ADRENOCHROME UPTAKE AND SUBCELLULAR DISTRIBUTION IN THE ISOLATED RAT HEART

A Thesis

Presented to the

University of Manitoba

In Partial Fulfilment of the

Requirements for the degree

MASTER OF SCIENCE

IN

PHYSIOLOGY

by

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April, 1980

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Acknowledgements

I would like to thank all those people who made possible and helped me with the completion of this thesis. This includes my parents, Maureen Otten, Micheal Daley, Dr. V. Panagia and all the others who helped. I would especially like to thank Dr. Satoshi Takeo for his kind help and I would like to thank Dr. Dhalla for his help and the opportunity given to me to do my work.

This thesis is dedicated to my mother and father, with great love and appreciation.

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

I Abstract

Adrenochrome, an oxidation product of epinephrine, has been implicated in the production of myocardial cell damage in catecholamine-induced cardiac necrosis. This study was undertaken to investigate the uptake and subcellular distribution of [C^{14}]adrenochrome in the isolated rat heart. The results revealed that adrenochrome was taken up readily by a low affinity high capacity system somewhat similar to that for extraneuronal catecholamine uptake. The uptake was concentration and time dependent, and obeyed Michaelis-Menton kinetics with a Km of 258 x 10^{-6} M and a Vmax of 54.6 ug/min/gm. Adrenochrome bound strongly to the myocardium and a study on subcellular distribution showed that the highest specific and total activity was found in the sarcolemmal fraction. Adrenochrome caused decreases in contractile force and resting tension of perfused hearts and the uptake of adrenochrome could be dissociated from these effects on heart contractile force and resting tension suggesting the biochemical and structural changes have an important role in these alterations. Adrenochrome uptake was inhibited somewhat by both neuronal and extraneuronal catecholamine uptake inhibitors. Propranolol and iproniazid, which decrease the cardiotoxicity of adrenochrome, reduced a large proportion of adrenochrome uptake. Corticosterone and 17-beta-oestradiol also strongly augmented adrenochrome induced increases in resting tension. The results suggest no simple relationship between adrenochrome uptake and its cardiotoxic

effects, but rather a more complex one involving the binding to various fractions of the heart and affecting their function.

II Introduction and Statement of the Problem

Evidence has been presented that suggests that excessive amounts of catecholamines play a role in heart disease in man (1-3) and catecholamine-induced cardiac necrosis has long been used as a model of catecholamine induced heart failure. Injections of relatively large amounts of epinephrine, norepinephrine or the synthetic catecholameine isoproterenol cause focal necrosis in the heart, along with a variety of ultrastructual, biochemical and functional damage (3-9). These effects have been observed in vivo (3-9), in isolated hearts (10-14), in cultured heart cells (15) and in humans when norepinephrine was used to maintain blood pressure (16,17).

The deleterious actions of exogenously administered catecholamines are also often compared to those of stress (3, 18-30). Various types of stress, either psychological or physiological, can result in increased output of and/or increased sensitivity to catecholamines (28, 30) and thereby produce pathological changes in the myocardium. The increased sensitivity to catecholamines is thought to be related to increased secretion of corticosteroids which is believed to be due to stress (19-21, 29). The various types of stresses can themselves result in pathological changes in the myocardium (19-21) and stress and corticosteroids in combination are particularly detrimental (19-21, 30).

Recently it has been suggested that catecholamines themselves do not induce cardiac necrosis, but rather their

oxidation products are involved (14, 32-39). This view is based on observations that showed that oxidized isoproterenol produces cardiac necrosis in the isolated perfused heart, while fresh isoproterenol does not (14, 33 39). The oxidized isoproterenol was found to have an absorbtion spectrum similar to that of adrenochrome. Subsequently adrenochrome was tested and found to produce myocardial cell damage and contractile failure while other metabolites of epinephrine did not (33, 39). In addition adrenochrome itself is known to have a variety of cellular actions which may be considered detrimental to the heart. These include increased oxygen consumption (40, 40), uncoupling of mitochondrial oxidative phosphorylation (42-44), vasoconstrictor properties (45, 46), inhibition of glycolysis (47-49), inhibition of myosin ATPase activity (50, 51) and still other effects (52-57).

