3.13	Epi 50γ/Kg.	171 ±1.7	52 ±3.73	47 ±4.67
4	ATP 200 mg/Kg (s.c.)	8	40 1.22	Epi 50γ/Kg.	85 5.28	45 4.6	109 11.7
5	ATP 200 mg/Kg(s.c.) +Thyroxine* 0.1 mg/Kg (s.c.)	8	102 ±9.89	Epi 50γ/Kg.	154 ±7.0	53 ±9.86	62 ±18.0
6	Iproniazid 30 mg/Kg (s.c.)	7	76 ±4.1	Epi 50γ/Kg.	116 ±11.5	42 ±5.4	56 ±9.3
7	Phentolamine (Regitine) 5 mg/Kg (s.c.)+5 mg/Kg i.v.	8	51 ±1.97	Epi 50γ/Kg.	65 ±3.43	13 ±2.43	26 ±3.8
8	Phenoxybenzamine (Dibenzyline) 2 mg/Kg (s.c.)+1 mg/Kg i.v.	8	61 ±2.5	Epi 50 /Kg.	68 ±5.2	10 ±1.98	16 ±2.94

* Thyroxine was administered daily for a period of 2 weeks prior to experiments.
All other subcutaneous (s.c.) administrations were made 30 minutes prior to epinephrine administration.
Iproniazid (s.c.) was in addition administered 24 hrs. prior to group #6 experiments.
Intravenous (i.v.) drug pretreatment was administered 5 minutes prior to epinephrine infusions.

Statistical Analysis of Mean Blood Pressure

Changes in Table 3a

Group	Pressor Level	Net Increase	% Increase
1 vs 2	N.S.	N.S.	N.S.
1 vs 3	0.01	N.S.	N.S.
1 vs 4	0.01	N.S.	
1 vs 6	N.S.	N.S.	N.S.
1 vs 7	<0.001	<0.001	0.01
1 vs 8	<0.001	<0.001	<0.001
3 vs 5	0.05	N.S.	N.S.
7 vs 8	N.S.	N.S.	N.S.

N.S. = Not Significant - probability level greater than 0.05 (5%).

Table 4

Levels of Glycogen in Aorta and Skeletal Muscle Following Acute I.V. Epinephrine (50γ/Kg.) in 24 hr. fasted Rabbits.

	n	Aorta	Skeletal Muscle*
1. Saline Controls	9	67 ± 8.6 mgs. %	122 ± 18.1 mgs. %
2. 2 hrs. after Epinephrine	8	51 ± 3.9 "	83 ± 21.3 "
3. 4 hrs. after Epinephrine	8	40 ± 3.46 "	41 ± 7.1 "
4. 8 hrs. after Epinephrine	8	59 ± 6.6 "	71 ± 15.8 "

P Values		Aorta	Skeletal Muscle
1 vs 2		N.S.	N.S.
1 vs 3		0.05	.001
1 vs 4		N.S.	.05

* Source of skeletal muscle was inner aspect of the thigh.

N.S. = Not Significant or a p value $> 0.05(5\%)$.

Table 5

Effect of Subacute (2 wk.) Administration of Epinephrine and Epinephrine + Thyroxine on Tissue Levels of Glycogen in the Rabbit

	n	Aorta	Skeletal Muscle*
1. Saline Controls (I.V.) 1 cc/Kg.	8	104. ± 11.2 mgs.%	182. ± 47.8 mgs.%
2. Thyroxine (s.c.) 0.1 mg/Kg.	8	84. ± 13.6 "	253. ± 20.9 "
3. Epinephrine (I.V.) 50γ/Kg.	8	108. ± 11.4 "	172. ± 34.3 "
4. Epinephrine (I.V.) 50γ/Kg. + Thyroxine (s.c.) 0.1 mg/Kg.	8	59. ± 4.56 "	138. ± 23.8 "

P Values	Aorta	Skeletal Muscle
1 vs 2	N.S.	N.S.
1 vs 3	N.S.	N.S.
1 vs 4	.01	N.S.

* Source of skeletal muscle was inner aspect of thigh.

I.V. = intravenous

s.c. = subcutaneous

N.S. = Not Significant or p value > 0.05(5%).

Table 6

Effect of Subacute (2 wks.) Administration of Epinephrine and Epinephrine + Thyroxine on Tissue Glucose in the Rabbit

	n	Aorta	Skeletal Muscle
1. Saline Controls (i.v.) 1 cc/Kg.	8	41.7 ± 3.31 mgs.%	97.8 ± 17.5 mgs.%
2. Thyroxine (s.c.) 0.1 mg/Kg.	8	55.9 ± 6.96 "	136. ± 20.9 "
3. Epinephrine (i.v.) 50γ/Kg.	8	97.0 ±10.6 "	156. ± 18.1 "
4. Epinephrine (i.v.) 50γ/Kg. + Thyroxine 0.1 mg./Kg. (s.c.)	8	70.1 ± 7.87 "	116. ± 9.52 "

P Values	Aorta	Skeletal Muscle
1 vs 2	N.S.	N.S.
1 vs 3	.01	.05
1 vs 4	.01	N.S.

i.v. = intravenous

s.c. = subcutaneous

N.S. = Not Significant or p value > 0.05 (5%).

Table 7

Effect of Subacute (2 wk.) Administration of Epinephrine and Epinephrine + Thyroxine on Tissue Lactic Acid in the Rabbit

	n	Aorta	Skeletal Muscle
1. Saline Controls (i.v.) 1 cc/Kg.	8	53.8 ± 10.4 mgs.%	434. ± 25.1 mgs.%
2. Thyroxine (s.c.) 0.1 mg/Kg.	8	59.2 ± 7.38 "	388. ± 28.4 "
3. Epinephrine (i.v.) 50 /Kg.	8	49.4 ± 12.5 "	408. ± 33.9 "
4. Epinephrine (i.v.) 50 /Kg. + Thyroxine (s.c.) 0.1 mg/Kg.	8	74.7 ± 3.9 "	377. ± 25.2 "

P Values	Aorta	Skeletal Muscle
1 vs 2	N.S.	N.S.
1 vs 3	N.S.	N.S.
1 vs 4	N.S.	N.S.

i.v. = intravenous

s.c. = subcutaneous

N.S. = Not Significant or p value > 0.05 (5%)

Table 80

Effect of Subacute (2 wk.) Administration of Epinephrine and Epinephrine + Thyroxine on Aortic IDH (Lactic Dehydrogenase) Levels in the Rabbit

	n	Units/100 mg. Wet Wt.	Units/mg. Protein
1. Saline Controls (i.v.) 1 cc/Kg.	8	7.44 ± 0.880	1.46 ± 0.175
2. Thyroxine (s.c.) 0.1 mg/Kg.	8	5.23 ± 0.574	0.989± 0.126
3. Epinephrine (i.v.) 50/Kg.	8	6.80 ± 0.703	1.29 ± .0246
4. Epinephrine (i.v.) 50 /Kg. + Thyroxine (s.c.) 0.1 mg/Kg.	8	6.80 ± 0.0211	1.39 ± 0.119
P Values			
1 vs 2		N.S.	N.S.
1 vs 3		N.S.	N.S.
1 vs 4		N.S.	N.S.

i.v. = intravenous

s.c. = subcutaneous

N.S. = Not Significant or p value > 0.05 (5%).

Table 9

Effect of Subacute (2 wk.) Administration of Epinephrine and Epinephrine + Thyroxine on Aortic Phosphate Levels in the Rabbit

	n	
1. Saline Controls (i.v.) 1 cc/Kg.	8	14.6 ± 0.87 μM/100 mgs.
2. Thyroxine Controls (s.c.) 0.1 mg/Kg.	8	10.74 ± 1.52 "
3. Epinephrine (i.v.) 50γ/Kg.	8	14.1 ± 1.41 "
4. Epinephrine (i.v.) 50γ/Kg. + Thyroxine (s.c.) 0.1 mg/Kg.	8	10.9 ± 0.76 "

P Values	
1 vs 2	0.05
1 vs 3	N.S.
1 vs 4	.01

i.v. = intravenous

s.c. = subcutaneous

N.S. = Not Significant or p value > 0.05 (5%).

Table 10

In Vitro $C^{14}O_2$ Production by Aortic Tissue

From Glucose-6-C^{14} — Krebs-Ringer Phosphate Buffer

	n	DPM/100 mg Wet Wt. x 10^3 per hr.	DPM/100 mg Dry Wt. x 10^3 per hr.
1. Controls	14	20.28 ± 2.13	77.906 ± 8.2
2. Epinephrine	14	28.1 ± 3.1	109.70 ± 11.6
3. Epinephrine + ATP	8	12.28 ± 1.4	55.85 ± 3.87
4. Epinephrine + Phentolamine	8	11.79 ± 1.32	53.94 ± 7.2
5. Epinephrine + Phenoxybenzamine	8	18.21 ± 2.81	80.94 ± 13.6

Table of P Values Group	Wet Wt. Basis P Value	Dry Wt. Basis P Value
1 vs 2	.05	.05
1 vs 3	<.001	<.001
2 vs 4	<.001	<.001
2 vs 5	.05	N.S.
2 vs 3	.01	.05
1 vs 4	.01	.05
1 vs 5	N.S.	N.S.
4 vs 5	N.S.	N.S.
3 vs 5	N.S.	N.S.