Adrenochrome has also been demonstrated to exert a variety of subcellular effects which might also interfere with the normal functioning of the heart cell. Adrenochrome was found to inhibit sarcolemmal Na^+-K^+ ATPase <u>in vitro</u> or in the perfused heart (38). Adrenochrome decreases both mitochondrial and microsomal calcium uptake and binding (37) <u>in vitro</u> though little is known about whether plasma adrenochrome can enter the heart cell or not. It is therefore the purpose of this study to investigate whether adrenochrome is taken up by the cardiac cell by using the isolated perfused heart, the model most often used to study catecholamine uptake. The subcellular distribution of

adrenochrome will also be looked at by isolating various subcellular fractions, and looking at their adrenochrome content. Since propranolol and iproniazid both been reported to reduce adrenochrome induced cardiotoxicity (31), the effect of these agents on adrenochrome uptake will also be studied

It is well known that catecholamines are selectively removed from the blood stream by the heart and are very concentrated by the myocardium and its adrenergic nerve endings (58, 59). Also catecholamines released from nerve terminals are returned to the neuron by neuronal uptake mechanisms or may be taken up extraneuronally by the heart muscle cells themselves (60). To help understand the nature of adrenochrome uptake the Km and Vmax of adrenochrome uptake will be determined. Also the effects of specific inhibitors of neuronal and extraneuronal catecholamine uptake will be looked at to further compare the adrenochrome uptake to that of these catecholamines. Changes in heart contractile force and resting tension will be monitered and compared to the adrenochrome uptake, to look for any relationship between adrenochrome uptake and adrenochrome effects on contractile force and resting tension. The reversibility of adrenochrome uptake and the revesibility of its effects on contractile force and resting tension will be looked at in order to study the association between adrenochrome content and these two parameters and to investigate how strongly adrenochrome binds to the myocardium.

III Review of the literature

A. Catecholamine Induced Cardiac Necrosis

Injections of high doses of catecholamines have long been known to produce myocardial cell damage (3-9). With injections of epinephrine and norepinephrine, early workers showed that rabbits and dogs developed subendocardial and endocardial hemorrhages, focal lesions, edema, degeneration of myofibrils, arrhythmias and other charachteristic changes (17, 61-63). Such experimental results shed light on a clinical problem involving the use of norepinephrine to maintain blood pressure. Patients were receiving norepinephrine for long periods of time at relatively high concentrations (17) and it was found that the detrimental effects of catecholamines reported in animals were present in humans. The use of norepinephrine was correlated with a nonspecific myocarditis found in hospital patients. Similarly other work showed that norepinephrine therapy decreased the survival rate of dogs in hemorrhagic shock (64). Subsequently both epinephrine and norepinephrine were found to cause lesions in the isolated perfused heart (65).

Since the earlier observations of the deleterious effects of catecholamines isoproterenol, a synthetic catecholamine, has been used for inducing myocardial damage. The initial work was carried out mainly by a group led by G. Rona (6, 66-68). Dose dependent lesions were made using isoproterenol and it was postulated that a "relative myocardial ischemia" was the pathological mechanism with the heart using more energy than it could supply itself with

from the surrounding medium (68).

A number of investigators have now characterized the typical damage produced, though there are some variations depending on the doses and animals used. The first morphological changes appear in as little as two min with large doses of isoproterenol used subcutaneously (69), or in up to 30 min with smaller doses of natural catecholamines (70). Briefly these include focal necrosis of specific areas which involves: a striking hypercontraction and dissarrayment of the myofibrils called myocytolysis (10, 13, 69-76), contracture of sarcomeres (13, 69, 74), swelling of the mitochondria with disruption of the christae and deposition of electron dense bodies which may be calcium salts (13, 69-71, 74, 76, 77) swelling of the sarcoplasmic reticulum (SR) and t-tubles (70, 71, 76), increased number of ribosomes, depletion of glycogen granules and hypertrophy of the golgi apparatus (70). The sarcolemma appears superfically normal but in the early stages there is a dramatic increase in sarcolemmal permeability (76, 78) and tissue enzymes are reported to be released (79).

Various biochemical and metabolic changes have been reported to occur during catecholamine-induced myocardial necrosis. Oxidative phosphorylation is uncoupled, creatine phosphate and ATP stores decrease with an increase in phosphate occuring (80, 81), glycogen stores are depleted (70) and oxygen uptake increases (82). The uncoupling of oxidative phosphorylation is thought to be one of the more important changes and it may be

related to the reported increases in calcium uptake and content (69, 80, 81, 83-89). Magnesium and potassium in contrast, have been reported to decline (75, 87, 88).