The following drug concentrations were employed in the incubation medium Epinephrine 0.5γ/ml, ATP 2 mg/ml, phentolamine 0.1 mg/ml, and phenoxybenzamine 0.03 mg/ml.

N.S. denotes Not Significant or a "p" level greater than 5%. (0.05)

Table 11

In Vitro $C^{14}O_2$ Production by Aortic Tissue

From Glucose-6-C^{14} — Krebs-Ringer Bicarbonate Buffer 0.5 μM/ml of Glucose Added

	n	DPM/100 mg Wet Wt. x 10^3 per hr.	DPM/100 mg Dry Wt. x 10^3 per hr.
1. Controls	8	2.746 ± 0.340	11.62 ± 1.710
2. Epinephrine	8	11.90 ± 1.42	46.77 ± 6.5
3. Epinephrine + ATP	8	4.730 ± 0.508	18.52 ± 1.65
4. Epinephrine + Phentolamine	8	1.072 ± 0.174	4.268± 0.64
5. Epinephrine + Phenoxybenzamine	8	2.502 ± 0.217	9.397± 0.924

p values	Wet Wt. Basis		Dry Wt. Basis	
		P value		P value
	1 vs 2	<.001	1 vs 2	<.001
	2 vs 3	<.001	2 vs 3	<.001
	2 vs 4	<.001	2 vs 4	<.001
	2 vs 5	<.001	2 vs 5	<.001
	4 vs 5	<.001	4 vs 5	<.001
	1 vs 4	<.001	1 vs 4	<0.01
	1 vs 5	N.S.	1 vs 5	N.S.

The following drug concentrations were employed in the incubation medium Epinephrine 0.5μ/ml, ATP 2 mg/ml, phentolamine 0.1 mg/ml, and phenoxybenzamine 0.03 mg/ml.

N.S. denotes Not Significant or a "p" level greater than 5%. (0.05)

Table 12

In Vitro $C^{14}O_2$ Production by Aortic Tissue
(2 wk. Thyroxine Pretreated Animals)

Glucose-6-C^{14} — Krebs-Ringer Bicarbonate Buffer
0.5 M/ml of Glucose added

	n	DPM/100 mg Wet Wt. x 10^3 per hr.	DPM/100 mg Dry Wt. x 10^3 per hr.
1. Controls	8	6.072 ± 0.614	22.85 ± 2.34
2. Epinephrine	8	11.38 ± 1.49	41.71 ± 4.62
3. Epinephrine + ATP	8	12.39 ± 1.41	46.70 ± 5.61
4. Epinephrine + Phentolamine	8	3.203 ± 0.345	12.49 ± 1.30
5. Epinephrine + Phenoxybenzamine	8	6.091 ± 0.515	24.34 ± 2.11

Table of P values	Wet Wt. Basis	Dry Wt. Basis
Group		
1 vs 2	0.01	0.01
2 vs 3	N.S.	N.S.
2 vs 4	<0.001	<0.001
2 vs 5	0.01	0.01
1 vs 4	0.01	0.01
1 vs 5	N.S.	N.S.
4 vs 5	<0.001	<0.001

The following drug concentrations were employed in the incubation medium Epinephrine 0.5γ/ml, ATP 2 mg/ml, phentolamine 0.1 mg/ml and phenoxybenzamine 0.03 mg/ml.
Thyroxine 0.1 mg/Kg was administered subcutaneously each day for two weeks.

N.S. denotes Not Significant or a "p" level greater than 5%. (0.05)

Table 13

In Vitro $C^{14}O_2$ Production by Aortic Tissue

From Glucose-6-C^{14} — Krebs-Ringer Bicarbonate Buffer / Krebs-Ringer Bicarbonate Buffer +0.5 M/ml. Glucose

I	n	DPM/100 mg. Wet Wt. x 10^3 per hr.	DPM/100 mg. Dry Wt. x 10^3 per hr.
1. C (KRB)	6	11.89 ± 1.73	31.51 ± 4.86
2. Epi + (KRB)	6	12.17 ± 2.33	34.65 ± 5.90
3. Epi + ATP (KRB)	6	9.26 ± 0.86	23.46 ± 0.63
II			
4. C (KRB)*	6	2.84 ± 0.61	8.133± 1.04
5. ATP (KRB)*	6	3.67 ± 0.26	11.36 ± 1.53
6. Phentolamine (KRB)*	6	1.45 ± 0.17	4.031± 0.58
7. Phenoxybenzamine (KRB)*	6	2.49 ± 0.43	6.860± 1.19
8. Norepinephrine	6	3.47 ± 0.51	12.86 ± 2.3

P Values	Group	Wet Wt. Basis p Value	Dry Wt. Basis p Value
	1 vs 2	N.S.	N.S.
	1 vs 3	N.S.	N.S.
	2 vs 3	N.S.	N.S.
	4 vs 5	N.S.	N.S.
	4 vs 6	N.S.(0.1)	0.05
	4 vs 7	N.S.	N.S.
	4 vs 8	N.S.	N.S.

The following drug concentrations were employed in the incubation medium Epinephrine 0.5γ/ml, ATP 2 mg/ml, Phentolamine 0.1 mg/ml and Phenoxybenzamine 0.03 mg/ml, Norepinephrine 0.5γ/ml.

N.S. denotes Not Significant or a "p" level greater than 5%. (0.05)

Table 14

In Vitro $C^{14}O_2$ Production by Aortic Tissue

From Glucose-1-C^{14} — Krebs-Ringer Bicarbonate Buffer + 0.5 μM Glucose/ml.

Group	n	DPM/100 mg Wet Wt. x 10^3 per hr.	DPM/100 mg Dry Wt. x 10^3 per hr.
1. Controls *	8	25.6 ± 3.25	94.4 ± 11.5
2. Epinephrine *	8	27.4 ± 3.76	100.8 ± 11.3
3. Epinephrine + ATP *	8	23.0 ± 2.42	86.9 ± 8.6
4. Epinephrine + Phentolamine *	8	18.0 ± 2.73	66.1 ± 10.5
5. Epinephrine + Phenoxybenzamine *	8	24.4 ± 2.88	94.75 ± 13.0
6. Control	6	5.97 ± 0.677	25.62 ± 4.07
7. Epinephrine	6	7.74 ± 1.23	30.8 ± 4.90

P Values	Wet Wt. Basis	Dry Wt. Basis
1 vs 2	N.S.	N.S.
1 vs 3	N.S.	N.S.
1 vs 4	N.S.	N.S.
1 vs 5	N.S.	N.S.
1 vs 6	0.001	0.001
1 vs 7	.001	.001

The following drug concentrations were employed in the incubation medium Epinephrine 0.5γ/ml, ATP 2 mg/ml, phentolamine 0.1 mg/ml, and phenoxybenzamine 0.03 mg/ml.

* Obtained from rabbits pretreated with 0.1 mg/Kg. thyroxine s.c. for 2 wks.

N.S. denotes Not Significant or a p level greater than 5%. (0.05)

Table 15

In Vitro Lysine U.L. C^{14} Incorporation into protein by Aortic Tissue from Normal and 2 Wk. Thyroxine Pretreated Animals

	n	DPM/100 mg. Wet Wt. x 10^3 per hr.
1. Normal Controls	4	32.72 ± 4.75
2. Epinephrine (Normal Animals)	4	12.65 ± 1.67
3. Thyroxine Pretreated Controls*	8	11.205± 2.5
4. Epinephrine*	8	7.058± 1.22
5. Epinephrine + ATP*	8	10.01 ± 2.27
6. Epinephrine + Phentolamine	8	6.677± 1.56
7. Epinephrine + Phenoxybenzamine	8	7.217± 1.43

P Values

1 vs 2	0.01
1 vs 3	0.01
2 vs 3	N.S.
3 vs 4	0.001**
3 vs 5	N.S.
4 vs 5	0.05**
4 vs 6	N.S.
4 vs 7	N.S.

** Using paired differences method

* Tissue obtained from animals pretreated for 2 weeks with 0.1 mg./Kg. s.c. of Thyroxine

The following drug concentrations were employed in the incubation medium Epinephrine 0.5 /ml, ATP 2 mg/ml, phentolamine 0.1 mg/ml, and phenoxybenzamine 0.03 mg/ml.

N.S. denotes Not Significant or a "p" level greater than 5% (0.05).

Chapter V

Discussion

Medial Sclerosis and Vasopressor Phenomena

Several investigators have suggested that the medial sclerosis following an epinephrine regimen is a consequence of the vasopressor effects of this drug (Lorenzen, 1961; Taylor, 1954; Waters and de Suto-Nagy, 1950a; and O'Sullivan, 1962). Although this position is not without merit, it is questionable whether such a narrow interpretation is adequate to explain the various observations made in this report and by other investigators. The present studies on vasopressor and metabolic effects of epinephrine and thyroxine suggest that a broader assessment of the role of these two drugs in medial sclerogenesis might be more consistent with experimental data.