The mechanisms by which catcholamines cause myocardial necrosis have been intensively investigated. Aside from the involvement of oxidation products, several different theories have been presented. These can roughly be divided into several groups; one is a relative ischemia or hypoxia theory and a closely related metabolic theory involving energy production, another is a hemodynamic theory implying interference with coronary circulation, a different theory implies electrolyte derrangements as the cause of cell necrosis, while depletion of endogenous norepinephrine stores is cited by some as being a key factor. Several other now less plausible theories also exist such as those involving thrombus formation, congestive heart failure and increases in plasma free fatty acids.

Among the earliest explanations for the cardiotoxicity of catecholamines was that by Raab and coworkers (3, 18). These workers thought that catecholamines, administered exogenously or released endogenously due to stress, caused a relative hypoxia. Catecholamines are well known to greatly increase the work, and hence the energy demand of the heart, but it was believed that the coronary circulation was not able to compensate for the increased oxygen demand. Thus there was a disproportion between supply and demand, which led to the necrosis. Much of Rona's (6, 7, 81) early work supported this idea. Their finding of the

greater myocardial damage due to isoproterenol in comparison to natural catecholamines, was explained in terms of its greater positive chronotropic and inotropic effects. This would greatly increase the cardiac need for oxygen and in addition, isoproterenol in contrast to other catecholamines, caused a decrease in blood pressure which would lead to decreased coronary perfusion and further aggravate the situation. Some authors support this idea (90, 92-94) citing the fact that high energy phosphate stores become depleted and that the increased heart rate decreases the duration of diastole and therefore decreases coronary flow. Furthermore, ischemia is believed to develop in the least perfused areas of the myocardium (90).

Handforth has pointed out that these are mainly unsubstantiated theories, with the actual supply of oxygen to the hearts and the demand not measured (95, 96). Handforth's work supports the idea that local coronary constrictions and dilation of precapillary shunts cause local ischemias and focal necrosis. These experiments involved injection of India ink retrogradely into hearts of animals treated with isoproterenol. The injections were at very early and later stages after isoproterenol administration and revealed that in the treated animals the ink did not perfuse well into all vessels and these correlated with the areas of necrosis. Also some vessels appeared to be constricted after the isoproterenol injections (95, 96). But several workers have directly contrasted these results. Ostadal *et al.* (97) looked at the turtle heart, an unusal but very useful

model for studying isoproterenol induced cardiac necrosis. In this heart, the spongy inner layer is supplied by diffusion from the ventricular lumen. This layer was greatly affected in isoproterenol induced necrosis in these hearts though it should be unaffected if changes in coronary circulation are the cause of the necrosis. Also, Belov and Khastova (10) found an equal distribution of foci of injury throughout the heart, in all cases the foci showed no tendancy whatsoever to correlate with the topography of the coronary vessels. Waldenstrom et al. (13) also found the cappilleries always appeared normally open and had a normal ultrastructure in necrosis induced by norepinephrine administration.

The metabolic theory, involving interference with energy prodcution, is related to both the relative ischemia and electrolytes theory. Interference with energy production in the cell can, especially when coupled with increased demand, seriously deplete ATP and creatinne phosphate stores. If not enough high energy phosphate stores are left for normal metabolic maintenance, serious damage can take place in the cell (83, 84). The relative ischemia theory states that energy supply cannot keep up with demand even at maximal production while others have suggested here that a defect occurs in the production of energy stores which is subsequently subnormal (80, 81, 85). As evidence, mitochondria from catecholamine treated hearts are shown to be uncoupled and high energy phosphate stores to be decreased (80, 81, 85). The causes though are in dispute, some suggest the

mitochondria are affected by catecholamines through electrolyte derrangements (81, 83, 84, 92) while others suggest an important and somewhat different role of endogenous norepinephrine (80, 85).

The theories on electrolyte involvement are fairly well developed. Several authors have proposed that increased calcium uptake by the myocardium leads to decreased energy production and increased usage (69, 80, 81, 84-86). The cause of the intracellular calcium overload may be excessive beta adrenergic stimulation, which results in excessive cAMP levels and is postulated to lead to excessively high calcium entry through the slow inward channels (86, 87, 92, 93). This view is supported by the fact that the more beta receptor stimulating an agent is, the greater is its ability to produce cellular necrosis (98) and that beta blockers prevent catecholamine induced cardiac necrosis (69, 74). Other reports suggest the increased calcium is due to a more general defect somehow induced in the sarcolemma. Soon after isoproterenol administration it was found that the sarcolemma became generally more permeable to the fine sturctural tracer horseradish peroxidase (76, 78).

Regardless of the mechanism, the increased intracellular calcium is believed to cause the necrosis through a variety of mechanisms. Many enzymes, which use or handle calcium, become activated thus using up high energy phosphate stores (86, 87). Also, mitochondria will maintain intracellular calcium homeostasis by sequestering excessive amounts of calcium. This

process requires energy and may occur in preference to ATP formation (99, 100). Such mitochondrial calcium overloading has been shown to impair high energy phosphate production, ultimately leading to irreversible damage to the mitochondria (81, 101). In support of this theory it has been shown that various calcium antagonists reduce the detrimental effects of catecholamine administration (86, 87) and calcium is necessary for the formation of the electron dense deposits found in the mitochondria during the development of the necrosis (77). Some inconsistancies are known in this theory. For example, increasing the dose of isoproterenol consistantly increased cell damage in some experiments, but did not continue increasing calcium content past a certain point. It was suggested that alterations in the subcellualr distribution of calcium were responsible for some of the necrosis (69). Propranolol also, completely prevented the increases in calcium but only partially prevented the necrosis (69). Also, some though not all, of the reports have measured calcium content of the myocardium by looking at content of tissue homogenates. This does not take into account changes in extracellular space and edema, which are known to occur in this type of necrosis (79). Still this theory on calcium involvement remains a strong one, though work is needed on the causes of calcium overload.

Magnesium and potassium are also reported to change in catecholamine induced cardiac necrosis (86-88). Both decrease in contrast to calcium. However it is not yet certain weather they

play a causative role or are secondary changes (86-88). Though potassium and magnesium salts can prevent or reduce the myocardial lesions and high potassium diet can reduce the lesions, these alterations may act through their antagonsim of calcium (86, 87).

Another type of mechanism sometimes suggested and one related to both calcium overload and relative ischemia is that involving endogenous norepinephrine stores. Several authors have stated that the endogenous norepinephrine stores of the heart may be causing the necrosis, with exogenous catcholamines simply causing release of these stores. This results in an upset in the normal cell metabolism (13, 80, 85). Several lines of evidence support this mechanism. Zavodskayan et al. (80) compared the effects of excessive electrical stimulation, which depletes endogenous norepinephrine, to those of exogenous catecholamine administration. The effects were the same with both treatments causing typical necrosis, uncoupled mitochondria and decreased high energy phosphate stores. Waldenstrom et al. (13) used tyramine on isolated hearts to deplete endogenous norepinephrine and found similar results, which could also be prevented by beta blockers. Further work is needed to clarify this role of endogenous norepinephrine.

Several other theories have been proposed to explain catecholamine induced cardiac necrosis but are now less accepted. Thrombus formation was once thought possible but direct evidence in favor of this is lacking and some authors note a lack of

thrombi formation (95, 96). Increases in plasma free fatty acids could be a cause but the production of the same lesions in isolated hearts goes against this theory. There is also no measurable increase in fatty acid content of mitochondria from treated animals and the mitochondria from these animals did not respond to the addition of albumin, which would nullify the effect of free fatty acids (85). Congestive heart failure is also not a factor since this is not shown in treated animals (85), and necrosis is known to occur in the absence of myocardial hypertrophy (102). One report suggested damage to the mitral valve as a factor but others have never substantiated this (94). Another report suggests an involvement of lysosomes, which are made fragile by isoproterenol, but questions arise as to weather these changes are of secondary nature (102).

It is worthwhile also to briefly discuss the physiological signifigance of the cardiac impairment produced by catecholamines and to compare it to other forms of heart diesease. As mentioned, the doses of catecholamines used to induce necrosis are high in comparison to plasma levels, but early workers found that lower physiological doses over prolonged time had the same effects (3, 17, 103). Waldenstrom et al. (13) used catecholamine concentrations of 10^{-6} to 10^{-4} molar in the perfusion medium of their isolated hearts. These workers have stated that these are much higher than the plasma concentrations of 10^{-6} to 10^{-8} molar but the concentrations of catecholamines are 10^{-6} or higher locally when myocardial stores are rapidly depleted under

conditions such as anoxia or ischemia (13, 104).