Our own assessment of the vasopressor theory of medial arteriosclerosis has been conditioned by a paucity of critical examinations of the vasopressor relationships involved. Qualitative mention of the potentiation of epinephrine's pressor action by thyroxine has been invoked by O'Sullivan (1962), Lorenzen (1961a and b) and others as categorical evidence that epinephrine induced medial sclerosis is primarily a consequence of the pressor actions of this drug. Secondly, it was assumed that drugs which potentiate this pressor effect will increase the incidence and severity of the arterial lesions. Conversely, it was assumed that drugs which interfere with the pressor actions of epinephrine will provide protection against arterial lesions (O'Sullivan, 1962; Waters and de Suto-Nagy, 1950a; and Lorenzen, 1961a, b, 1962a, b).

Examination of the present data suggest that some discrepancies exist between the ability of a drug to prevent rise in systolic or diastolic

pressure in acute experiments and its ability to prevent epinephrine induced medial sclerosis. Pertinent to this observation is the data in Figs. 1 - 9 and Tables 1 - 3. Thus iproniazid pretreatment at the dose employed decreases the net and percentage increase in systolic pressure following epinephrine but according to O'Sullivan (1962) on a subacute basis greatly augments both the incidence and severity of epinephrine induced lesions. Conversely ATP which does not significantly inhibit the net or percentage increase in systolic pressure caused by epinephrine infusion is perhaps one of the most effective agents known in preventing medial sclerosis induction by epinephrine (Davis et al. 1955). Phentolamine and phenoxybenzamine produced an inhibition of pressor response to epinephrine to an almost equal degree (Figs. 2, 3, Tables 1a, b). Yet, according to O'Sullivan (1962), when administered prophylactically on a subacute basis phenoxybenzamine is rather ineffectual in preventing epinephrine induced medial sclerosis while phentolamine shows good activity in this regard. Norepinephrine, which in the normal animal produces a significantly lower incidence of medial damage than epinephrine, Friedman et al. (1955a, b) produced net changes in systolic and diastolic pressure which were similar to those observed with epinephrine (Figs. 2, 4, Tables 1, 2).

Acute diastolic pressure data similarly do not appear to be fully consistent with a pressor theory of medial sclerogenesis. Iproniazid pretreatment at a dose reported by O'Sullivan (1962) to intensify epinephrine induced medial sclerosis did effect a net increase in diastolic pressure after epinephrine challenge as compared with controls; however, this increase was not statistically significant (Tables 2a, b).

Net increase in diastolic pressure produced by epinephrine, in ATP pretreated animals, was virtually identical to that observed in saline pretreated controls (Tables 2a, b). If the increase in diastolic pressure in ATP pretreated animals is expressed as a percentage increase the rise following epinephrine in both systolic and diastolic pressures exceeds the values for thyroxine, which is reported to accentuate the medial damage produced by epinephrine (Michulicich and Oester, 1951). Although the above data raise questions regarding the sole importance of systolic and diastolic blood pressure changes following infusion of epinephrine as etiological factors in medial arteriopathy it should be kept in mind that these results were obtained in acute experiments. It is entirely possible that subacute administration of these various drugs could conceivably alter interpretation of the etiological role of pressor effects.

Analysis of hemodynamic data based on the pre-infusion systolic and diastolic pressure suggests some correlation may exist between the ability of a given drug to increase or decrease systolic and diastolic pressures and the ability of a drug to intensify or prevent medial arteriopathy. Thus ATP and phentolamine, reported to be of value in preventing epinephrine arteriopathy, (Davis et al., 1955; O'Sullivan, 1962) decreased systolic and diastolic pressures to a greater extent than the other drugs studied (Tables 1a, 2a). Conversely, thyroxine which according to Michulicich and Oester (1951) augments epinephrine arteriopathy was most potent in increasing systolic and diastolic pressure prior to epinephrine infusion. However, iproniazid at a dose reported by O'Sullivan (1962) to augment epinephrine arteriopathy, decreased pre-infusion systolic and diastolic pressures (Tables 1a, 2a).

Thus, modification of the epinephrine pressor response following various drugs does not appear to be wholly consistent with a hemodynamic etiology of epinephrine arteriopathy; yet, the possibility cannot be excluded that other facets or extensions of cardiovascular studies could show positive correlations.

Recent studies from another laboratory question the sole importance of vasopressor effects in producing arterial lesions. Gutstein and Cooper (1964) have reported that intravenous administration of Vasopressin either intravenously or intraperitoneally three times weekly for a period of 6 to 11 weeks did not produce any observable arterial lesions. When, however, this regimen was supplemented by concomitant cholesterol feeding, medial and intimal calcification was observed in about 50% of the animals. Gutstein (personal communication) has indicated "We are in complete agreement with your statement that, factors other than vasopressor effects alone may play an important role in the development of this type of pathology."

A similar position in regard to medial sclerosis has been taken by Milch and Loxterman (1964) who have taken issue with Lorenzen's (1959, 1961a, b) suggestion that medial sclerosis following epinephrine is a consequence of the vasopressor effects of this drug. Milch and Loxterman (1964) have recently stated that in their opinion "Lorenzen's data are subject to additional interpretation in that his results also support the hypothesis that epinephrine initiates and aggravates a defect in the "biochemical architecture" of aortal wall which renders it susceptible to degenerative change, and that the defect is manifested by an alteration in aortal mucopolysaccharide metabolism". In the analysis of their studies on

mucopolysaccharide metabolism of the rabbit aorta Milch and Loxterman (1964) conclude, "the data support the hypothesis that epinephrine induces a defect in the biochemical architecture of the arterial wall which renders it susceptible to degenerative change".

Further analysis of significance of pressor effects suggests that if arterial damage is a sole or primary consequence of vasopressor effects a correlation should exist between areas of maximal pressure and localization of lesions. The pressure pulse moving toward the periphery at a velocity considerably in excess of the velocity of corpuscular elements of the blood causes a rebound of blood from regions of sharply increasing resistance, thus giving rise to a decrementing diphasic centripetal wave, (Wiggers, 1952). Since this centripetal wave and the pressure pulse are both present in the same system of "tubes", they summate effecting a major alteration in contour of the pressure pulse. The propagation of this reflected pulse is decremental. Therefore, a greater amplitude is available at the more distal portions of the arterial tree giving rise to a greater net wave amplitude. At points more proximal to the ventricle the attenuated reflected wave causes less of a modification of the ventricular pulse and the resultant arterial pulse is lower in magnitude.

Thus contrary to expectations the amplitude of the pressure pulse recorded at points more distal to the heart is increased in amplitude and therefore the pressure recorded at more distal points is greater in magnitude than at points more proximal to the heart.

Thus if the mechanical effects of the pulse wave were the sole factors operant in medial damage it would be expected that the lesions would occur at maxima of the reflected ventricular waves. Such maxima would place medial lesions in the more peripheral arteries. Roszkowski (1956) in discussing localization of epinephrine induced lesions stated "In general, only the thoracic aorta was involved." He further states "Apparently large trunks are the vessels primarily affected, small arteries and arterioles did not appear to be altered." This apparent lack of correlation between pulse wave maxima and localization of arterial lesions does not however completely rule out the importance of hemodynamic factors. It is entirely possible that mechanical effects of the arterial pulse wave might exert their damaging effects not at their maxima, but rather at sites in the arterial wall which by their structural or biochemical characteristics are particularly susceptible to mechanical damage. Such an interpretation is consistent with Blumenthal's (1959) hypothesis.

A recent study by Wolinsky and Glagov (1964) examined the effects of intramural tension on the static mechanical properties of the rabbit aorta. In their experiments the rabbit aorta was removed under anesthesia and all branches tied. The aorta was washed in saline and attached to a cannula at the proximal end, while the distal end was closed by a pinch clamp. After placing the preparation into a small airtight chamber, various hydrostatic pressures were applied to the isolated arterial segment. Then, the saline was flushed out by a fixative solution and after a suitable period (usually 2 hours), the segment was removed and studied by both conventional histological techniques as well as via electron microscopy. Several observations pertinent to the present discussion were made.

Wolinsky and Glagov (1964) found that at physiological pressures the aortic media functions as a two phase system in which the "circumferentially aligned collagen fibers bear the tangential stressing forces while the elastin net distributes the stressing forces uniformly throughout the wall". The intraluminal pressures employed in their studies varied between 5 and 200 mmHg.

Similarly Roach and Burton (1957) MacDonald (1960) and Bergel (1961) have suggested on the basis of their studies that the circumferentially arranged medial collagen is the effective structural component of the aorta at and above physiological pressures. O'Sullivan's (1962) description of rupture of longitudinal elastic fibers the first day after administration of epinephrine is difficult to reconcile with these reports. Wolinsky and Glagov (1964) state that because of the two phase structural system present in the rabbit aorta the more extensible elastin component distributes the stresses uniformly and that stresses around flaws are transferred to other parts of the arterial wall. Also mentioned is the observation that "the aorta rarely breaks despite numerous irregularities and flaws of composition and architecture which increase with age." In their concluding remarks Wolinsky and Glagov (1964) state "focal medial defects and medial pressure and tension gradients may be relatively insignificant factors in the development of aortic disease". Unfortunately these studies were performed using static rather than pulsating pressures and therefore do not correlate as closely with in-vitro conditions as might be desired. It should also be emphasized that the studies of Wolinsky and Glagov do not preclude the occurrence of focal medial lesions in the aorta.