Catecholamine induced myocardial necrosis has both similarities and dissimilarities to many types of heart failure. Some authors (70, 74, 102) report that at the periphery of experimentally induced myocardial infarcts the same type of contracture bands and degeneration are found. But ischemia produces whole necrotic zones, especially when made by coronary artery occlusion, in contrast to the focal necrosis produced by catecholamine administration (70, 80). The centre of the ischemic zone also differs in that relaxation and not contraction, is shown. Regan et al. (75) noted signifigant differences from ischemia and epinephrine induced myocardial necrosis with regard to potassium ions and triglyceride content, though these might be due to differences in degrees of progression of the two damages. In angina, contracture bands are found in patients which are similar to those produced by norepinephrine in rats (70) and similar myofibrilar degeneration is also shown in stone heart and in patients after heart surgery (76). The first few hours after neurogenic degeneration produces uncoupling of oxidative phosphorylation and decreases in high energy phosphates similar to those caused by norepinephrine injections (80). Overall, it appears that several aspects of catecholamine induced cardiac necrosis are similar to some aspects of a variety of pathological states of the myocardium.

B. Stress and Catecholamines

Isoproterenol induced cardiac necrosis may also be compared to to that due to abnormal catecholamine production or an abnormal increase in the sensitivity to catecholamines. The syndrome pheochromocytoma is caused by an adrenal medullary tumour which excretes excessive catecholamines and results in myocardial necrosis similar to that seen after injections of exogenous catecholamines (17,18,31). This cardiomyopathy is not caused by hypertension and is likely a more direct effect, since the same effects are seen when hypertension is not present (105).

Stress and catecholamines are also thought to be associated with several detrimental effects. Stress causes increased output of catecholamines such as in the "fight or flight" responses and long term increases in excreted catecholamines are associated with stressful work and severe emotional strain (106-109). Persons in such stressful situations are more likely to suffer from coronary heart disease (109-111). Acute myocardial infarction and sudden death are sometimes preceded by severe or prolonged stress (112). Subjects with angina have a higher than normal urinary catecholamine secretion in response to stressful stimuli (28) and signifigant differences are known between emotional and excercise stress, thus accounting for their different effects on the cardiovascular system (113). However, problems arise in quantifying behavior and stress in humans which may lead to faulty conlusions or to the ignoring of these factors completely by some investigations (113). Some authors though,

have recently begun systematic investigations into this problem, looking at behavior patterns of subjects and their responses to stress. Results have shown that some stressful behavior types may be more prone to coronary heart disease, a relationship independent of other risk factors (114).

Due to the relative ease of manipulation, work with animals has progressed more than that with humans. The earlier works of Selye (30,115) have long shown an interaction with stress and various steroids. Stresses such as pronlonged restraint, surgical, bacterial, caloric and vagotomy, in combination with steroids were shown to cause damage in the myocardium. Steroids in combination with epinephrine and norepinephrine were also more effective in producing heart damage than catecholamines themselves. Raab (18,26) did related work on rats and also found that corticosteroids in combination with catecholamines were similarly more potent. Prolonged isolation, one type of stress, has been shown by Raab (26) and many others (116-119) to increase serum corticoids and senistivity to isoproterenol. The mean lethal dose in rats decreases 1,000 - 10,000 times by 3 months of isolation. Bassett and Cairncross (19-21, 120-122) have recently done a large body of related work. They used irregular signalled footshock (with equal amounts of regular footshock used in control experiments) and found an increase in plasma corticosterone (19), detrimental changes in microcirculation of the heart (19,20,120), greatly increased sensitivity to exogenous catecholamines (21) and disturbances in the uptake and handling

of catecholamines (122).

Other workers have looked at the mechanism by which catecholamines interact with the steroids. It is known that the synthesis of the enzyme PNMT (phenylethanolamine - N - methyltransferase) of the adrenal medulla (which catalyses the conversion of norepinephrine to epinephrine) is regulated by the glucocorticoids of the adrenal cortex (123). The ability of the adrenal medulla to synthesize and release epinephrine and norepinephrine, was found to increase in stressful experimental situations (106). Fleckenstein (81, 87) proposed that corticosteroids act by augmenting calcium accumulation in catecholamine induced cardiac necrosis accounting for their potentiating effects.