Although the preceding discussion raises a number of questions concerning the significance of pressor effects, it does not obviate the

participation of all cardiovascular phenomena in the genesis of epinephrine induced arteriopathy. One such aspect, hydrostatic tension, may be of significance. Tension in a given vessel is an expression of the stretching force exerted by the hydrostatic pressure on the vessel wall, which in turn is opposed by the tension developed in the wall of the vessel by its smooth muscle and elastic components. Hydrostatic tension in a vessel may be calculated by applying the law of Laplace, represented by the equation $T = P \times R$; in which P is the excess of the hydrostatic pressure inside the vessel over the local surrounding tissue pressure in dynes/cm.^{2}, T, the tension developed in the wall in dynes per square centimeter; and R, the radius of the cylinder in centimeters (MacDonald, 1960). Using literature data Blumenthal (1956) has calculated levels of hydrostatic tension in various arteries. Thus, in an aorta with a radius of 1.3 cm. and a mean pressure of 100 mm.Hg., the tension on the wall was calculated to be 170,000 dynes/cm.^{2}. In arteries such as the hepatic or splenic having a smaller radius, eg. 0.15 cms., the tension was calculated to be 20,000 dynes/cm.^{2}. In the rabbit aorta the hydrostatic tension acting on the vessel wall should therefore be considerably higher than that found in vessels of smaller radius as the femoral artery. Such a tension gradient would parallel the localization of epinephrine induced lesions reported by Roszkowski (1956). This line of reasoning suggests that protection against possible mechanical aspects of epinephrine induced arterial damage could be produced by reduction of arterial pressure changes via decrease in peripheral resistance, accompanied by no change or a decrease in aortic radius. Regretably such qualitative data on these parameters was not obtained in the present study, due to the difficulties attendant in their determination.

Biochemical Aspects

From the very outset of this examination of epinephrine induced medial sclerosis the uniqueness of the metabolism of arterial tissue became evident. One of the striking points noted was that aortic tissue exhibits a rather low respiratory rate. Kirk et al. (1953, 1954) have reported that the respiratory rate of human aortic tissue is only about 2% that of liver tissue Briggs et al. (1949) have reported that in the rat aortic respiration proceeded in vitro at a rate about one-tenth that of liver slices from the same animal. Regrettably these two studies did not offer comparisons of aortic tissue with intestinal smooth muscle or skeletal muscle. Fontaine et al. (1960) have reported that in the ox aorta sixty to ninety percent of glucose is metabolized via glycolysis, while most of the remainder varying from 10 to 30% is metabolized via the Krebs cycle. To the contrary, the liver exhibits an aerobic glycolysis of about 30 to 40% and oxidative metabolism of about 60 to 70%. Thus the efficiency of carbohydrate metabolism in aortic tissue is only about 40% of that observed in liver (Fontaine et al., 1960). Kirk (1963) has calculated diffusion coefficients for oxygen in human aortic tissue and states "These estimated depths to which oxygen can penetrate through diffusion indicate a low margin of reserve for the oxygen supply of the normal aortic wall." Lehninger (1959c) has also indicated that "If the diffusion of oxygen to the cells in the media is at all limited, then it can reasonably be expected that the diffusion of glucose, the substrate for glycolysis must also be limited, since both are brought into the cells by physical diffusion, so far as is known." Fontaine et al. (1960) has indicated that distances between adjacent capillaries "in parenchymatous organs averages from ten to thirty microns, but it increases to five hundred microns for arterial walls, thus

illustrating the poor vascularization coming from the vasa vasorum." This compares with mean capillary distances of 17 to 83 microns in skeletal muscle (Pearson 1962). A similar position is held by Kirk et al. (1955) who state that the arterial wall fed either by diffusion from the lumen or by the vasa vasorum in the adventitia and media presents extended diffusion distances for the inward passage of substrate and oxygen and outward diffusion of metabolites. Another peculiarity of arterial tissue is its low concentration of ATP which in the ox aorta is approximately 1.34 uM/100 gms. (Kempf et al. 1961). This level is only about two to four percent of that reported in skeletal muscle by Bendall and Davey (1957).

The rather low metabolic efficiency and extended diffusion paths for metabolic substrates in the aorta suggested that this tissue might be susceptible to biochemical defects. Therefore, several exploratory studies were undertaken in an effort to survey biochemical aspects of arterial metabolism which might be profitably explored to gain insight into possible biochemical aspects of epinephrine arteriopathy. In the course of such pilot experiments it was found that increases in blood glucose and lactic acid following epinephrine levelled off approximately two to three hours after administration of this drug. Subsequently tissue experiments were conducted in which aortic levels of glycogen, phosphate, and lactic acid were determined at two, four and eight hours after epinephrine administration in fasted rabbits. A significant depletion in aortic glycogen was observed four hours after epinephrine administration. This decrease in glycogen of approximately 40% was paralleled by a decrease of approximately

66% in skeletal muscle. At the peak depletion time aorta contained 40 mgs.% and skeletal muscle 41 mgs.% glycogen (Table 4). The smaller percentage decrease in aortic glycogen is interpreted as being in part a consequence of a lower level of epinephrine controlled glycolytic activity in this tissue. Such an interpretation is consistent with the report by Klainer et al. (1962) who reported a very low level of cyclase in dog aorta as compared with dog skeletal muscle.

Since Fouts (1962) has reported cumulative effects on drug metabolism following repeated epinephrine administration, subacute experiments were conducted in which epinephrine, thyroxine, and epinephrine plus thyroxine were administered daily for a period of two weeks, in accordance with the procedures used by Oester et al. (1954, 1959). Drug administration was terminated twenty-four hours previous to autopsy with the expectation that any alterations in levels of tissue constituents would be of a sub-acute rather than acute nature. Aortic glycogen levels which on an acute basis were decreased by epinephrine, were not significantly changed by subacute epinephrine administration. Thyroxine administration for a two week period did result in a slight decrease in aortic glycogen, an effect which appeared to be potentiated by concomitant administration of epinephrine (Fig. 10, Table 5). Comparative determinations on skeletal muscle indicated no statistically significant changes of glycogen level in any of the groups. Aortic glucose levels were significantly elevated in animals treated with epinephrine or epinephrine plus thyroxine (Fig. 11, Table 6). Skeletal muscle glucose was significantly elevated only in animals receiving epinephrine alone. Subacute epinephrine alone or in combination with thyroxine did not produce any significant changes in lactic dehydrogenase or lactic acid levels in the

aorta (Tables 7, 8).

The lower levels of aortic phosphate (Table 9) observed in both thyroxine treated groups are difficult to reconcile with Hoch's (1962) statement that tissue levels of inorganic phosphate usually rise in animals treated with thyroxine and are contrary to our expectations. Thus in the biochemical studies a few changes occurred which appear to be related to effects of epinephrine on carbohydrate metabolism. The acute depletion of aortic glycogen following epinephrine appears to be a consequence of stimulation of glycolysis. The subacute increase in aortic glucose could be interpreted as a decrease in utilization or a consequence of increased uptake from the blood.

Metabolic Studies Using Radioisotopically Labelled Substrates

These studies disclosed several points which may be important (Fig. 16-25, Tables 10-14). The first of these, stimulation of $C^{14}O_2$ production by epinephrine, was a rather consistent finding, when aortic tissue was incubated in a medium containing glucose-6-C^{14} (Fig. 16-19, Tables 10, 11, 13). Pretreatment of animals for two weeks with thyroxine prior to experiments more than doubled $C^{14}O_2$ production from glucose-6-C^{14} (Figs. 16, 17). When aortic tissue was incubated in a medium consisting of Krebs-Ringer bicarbonate + 0.5μM/ml. of non labelled glucose and glucose-6-C^{14} as a labelled substrate an accentuation of the effects of epinephrine were noted. Under these conditions epinephrine addition increased the formation of labelled carbon dioxide nearly fourfold, (Figs. 19, 22, Table 11) while norepinephrine appeared impotent (Table 13). ATP, phentolamine and phenoxybenzamine appeared to inhibit this stimulating effect of epinephrine (Table 11). Phentolamine,

which according to O'Sullivan (1962) is more effective in preventing epinephrine arteriopathy than phenoxybenzamine was the more effective of the two drugs in this regard, even lowering $C^{14}O_2$ production to sub-control levels (see Fig. 19). ATP which according to Davis et al. (1955) is also an excellent prophylactic agent against epinephrine arteriopathy did not inhibit $C^{14}O_2$ production to as great an extent as either phenoxybenzamine or phentolamine suggesting that its role as a preventive agent may be unrelated to its effects on carbohydrate metabolism.

Under similar conditions aortic tissue from animals pretreated with thyroxine for two weeks exhibited a rate of $C^{14}O_2$ production twice that observed in control tissues from normal animals (Figs. 20, 23, Table 12). However, the rate of formation of labelled carbon dioxide by normal aortic tissue under the influence of epinephrine was nearly double that found in the case of the thyroid controls. Aortic tissue from thyroxine pretreated animals was also susceptible to the metabolic stimulating effect of epinephrine in that addition of epinephrine nearly doubled $C^{14}O_2$ production in these tissues (Figs. 20, 23, Table 12). As in the case of aortic tissue from normal animals both phentolamine and phenoxybenzamine inhibited the epinephrine stimulating effect, the latter drug being less effective in this regard. ATP did not appear to affect the epinephrine induced stimulation of metabolism in tissue from thyroxine pretreated animals.

From the above discussion it is apparent that there may be a correlation between stimulation or inhibition of oxidation via the Krebs cycle and production or prevention of epinephrine arteriopathy. Thus, epinephrine which when administered alone to rabbits on a subacute basis produced medial arterial sclerosis (Oester 1959) also causes a substantial rise in rate of

oxidation via the Krebs cycle.

Thyroxine administered alone on a subacute basis does not produce medial sclerosis, but when administered concurrently with epinephrine appears to greatly potentiate the sclerogenic effect of the latter drug (Michulicich and Oester 1951). In the studies where unlabelled glucose was present in the medium the rate of oxidation of labelled glucose although higher in thyroxine pretreated animals did not reach the levels produced in normal animals by epinephrine (Figs. 20, 23, Tables 11, 12). There also appears to be a consistent relationship between the potency of the two adrenergic blocking agents in inhibiting oxidation by arterial tissue under the influence of epinephrine and the ability to protect against epinephrine sclerosis in the intact animal. Phentolamine which is the more potent of the two drugs in inhibiting epinephrine sclerosis (O'Sullivan 1962) is also more potent in inhibiting oxidation by aortic tissue treated with epinephrine. It may be recalled that phentolamine and phenoxybenzamine showed similar potency in inhibiting the pressor effect of epinephrine in the intact animal. Thus in this case there appears to be a better correlation between metabolic inhibition and ability to protect against epinephrine sclerosis than is the case in regard to inhibition of pressor effects.

Hexose Monophosphate Shunt

In aortic tissue from normal animals the yield of $C^{14}O_2$ obtained from glucose labelled in the number one position was nearly twice that obtained from glucose labelled in the number six position (Figs. 19, 20, 22, 23). Similar observations have been made by Winegrad et al. (1958) and Lynn et al. (1960) in adipose tissue. Sbarra et al. (1960) have reported that production of labelled carbon dioxide by guinea pig aorta is greater when the glucose

substrate is labelled in the number one position than when the label is in the number six position, paralleling our results. The yield of $C^{14}O_2$ from glucose-6-C^{14} is believed to reflect oxidation via the Krebs cycle, while the increase in yield of labelled CO_2 from glucose-1-C^{14} is believed to be a consequence of the participation of the hexose monophosphate shunt (phosphogluconate oxidative pathway).

If the hexose monophosphate shunt is not present or operant in a particular tissue the yield of radioactive carbon dioxide is the same regardless whether the label is in the one or six position. The differential observed in our experiments thus, suggests that, since the yield of radioactive carbon dioxide is greater when glucose-1-C^{14} is used as a substrate as compared with glucose labelled in the six position the hexose monophosphate shunt is operant in rabbit aorta.

In aortic tissue from normal animals the ratio of $C^{14}O_2$ formed from glucose-1-C^{14} as compared with that formed from glucose-6-C^{14} was about two (Fig. 19, Table 14). This same ratio in aortic tissue from animals pretreated for two weeks with thyroxine was approximately six, thus indicating a rather significant stimulation of the hexose monophosphate shunt by thyroxine in this tissue (Fig. 23, Table 14).

When epinephrine was added to aortic tissue from normal animals (Fig. 19), yield of labelled carbon dioxide from glucose-6-C^{14} was increased almost four fold. On the other hand, in similar experiments where glucose-1-C^{14} was used as a substrate the yield of labelled carbon dioxide was increased by only a factor of less than fifty percent. The added increment is interpreted as stimulation of oxidative phosphorylation in the Krebs cycle with no

apparent stimulation of the hexose monophosphate shunt, but rather in inhibition of catabolism via this route.

Aortic samples from thyroxine pretreated animals challenged with epinephrine exhibited a level of $C^{14}O_2$ from glucose-1-C^{14} which was not significantly different from that observed in controls (Figs. 20, 23, Table 14). This observation indicates that an inhibition of the hexose monophosphate shunt occurred in thyroxine pretreated animals.

It is felt that the data obtained on the participation of the hexose monophosphate shunt in normal and thyroxine pretreated aorta may assist in the resolution of certain inconsistencies in interpretation of the pharmacological role of thyroxine in experimental arteriosclerosis. It is a well known fact that concomitant administration of thyroxine to animals on a high cholesterol diet provides protection against atheroma formation (Constantinides 1960). Conversely rabbits treated with epinephrine and thyroxine appear to be more prone to development of arterial lesions when cholesterol is administered intravenously [Oester et al. (1955)] or when fed in the diet [Constantinides et al. (1960)].

Koritz and Peron (1957) have reported that TPNH and hexose monophosphate shunt enzymes are required for steroid biosynthesis in cell free systems. Thus, it is possible that the TPNH generated in the hexose monophosphate shunt is utilized in the "detoxification" of cholesterol making it more water soluble and thus increasing its potential for removal and excretion via the kidney. This supposition is further strengthened by the report by Talalay and Williams-Ashman (1958) that estradiol appears to function as an essential co-factor in a DPN-TPNH linked transhydrogenation reaction. Also, the lower incidence of coronary arteriosclerosis in premenopausal females has been

ascribed to endogenous estrogen. Thus, if cholesterol atherosclerosis is looked upon as a consequence of insufficient hexose monophosphate shunt activity, drugs which stimulate shunt activity should provide protection against atherosclerosis. Our findings which show a rather striking stimulation of the hexose monophosphate shunt activity by thyroxine suggest that this may be the case. The potentiation of cholesterol atheroma formation by epinephrine-thyroxine regimen may be a consequence of the inhibition of this shunt by epinephrine. Our studies indicate that such an inhibition does occur.

From our discussion of the stimulating effect of epinephrine on $C^{14}O_2$ production from glucose-6-C^{14} by arterial tissue it is apparent that this increase in metabolism is primarily if not solely due to effects on the Krebs cycle. Since it is generally believed that the mitochondria are the site of oxidative phosphorylation via the Krebs cycle we feel that it might be reasonable to postulate a hypothetical site of action for epinephrine on the mitochondrion in arterial tissue.

If the increase in oxidative phosphorylation is due to a swelling of arterial mitochondria we might expect some parallelism between results which we have obtained and data reported on the swelling of mitochondria by Lehninger (1959a, c) and others. Chappel (1958) has reported that the swelling of hepatic mitochondria is potentiated by addition of substrate and does not occur in the presence of metabolic inhibitors or absence of substrate. If we compare the results obtained in aortic samples incubated in a medium free of non-labelled glucose with studies in which 0.5μM/ml. of non-labelled glucose was added (Figs. 18, 19) we will note that in the latter case (of added substrate) the effect of epinephrine is vastly enhanced. Results observed with KRP alone were similar to those which KRB alone was used as a

medium. Thus, if epinephrine acted in a manner similar to that of thyroxine we would expect that the extent of swelling and subsequent increase in metabolism would be greater in a medium containing glucose than in its absence. Such an effect was noted in the KRB and KRB + non-labelled glucose experiments (Figs. 18, 19, Tables 10, 11, 13). Lehninger (1959b) has reported that thyroxine induced swelling of mitochondria can be prevented or reversed by addition of ATP. Inhibition of epinephrine induced increase in oxidation by ATP could possibly be explained by assuming that as in the case of thyroxine, ATP inhibits the mitochondrial swelling produced by epinephrine and thus inhibits the increase in oxidative phosphorylation. The effects of phentolamine and phenoxybenzamine are less clear. It might be proposed that these drugs act by interfering with the postulated swelling effect of epinephrine of arterial mitochondria or that they may cause a contraction of mitochondria either directly or indirectly. In any event we do feel that our findings suggest that studies of possible effects of epinephrine on arterial mitochondria might be profitably explored.

According to Zamecnik (1954) addition of ATP to tissue slices increases the incorporation of isotopically labelled amino acids into protein. Conversely Hoch (1962) indicated that thyroxine in high doses is believed to inhibit protein synthesis.

Thus, it was felt that investigation of the effects of epinephrine, thyroxine and ATP on the incorporation of C^{14} uniformly labelled lysine into aortic protein might provide some suggestion as to a possible mechanism by which epinephrine causes medial arteriopathy.

The studies which were performed indicated that epinephrine significantly decreased the incorporation of uniformly labelled lysine C^{14} into

arterial protein (Figs. 24, 25, Table 15). Incorporation of labelled lysine into protein was also significantly inhibited in aortic tissue obtained from animals pretreated for two weeks with thyroxine. Noteworthy was the observation that protein synthesis significantly depressed by thyroxine pretreatment of animals was further depressed by addition of epinephrine to the incubation flasks (Fig. 25, Table 15). Neither phentolamine or phenoxybenzamine appeared to significantly inhibit the depression of protein synthesis caused by epinephrine, which was nullified by ATP.

Data accumulated on both protein synthesis and carbohydrate metabolism suggest that drugs involved in induction, potentiation or prevention of epinephrine induced medial arteriopathy exert an effect on the metabolism of arterial tissue which in general is consistent with their role in arteriopathy. Epinephrine, sine qua non, appears to affect not only oxidative phosphorylation but also appears to exert an inhibitory effect on protein synthesis. The inhibition of protein synthesis observed may be a consequence of the effect of epinephrine on ATP availability. Since ATP has been found to be essential for amino acid activation, reduction in tissue ATP should result in reduced protein synthesis.