C. Catecholamine Binding and Uptake

Exogenous and endogenous catecholamines are accumulated by the myocardium both *in vivo* and *in vitro*. Raab and Gigee (59) found that norepinephrine made up the bulk of normal stores of catecholamine in the heart but the heart had an "amazing ability" to accumulate in an active form, large quantities of catecholamines, in particular epinephrine. Szakács and Mehlman (103) found similar results and also showed that prolonged infusion over a period of time with lower doses, still results in accumulation of toxic amounts in the myocardium. Axelrod and coworkers (124) observed similar uptake of exogenous catecholamines but suggested that some of the catecholamine was fairly rapidly metabolized, though another part appeared to be protected from being metabolized, possibly by being bound to tissue.

The distribution of exogenously administered isoproterenol was looked at by Herting (125) and compared to that of norepinephrine. In contrast to norepinephrine, only very small amounts of intravenously injected isoproterenol bound in the tissues. Most of the labelled isoproterenol was o-methylated within ten minutes after injection. By two hours most was gone from most tissues, including the heart, in contrast to norepinephrine where the concentration in the tissues was down only moderately. However the doses used in this study were very small and may not reflect what happens with the much larger doses used to induce isoproterenol induced cardiac necrosis.

The above workers suggest a relationship between the uptake and binding of exogenously administered catecholamines and their toxic effects but other workers disagree (126 - 129). Several investigators have reported that cardiac catecholamine stores decrease in congestive heart failure (126 - 128) while plasma levels of catecholamines increase (129). However, congestive heart failure differs from catecholamine induced cardiac necrosis and other models of stress induced cardiac necrosis, and their mechanism of genesis and relationships to catecholamines need not be the same. Also some workers question weather the decrease in norepinephrine content is a causative factor or simply a later development, possibly the result of an already damaged myocardiums inablity to store catecholamines properly (129). In any event, these observations are not inconsistant with the theories that plasma or exogenously administered catecholamines cause release of endogenous catecholamines, which then cause myocardial damage (13, 80, 85).

In other pathological situations such as angina and myocardial ischemia, catecholamine content increases. Lund (130) reported very high catecholamine levels in post - mortem studies of catecholamine concentrations in blood, following sudden death. Angina attacks have also been associated with acute elevations of serum catecholamine levels (131) which may be causative in some cases of sudden death (132 - 135).

Catecholamines secreted at the nerve terminal are certainly taken up by at least two mechanisms, neuronally and

extraneuronally (18, 60) but these are only now being fully understood and weather or not extraneuronal uptake is the mechansim of circulating catcholamines uptake, is not clear. Most people have worked with isolated organ preparations to study neuronal and extraneuronal uptake and determine their characters. Neuronal uptake (sometimes called uptake 1) is the uptake mechanism which takes norepinephrine released from the nerve terminal, back up into the nerve ending, thus shortening the life span of the transmitter in the extracellular space. It obeys Michaelis - Menton kinetics with and average Km of about 0.06 uM and a Vmax of 234 ng/min/gm (137). It exhibits stereoselectivity - (-)-norepinephrine > (+)-norepinephrine - and can take up epinephrine also, though the affinity is not as high (Km - 1.4 uM). In an isolated perfused heart neuronal uptake operates between about 20ng/ml to 0.5 or 1.0ug/ml (138, 139). At higher concentrations the rate of uptake does not increase. Neuronal uptake is an inactivation mechanism and the site of binding for this uptake is not well known, but is suggested not to be an adrenergic receptor or a metabolizing enzyme (138). The high affinity of the system is contrasted by its low capacity. Upon perfusing the heart with various concentrations (20 - 500ng/ml) and times, heart catecholamine content varied from 0.1 ug/gm to 0.6 ug/gm (137 - 139).

Cocaine is one good inhibitor of neuronal uptake. It has been used to inhibit neuronal catecholamine uptake in organ preparations (137, 140 - 143) or <u>in vivo</u> (144). Inhibition of

neuronal uptake by cocaine or other inhibitors, results in potentiation of the actions of catecholamines (58, 138, 144). The concentrations used to inhibit neuronal uptake vary, with most authors using 30 uM (142, 145) and some as high as 100 uM (137, 140). These concentrations are usually added in with the perfusing catecholamine and 90 to 100 percent inhibition of the uptake is usually reported (137, 141, 143). While low concentrations of cocaine (10 uM) have no apparent effects on heart rate and contractile force, higher concentrations have been shown to interfere with the electrical stimulation of isolated left and right atria, probably by causing arrhythmias, and this was pronounced in the presence of catecholamines (145).