An increase in oxidative phosphorylation following addition of epinephrine to arterial tissue should result in a higher tissue ATP level, but only if the rate of destruction of ATP were unaltered. Two recent reports suggest that this may not be the case. Stamm and Honig (1962) have reported that epinephrine interferes with the inhibition of cardiac adenosinephosphatase activity by the so called relaxing substance (RS). According to Parker and Gergely (1960) RS is an important physiological modifier of myofibrillar ATP-ase activity and is thought to terminate contraction in heart muscle by

inhibiting ATP hydrolysis. In Stamm and Honig's (1962) experiments RS obtained from cardiac muscle inhibited cardiac ATP-ase by approximately forty percent. Epinephrine addition to the incubation mixture containing both ATP-ase and RS decreased this inhibition by approximately 45%. This effect appeared to be dose dependent since lower concentrations of epinephrine caused less inhibition of the RS activity. Lynn et al. (1960) have indicated that in adipose tissue "epinephrine and ACTH promote the synthesis of ADP from ATP". They also suggest that the stimulation of respiration by epinephrine in adipose tissue may be mediated via this effect, since it is generally accepted that tissue ADP level is one of the factors controlling rate of carbohydrate metabolism.

Inasmuch as epinephrine has been reported to have an "uncoupling" effect on ATP it is entirely possible that such an effect obtains in arterial tissue. If epinephrine increased the level of available ATP we might expect that protein synthesis would be stimulated when epinephrine was added to arterial tissue. Such an effect was not observed. If the tissue ATP level was a limiting factor in depression of protein synthesis by epinephrine we would expect ATP to reverse this inhibition. This effect was observed. Addition of ATP to arterial tissue where protein synthesis is depressed by epinephrine resulted in a rate of protein synthesis similar to that in controls. Thus, there is reason to believe that inhibition of protein synthesis by epinephrine may be linked to ATP availability.

A possible explanation of protective action provided by phentolamine may lie in substrate conservation. As indicated previously epinephrine produced depletion of aortic glycogen. This effect appeared to be accentuated by concomitant administration of epinephrine and thyroxine. Brewster (1956)

has reported that adrenergic blocking agents can prevent the thyroxine induced increase in metabolism. The results obtained in the present investigation are consistent with his findings since addition of phentolamine to arterial tissue from thyroxine and epinephrine pretreated animals resulted in $C^{14}O_2$ production which was of the same order of magnitude as that observed in aortic samples from normal animals not pretreated with thyroxine and epinephrine. The less potent activity of phenoxybenzamine in preventing epinephrine sclerosis parallels its lesser potency in regard to inhibiting oxidative phosphorylation by aortic tissue.

Failure of norepinephrine to significantly increase Krebs cycle oxidation by aortic tissue is consistent with its lesser potency in inducing medial arteriopathy as compared with epinephrine reported by Friedman et al. (1955a, b). Both drugs produce effects on systolic pressure which are similar.

Although the above discussion has presented evidence suggesting the existence of a metabolic component in the genesis of epinephrine arteriopathy it should be emphasized that in vitro data does not necessarily reflect changes which take place in the normal intact animal. In vitro incubation of tissue suffers from a number of shortcomings in that unavoidable trauma usually occurs in preparation of the tissue sample, the incubation medium only grossly approximates that present normally and in some cases reactions found to occur in isolated tissue proceed only to a limited extent in the intact animal. Despite these disadvantages in vitro incubation has in a number of instances been useful in providing simple models useful in the study of more complex systems.

Conclusions

In general, a poor correlation was observed between the ability of drugs studied to modify, on an acute basis, the pressor response to epinephrine, and their relative effectiveness in preventing or augmenting epinephrine arteriopathy as reported by other investigators. For example thyroxine which significantly augments epinephrine arteriopathy (Michulicich and Oester, 1951) did not potentiate the net rise in systolic and diastolic blood pressure following epinephrine. The net pressor rise following norepinephrine did not significantly differ with that observed following epinephrine; however norepinephrine administered on a subacute basis is reported to cause a lower incidence and severity of medial lesions than epinephrine (Friedman et al. 1955a, b). Although phentolamine and phenoxybenzamine were found to be similar in their ability to reduce the pressor response following epinephrine, phentolamine is reported to be more active as a prophylactic agent vs epinephrine arteriopathy (O'Sullivan, 1962).

ATP which is reported to be one of the most potent prophylactic agents vs epinephrine arteriopathy, was less effective in inhibiting the net epinephrine pressor effect than phenoxybenzamine which is relatively impotent in this regard.

On the other hand the decreases in pre-infusion systolic and diastolic pressures produced by ATP and phentolamine appear to parallel their prophylactic value in epinephrine arteriopathy as reported by Davis et al. (1955) and O'Sullivan (1962). The possibility that protection against mechanical aspects of epinephrine induced arterial damage could be a consequence of reduction of intravascular wall tension is raised. Although the present report questions the importance of increases in systolic and

diastolic pressures in the genesis of epinephrine arteriopathy, it is entirely possible that acute cardiovascular relationships noted in the present study may not reflect subacute cardiovascular effects.

Data in this report suggest that study of the relatively unexplored metabolic effects of epinephrine on arterial tissue may assist in the identification of a specific lesion or lesions which precipitate medial arteriopathy. The rather significant effects of epinephrine in potentiating Krebs cycle oxidation, inhibition of protein synthesis and inhibition of oxidation via the hexose monophosphate shunt suggest further areas for profitable investigation. It is the opinion of the author that one or more of these effects may be contributory factors in production of medial arteriopathy.

Correlation between effects of epinephrine, norepinephrine, thyroxine, phentolamine and phenoxybenzamine on Krebs cycle oxidation and the ability of these several drugs to modify incidence and severity of subacutely induced arterial lesions appears to be consistent with a biochemical hypothesis of medial sclerogenesis. Thus, thyroxine which augments epinephrine arteriopathy, induced an increase in oxidation via the Krebs cycle. Phentolamine which is reported to provide protection against subacute medial arteriopathy (O'Sullivan, 1962) was quite effective in inhibiting effects of epinephrine on Krebs cycle metabolism. Phentolamine was found to be more potent in this regard than phenoxybenzamine which is inferior as a prophylactic agent. Norepinephrine reported by Friedman et al. (1955a, b) to be less effective in inducing medial arteriopathy than epinephrine did not produce any significant effect on Krebs cycle oxidation. Although ATP was not particularly effective in inhibiting effects of epinephrine on carbohydrate metabolism, it was the most potent of the three drugs studied in reversing

inhibition of protein synthesis caused by epinephrine.

It is the opinion of this author that epinephrine arteriopathy is a rather complex phenomenon which is the resultant of several vectoral factors. Data in this report suggest that greater weight be given to biochemical factors than has been heretofore ascribed. The biochemical effects observed following in-vitro incubation of arterial tissue with epinephrine suggest that this drug may exert a biochemical action on the genesis of medial arteriopathy. Such biochemical effects if sufficiently intense could summate with other lesion inducing factors, thus contributing to the genesis of medial arteriopathy. It is possible that a biochemical lesion induced by epinephrine might exert a permissive action on the induction of lesions by hemodynamic factors.

Chapter VI

Summary

1. Subacute administration of thyroxine increased resting systolic and diastolic pressure in the unanesthetized rabbit but did not significantly augment the pressor effect of epinephrine.
2. ATP did not significantly decrease the net increase in systolic pressure following epinephrine in normal rabbits or significantly alter the pressor response to epinephrine in rabbits pretreated for two weeks with thyroxine.
3. Phentolamine and phenoxybenzamine, at doses employed, reduced pressor effects of epinephrine to a similar extent.
4. Iproniazid did not significantly alter the pressor response to epinephrine in the rabbit.
5. ATP and phentolamine reduced control systolic and diastolic pressures to a greater extent than did phenoxybenzamine.
6. Ability of drugs studied to modify the pressor response following epinephrine administration in the rabbit did not appear to correlate well with their ability to modify the incidence and severity of epinephrine arteriopathy as reported in the literature.
7. Epinephrine produced several metabolic effects on rabbit aortic tissue such as depletion of tissue glycogen, stimulation of oxidation via the Krebs cycle, inhibition of oxidation via the hexose monophosphate shunt, and inhibition of protein synthesis.
8. Stimulation of Krebs cycle oxidation following addition of epinephrine to rabbit aorta suggests a possible biochemical mode of action of this drug on vascular tissue.

9. Norepinephrine which is reported to possess lesser sclerogenic potency was less effective than epinephrine in stimulating oxidation in arterial tissue. Both drugs produced similar increases in systolic pressure.
10. Augmentation of hexose monophosphate shunt activity by thyroxine may explain reported protective action against cholesterol atherosclerosis by this drug.
11. Inhibition of hexose monophosphate shunt activity may explain augmentation by epinephrine of intimal lesions following cholesterol feeding reported by Constantinides (1958).
12. There appeared to be good correlation between the ability of drugs studied to augment or inhibit Krebs cycle oxidation and the ability of a drug to intensify or ameliorate epinephrine arteriopathy.
13. ATP which although less potent in inhibiting effects of epinephrine on carbohydrate metabolism, reversed epinephrine induced inhibition of protein synthesis in thyroxine pretreated animals. ATP may act at more than one point in inhibiting epinephrine arteriopathy.
14. Correlation between augmentation or inhibition of biochemical effects of epinephrine in rabbit aorta appears to be consistent with the role of a given drug in increasing or decreasing medial arteriopathy following subacute administration of epinephrine.
15. Data presented suggest that epinephrine produces medial arteriopathy in the rabbit by a mechanism which may include biochemical effects on aortic metabolism.

APPENDIX

SCINTILLATION FLUID

Bray, G. A., Anal. Biochem. 1: 279-285 (1960).

Scintillation fluid for radioisotope experiments, was prepared as follows:

60 gms. Napthalene

4 gms. PPO (2,5, diphenyloxazole) scintillation grade

0.2 gm. POPOP (1,4-bis-2(5-phenyloxazole)-benzene scintillation grade

100 ml. absolute methanol (AR grade)

20 ml. ethylene glycol (AR grade)

Q.S. to 1 liter with p-dioxane (scintillation grade) use purest quality available commercially.

PPO and POPOP were obtained from Packard Instrument Company. It was found that only the purest grade of dioxane could be used since analytical grade yellowed in the presence of Hyamine-10X and caused considerable quenching.

Buffer solutions used for incubation of aortic tissue in radioisotope experiments were prepared as described by Long (1961).

	Krebs-Ringer phosphate (KRP)	Krebs-Ringer bicarbonate (KRP)
0.9% NaCl	100 mls.	100 mls.
1.15% KCl	4 mls.	4 mls.
1.22% $CaCl_2$	-	3 mls.
2.11% KH_2PO_4	1 ml.	1 ml.
3.82% $MgSO_4$	1 ml.	1 ml.
0.1 M $Na2HPO_4$	21 mls.	-
1.3% $NaHCO_3$*		

* $NaHCO_3$ solution was saturated with CO_2 before addition to prevent precipitation of calcium.

Prior to use buffers were gassed with 100% oxygen for the Krebs-Ringer Phosphate buffer or 5% CO_2 95% O_2 for the Krebs-Ringer Bicarbonate buffer.

Inorganic and Labile Phosphate Determination

Method used was that described by Fiske and SubbaRow (1929). The principle of the method is based on the formation of a phosphomolybdate complex with inorganic phosphate and molybdate ion in the presence of sulfuric acid.

Reagents: 5 N Sulfuric acid (reagent grade).

2.5% ammonium molybdate.

Reducing reagent prepared in powdered form and stored in dessicator. Contained 0.2 gm. of 1 amino-2-napthol-4-sulfonic acid, 1.2 gm. sodium bisulfite and 1.2 gm. sodium sulfite. The mixture was ground to a fine powder with a mortar and pestle. For use, 0.25 gm. of powdered reagent was dissolved in 10 mls. of distilled water prior to use.

Standard solution concentrate containing 1.3613 gm. KH_2PO_4 in 1 L. of distilled water was prepared. For use as a standard this solution was diluted 1:10 so that it contained 1 micromole of phosphate per ml.

Tissues were deproteinized as described in lactic acid procedure. A 0.5 ml. aliquot of this supernatant was used. To the unknown sample 1 ml. of sulfuric acid was added, followed by 1 ml. of the ammonium molybdate solution. After mixing, 0.1 ml. of reducing solution was added and volume adjusted to 10 mls. by addition of distilled water. After 10 minutes optical density was measured at 660 mu. in a Beckman DU spectrophotometer. In procedure for analysis of labile phosphate sulfuric acid was added to unknown and volume adjusted to 5 mls.

Samples were then heated in a boiling water bath for seven minutes, cooled and then addition of molybdate and reducing solution was carried out. As a final step the volume was adjusted to 10 mls. Samples were read as described above after 10 minutes. In all analysis standards containing 1.0, 0.5 and 0.25 uM phosphate were also processed and calibration curve used to ex rapolate results. Labile phosphate was calculated by subtracting values for inorganic phosphate from labile phosphate values.

Lactic Acid Determination. Method of Barker & Summerson (1941)

The method is based on the quantitative conversion of lactic acid into acetaldehyde when heated in concentrated sulfuric acid. The acetaldehyde formed is determined via formation of a purple colored complex with hydroxy diphenyl.

Reagents: 4% Solution of $CuSO_4 \cdot 5H_2O$. Concentrated Sulfuric Acid (Reagent grade). 1.5% Solution of p-hydroxydiphenyl in 0.5% NaOH. Lactate standard.

Subsequent to removal of tissues from the animal the weighed sample was homogenized in a Potter Elvehem type glass homogenizer, immersed in an ice bath. In all cases the tissue sample was immersed in 2.5 cc. of ice cold distilled water for homogenization and the resultant supernatant freed of cellular debris was processed as suggested by Barker and Summerson. A 1 cc. aliquot was deproteinized by addition of 1 cc. of 10% TCA and the supernatant used for determination of tissue lactic acid. The size of aliquot used was usually 0.2 mls. This was adjusted up to a volume of 1 cc. by addition of distilled water. To the test tube containing this aliquot in a volume of 1 cc., 1 drop of 4% $CuSO_4$ was added, and the tube chilled in an ice bath. After equilibration, cold concentrated sulfuric acid was added from a pipetting syringe set at 6 mls. During addition of acid, tube was swirled to minimize heating.

This swirling was continued until tube and contents were approximately at room temperature. When addition of reagents to all tubes in the series was completed, the tubes were withdrawn from the ice bath in which they were stored and heated in a boiling water bath for 5 minutes.

On removal from the boiling water bath, they were again cooled to below 20°C. Two drops of the p-hydroxydiphenyl reagent were added from a 1 ml. pipette (0.1 ml.) and the reagent dispersed by stirring with a thin glass rod. The tubes were then "aged" for 30 minutes in a water bath at 25°C room temperature. During this period the precipitated p-hydroxydiphenyl reagent was resuspended at least three times. At the end of the 30 minute period the excess p-hydroxydiphenyl reagent was destroyed by heating the tubes in boiling water for 90 seconds and then recooling. Color development was measured in a Beckman DU spectrophotometer at 560 mu. Prior to reading of samples the instrument was adjusted to zero optical density by use of a sulfuric acid blank, in accordance with Barker and Summerson's recommendation. Along with each series of unknowns a calibration curve was established using known amounts of lactic acid. These were prepared to contain 2, 4 and 6 mcg. of lactic acid per tube. Any samples which gave readings in a range beyond an O.D. of 0.600 were rerun at higher dilution.

Lactic Dehydrogenase (LDH) Assay - for use in arterial tissue. Kittinger et al. (1960).

The principle of the method is based on the oxidation of DPNH and reduction of pyruvic to lactic acid. DPNH has a strong absorption band at 340 mu. while DPN absorbs very little at this wave lenth. Thus, change in optical density is proportional to oxidation of DPNH. Reagents: 4×10^{-3} M Na Pyruvate, 1.2×10^{-4} M DPNH, 0.1 M phosphate buffer pH 7.8.

Pieces of aortic or skeletal muscle tissue were homogenized as previously described in the lactic acid analytical method. Total volume of saline used was 2.5 mls. Homogenate was cleared by centrifugation for 10 minutes at 3000 rpm. The following reagents were added to a Beckman DU cuvette centrifuge tube 1) 0.1 ml. of NaPyruvate, 2) 0.1 ml. of DPNH and 3) 2.75 ml. phosphate buffer. This solution was stirred with a fine glass rod and 0.05 ml. of tissue homogenate supernatent were added and again the contents were stirred. The resultant solution was rapidly placed in a Beckman DU spectrophotometer and change in optical density recorded at 30 second intervals, for a period of three minutes. Rate of change in optical density was compared with standard curve obtained by adding varying amounts of DPNH to the buffer system described above. Activity was expressed as rate of substrate reacting per unit of time, one unit being equivalent to oxidation of 1 micromole of DPNH per minute. All data were corrected for dilution and tissue weight.

Glucose Determination (Glucose Oxidase Procedure)

This procedure is based on the oxidation of glucose via glucose oxidase forming H_2O_2 and gluconic acid. The peroxide formed oxidizes a chromogen which in reduced form does not absorb at 400 mu. Reagent: Glucostat reagent, Worthington Biochemical Corp., Freehold, N. J. was dissolved in distilled water and chromogen added. The final volume was adjusted to 90 ml. in accordance with the manufacturers instructions.

Deproteinized solution was obtained by treating tissue homogenate or blood with 1 ml. each of the Somogyi reagents (2.0% $ZnSO_4 \cdot 7H_2O$ and 1.8% $Ba(OH_2)$. Thus, blood was processed by adding 0.2 ml. of whole blood to 1.8 ml. of H_2O, and 1 ml. of each of the Somogyi reagents was added. Tissue extract was prepared by homogenization in distilled water in a volume of 5 mls. A 2 ml. aliquot of the filtrate was then processed as described for blood with addition of 1 ml. of each of the Somogyi reagents.

Color Development

Two mls. of the tissue extract were placed in a test tube, 8.0 mls. of glucose oxidose reagent were then added and the resultant solution was incubated at 37°C for 30 minutes. At this point one drop of 4NHCl was added to each tube to stop the reaction. Samples were then read in a Beckman DU. spectrophotometer at 400 mu. Instrument was set at 100% transmission by use of a reagent blank. A calibration curve was established using known amounts of glucose for each series of determinations.

Protein Determinations

Protein was determined by the method described by Gornal et al. (1948).

The method is based on the formation of a violet color when proteins or polypetides containing amide or peptide linkages react with copper sulfate in a strongly alkaline solution.