Extraneuronal uptake was discovered in the sixties and differs greatly from neuronal uptake. Iversen (141) was the first to observe this uptake, naming it uptake 2. It was found to operate at higher perfusion concentrations and had many different properties from uptake 1. The capacity was very high sometimes accumulating over 28ug./gr. or up to 10 times the endogenous content, but the affinity was low, it operated from 0.75 to 40.0 ug/ml The Km for the uptake for epinephrine and norepinephrine was 51.6 and 252 uM respectively, while maximum velocities were 11.8 and 17.0 ug/min/gm. The hearts concentrated the perfused catecholamine several times greater than the perfusing medium. The uptake favored epinephrine over norepinephrine and it exhibited no stereochemical specificity for the (+)- and (-)- stereoisomers of epinephrine or norepinephrine.

Iversen (141) also studied the disappearence of the catecholamines when perfused with catecholamine free solutions after ten minutes of 5 ug/ml perfusions. Levels of both epinephrine and norepinephrine rapidly decreased from 13 ug/gm to 2 ug/gm with a 20 minute perfusion.

Several other early works appeared which verified these results (138, 146), (though slight differences may have been noted) and a variety of works have further characterized it. Farnebo and Malmfors (147) looked histochemically at both high and low perfusion concentration of noradrenalin to examine extraneuronal and neuronal uptake. They found that after perfusions with low concentrations the amine was present only neuronally, while at higher concentrations the amine was also present extraneuronally.

Extraneuronal uptake occurs in the heart in vascular smooth muscle, myocardial cells and somewhat in connective and elastic tissue. However only the first two probably bind signifigant amounts and only those two exhibit the typical pharmacological characters of uptake 2 (136). Isoproterenol is taken up only signifigantly extraneuronally, probably because it configuration prevents monoamine oxidase from deaminating it (148 - 150).

Recently it was shown that the early work committed some omissions in considering extraneuronal uptake. They looked at only content of unchanged catecholamine in the myocardium and ignored any catecholamine taken from the perfusing solution and released back as metabolites. Using either isoproterenol, or

norepinephrine with cocaine present, it has been shown that two main compartments are involved in the extraneuronal uptake of catecholamines (148 - 152, 140). One is an o-methylating system involving catechol -o- methyl - transferase. This enzyme works at both the high concentrations previously reported or at lower ones. This system has a high affinity with a Km of 2.9 uM for isoprenaline (150), 1.7 uM for norepinephrine (140) and Vmax's of 1.2 nmoles/gm/min and 1.7 nmoles/gm/min for norepinephrine and isoprenaline respectively (140, 150). After the catecholamine is o-methylated it is quickly released, explaining why this extraneuronal uptake was not detected by earlier workers (150). This system exists in series with the uptake into the cell but in parrellel with the other compartment (136, 153).

This second compartment is that which was detected by earlier workers. It operates at higher perfusion concentrations, equalibriates slowly, has no catechol -o- methyl transferase involvement and has a high capacity for storage of unchanged amine (148). At low concentrations virtually all the catecholamines go to the o-methylating system while at higher amounts, this system is saturated and a substantial accumulation of unchanged amine occurs (150). The Km for exclusively unchanged accumulation of isoproterenol was determined and was found to be 71.3 uM, while the Km for removal of isoproterenol from the perfusion fluid was 63.2 uM (150).

Different types of inhibitors, particularly steroids, inhibit extraneuronal uptake. Metanephrine, normetanephrine and

phenoxybenzamine are some non steroidal inhibitors of uptake 2, but they also inhibit uptake 1 (143). The steroids are the most potent and selective of the inhibitors with corticosterone the most widely used and the most selective. Salt (143) reported that in the perfused heart a concentration of 10 ug/ml inhibited 95 percent of uptake 2, the same concentration of 17-beta-oestradiol inhibited about 83 percent. Other authors reported similar results using 87 uM (30ug/ml) to ensure blockade of extraneuronal uptake (145, 154, 155). Many, if not most investigators do not use a preperfusion period with the steroids, however some reports have used a 5 or 10 minute preperfusion period (140, 143) and Bonish (150) found that the inhibitory effects of corticosterone increase with preperfusion time up until 10 minutes, where they are maximum and remain such thereafter. The steroids 17-beta-oestradiol (20 uM) and cortisol (80 uM) have no effects on the resting rate or contractile force of cat atria (145). They are thought to reduce both types of uptake 2 by inhibiting the uptake of the amines into the tissue and do not inhibit the enzyme catechol -o- methyl transferase directly (140, 143, 154).