Reagents: Biuret reagent (Gornall et al, 1948) containing the following: 1.5 gm. cupric sulfate ($CuSO_4 \cdot 5H_2O$). 6.0 gm. potassium tartrate ($NaKC_4H_4O_6 \cdot 4\ H_2O$) dissolved in approximately 500 ml. of distilled water to which is added 300 ml. of 10% NaOH. Q.S. to 1 liter.

One ml. of tissue supernatant, obtained by homogenizing 40 to 100 mg. of tissue in 5 ml. of distilled water and centrifugation at 3000 rpm. for 15 minutes was placed in a 12 ml. centrifuge tube. To this was added 4 ml. of biuret reagent and the resultant solution was allowed to stand for 30 minutes at room temperature. Unknowns and standards were read in a Beckman DU spectrophotometer at 540 mu. adjusted to zero O.D. with distilled water. Reagent blank and protein standards were used as controls in each series of analysis. Protein standard was obtained from Dade reagents (Lab-Trol) and adjusted to contain .083%, 0.1675% and 0.2515% of protein in each respective sample.

Tissue Glycogen Analysis

Procedure followed was essentially that described by Seifter et al. (1950). This method is a modification of the procedure of Good et al. (1933).

Principle of the method involves the separation of glycogen from tissue and other contaminants, hydrolysis of glycogen to glucose and determination of glucose present by the development of color with anthrone reagent.

Reagents: 30% KOH, 95% ethanol, 60% ethanol, 0.2% anthrone in 95% sulfuric acid (reagent grade).

Aortic and skeletal muscle tissues were rapidly excised from rabbits, weighed on a Mettler balance, and placed in a test tube containing 1 ml. of 30% KOH. The test tube was then placed in a boiling water bath and heated for twenty minutes. At the end of this time, the tube was allowed to cool to room temperature and 1.25 mls. of 95% ethanol added. Contents were mixed with the aid of a thin glass rod which was rinsed with 60% ethanol which was added to the contents of the tube. The sample was then heated to boiling in a water bath, cooled to room temperature and centrifuged for 15 minutes at 3000 rpm. The supernatant was decanted and the precipitate redissolved in 1 ml. of distilled water and reprecipitated as described above. This procedure was repeated twice. In preparation for analysis the sample of glycogen was dissolved in 2.5 mls. of distilled water and cooled in an ice bath.

Cold anthrone reagent was then added from a pipetting syringe and the contents of the tube swirled in the ice bath. The tube containing glycogen and anthrone reagent were "capped" with glass marbles and heated in a boiling water bath for 10 minutes. Samples were then cooled and stirred with a thin glass rod and read at 620 mu. in a Beckman DU spectrophotometer. A reagent blank was used to adjust the instrument to zero O.D. Standards containing 10, 20 and 50 micrograms of glucose were also run with each set of unknowns. Tissue samples used in the analysis varied from about 40 to 100 mg. depending on availability.

Incubation Flasks

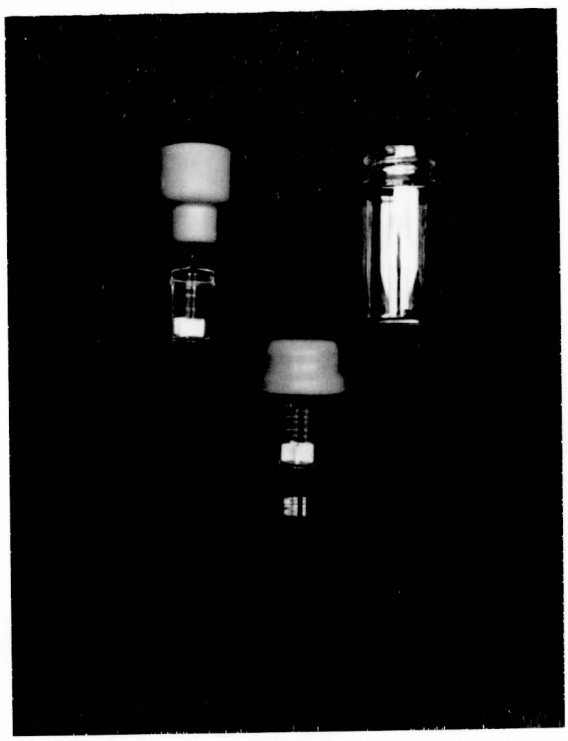
Problems associated with cleaning of Warburg flasks in radioisotope experiments are particularly disconcerting since not only must the vessel be chemically clean but must also be free of radioisotopes which when present in only trace quantities are likely to alter results significantly. During the course of pilot experiments preliminary to the radioisotope experiments described in this report the desirability of having a "disposable Warburg flask" became evident. As a result a disposable metabolism flask was developed, and is pictured on the subsequent page. The components are rather inexpensive, total cost being in the neighborhood of twelve cents, and are readily available as stock items from most scientific supply houses. These are:

1. Serum vial stoppers (rubber).
2. Disposable plastic "Dispo" beakers (2 ml.).
3. Copper wire 22 gauge.
4. Filter paper.
5. Disposable glass counting vials.

Assembly procedure is as follows:

1. Fold filter paper in shape of fluted stars approximately 3/16" high and with a radius of 7/16". A three layered fold was found to have satisfactory absorbancy.
2. Pierce disposable plastic beakers with a red hot 23 gauge needle so as to produce two holes at opposite ends of the the top of the beaker.

3. Place filter paper into bottom of beakers and "tie" the beaker with copper wire (as per photograph).
4. Thread remaining end of copper wire through hole previously punched through bottom of lower margin of rubber serum vial stopper. Snip off any remaining ends with wire cutter.



Prior to use 0.2 ml. of 4 N KOH is applied onto the filter paper in the beaker taking care not to touch sides. A 23 gauge hypodermic needle attached to a 1 ml. tuberculin syringe was used.

The counting vials were prefilled with previously gassed buffer and radioactive substrate prior to use. After tissue samples were cleaned and weighed the plastic cap on the counting vial containing buffer was removed and tissue samples placed in the medium. These glass vials were then placed in a metabolic shaker and gassed via a manifold to which 18 gauge needles were attached for entry of oxygen and a second needle for exit of air mixture. After appropriate gassing the serum vial stoppers (with attached beaker containing KOH) were rapidly affixed and addition of drugs was performed by injection through the rubber stopper. When it was desired to terminate the incubation 0.5 ml. of 4 N H_2SO_4 was added via a 23 gauge hypodermic needle and syringe. The vial was then shaken on an Eberbach shaker for twenty minutes at low speed. At this time the vials were removed from the shaking apparatus, the caps removed and the plastic beakers washed with 1 ml. of distilled water which was allowed to flow into the vial. Tissue samples were then removed and the vial containing medium and radioactive substrate was capped with a plastic screw cap and stored in a freezer for additional analysis.

To each beaker (hanging from the serum vial cap) 0.1 ml. of 1% Triton X200 was added to assure wetting and release of $C^{14}O_2$ trapped by the KOH. The serum vial stopper was then placed atop a second vial containing 0.5 ml. of Hyamine 10 X. Acid (0.4 ml. of 4 N H_2SO_4) was added to the beaker by injection through the rubber serum vial cap, to effect release of the $C^{14}O_2$ which was then bound by the Hyamine.

to effect release of the $C^{14}O_2$ which was then bound by the Hyamine. After shaking for an additional 30 minutes the serum vial cap was removed and 10 mls. of Bray's solution added. The vial was then capped with a plastic screw cap and counted in a Packard liquid Scintillation Counter.

During the course of development of this procedure yield and linearity of $C^{14}O_2$ trapping was determined by use of radioactive $Na_2C^{14}O_2$. Comparison of triplicate samples containing 0.0125, 0.025 and 0.05 uC per vial disclosed that the average variation between triplicate samples averaged 2.2%. Data comparing yield and reproducibility for this procedure and for an alternate method using conventional Warburg flasks is tabulated below:

	"Disposable Warburg Flask"		Conventional Warburg Flask	
	Precision*	% Yield	Precision*	% Yield
0.0125 C	1.0%	122	3.8	110
0.025 C	4.2%	117	0.8	76
0.050 C	0.4%	105	11.1	90
Average	1.9%	114	5.2	92

* Percentage variation between triplicate samples.

% yield was calculated vs $Na_2C^{14}O_2$ counted in Bray's solution. The difficult solubility of this salt in the scintillation medium is in our opinion responsible for the yield in excess of 100% observed.

In summary, the "disposable Warburg flask" described appears, from this author's experience to have the following advantages over and above the conventional Warburg flask when used for radioisotope experiments:

1. Greater accuracy. 2. Allows prefilling of vials which can be conveniently stored. 3. Although somewhat more expensive this procedure allowed economy of time normally spent cleaning ordinary Warburg flasks. 4. Greater simplicity of handling - elimination of volumetric transfers e.g. removal of KOH from center well of Warburg flask and the several rinsings necessary to effect complete removal of trapped $C^{14}O_2$. 5. Allowed a greater number of experiments to be conducted each working day.

It should be emphasized that the procedure described above is <u>not</u> suitable for use when manometric methods are employed.

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APPROVAL SHEET

The dissertation submitted by John F. Zaroslinski has been read and approved by five members of the faculty of the Graduate School.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

15 Jan 1965
Date

J. T. Oesterle
Signature of Advisor