The inhibition of extraneuronal uptake or of catechol -o- methyl transferase directly, accounts for some supersensitivity to catecholamines. This is well illustrated by Kaumann (156) in the cat, by Iversen and Salt (155) in rabbit aortic strips and by others in vivo (157, 158) and in vitro (156, 159).

Several authors speculate on the nature of both the uptake mechansims. A role for passive diffusion has generally been ruled

out because, 1) selective inhibitors limit uptake 2) the results follow Michaelis - Menton kinetics and 3) the amines can be concentrated several times greater than the surrounding medium (137, 151). Facilitated diffusion is suggested for extraneuronal transport by Gillespie et al. (161) since neither anoxia or substrate lack appeared to affect the uptake. Other authors question how facilitated diffusion could account for tissue gradient concentrations as high as 8:1 (141, 150). Though intrcellular binding might account for this (150) isoproterenol for example, is shown not to bind strongly intracellularly (125). Thus an active transport system was thought probable by some (141, 150). In this regard, Bonish (150) states that extraneuronal uptake of isoproterenol showed many charachters of a "pump leak system" though efflux may be associated with a carrier. As yet, no consenses has been reached on this point.

D. Adrenochrome

Though catecholamines are considered to be involved in the production of myocardial necrosis, various source have suggested that the catecholamines themselves are not resposible and rather their oxidation products are involved. As mentioned, work in our labratory showed that fresh unoxidized isoproterenol did not induce damage in isolated perfused hearts while oxidized isoproterenol does (14, 33, 39). The oxidation products and metabolites of epinephrine were tested and only the bright red oxidation product adrenochrome, produced damage to the hearts. Oxidized isoproterenol and adrenochrome perfusions produced the typical pathological changes in catecholamine induced cardiac necrosis including swollen mitochondria, impaired oxidative phosphorylation, disrupted myofibrils, swollen sarcoplasmic reticulum and decreased contractility (14, 33, 39). The damage adrenochrome produces can be reduced or inhibited by propranolol and iproniazid (35). Increasing calcium or potassium in the perfusion medium causes increased ultrastructural damage, while decreasing calcium reduced the myocardial necrosis (36). This observation was in contrast to those with catecholamines where increasing potassium antagonized the cardiac damage. The discrepancy is left unexplained.

Though it is well known that the major metabolic pathway of epinephrine and norepinephrine is <u>via</u> o-methylation (124, 162), the ease with which oxidation of catecholamines occurs suggests the role of oxidation products should not be ignored. When not

bound by tissue or stabalized chemically, the catecholamines can readily undergo oxidation (162). At 30°, pH 7.6, in five minutes a simple solution of epinephrine degrades signifigantly to an "oxidized pink solution", after 30 minutes most if not all the epinephrine has degraded (163). The oxidation of epinephrine to adrenochrome may be an autoxidation or may be catalized by trace metals (162). Adrenochrome can further oxidize to adrenolutin and eventually to melanin (162). This can occur spontaneously or can also be catalyzed by heavy metals (162) though adrenochrome is more stable when in pure conditon and can be stabalized by binding to tissues (163). Injected adrenochrome will be carried throughout the plasma and much will be degraded but some is excreted unchanged in some species (164).

In addition to spontaneous or heavy metal catlysis, adrenochrome formation is catalyzed enzymatically from epinephrine by several pathways in many tissues including enzymes of heart and skeletal muscle and heart mitochondria (56, 57, 162, 165-168). The oxidation products of epinephrine, adrenochrome and adrenolutin, have been identified in the heart, skeletal muscle, liver, brain and kidney of rabbits by paper chromatography and by their fluorescence properties (169 - 172). Adrenochrome formation from epinephrine can also be catlyzed by a variety of other tissues and tissue enzymes such as fresh hemoglobin (56), bovine heart muscle amine oxidase and cytochrome oxidase (173) and the microsomal fraction of the liver (57). The oxidation of dopamine, norepinephrine and epinephrine to adrenochrome and adrenochrome

like compounds may be regulated by serotonin, in brain enzyme preparations (174).

Adrenochrome and similar oxidation products have been shown to have a wide range of physiological activities many of which can be considered detrimental. Indeed, Hoffer and Osmond (52) stated that excessive amounts of adrenochrome appear to affect the metabolic function of nearly all cells of the body. Some of these effects include; an inhibition of glycolysis in brain tissue under various conditons, possibly by inhibiting hexokinase and phosphofructokinase (47, 175), inhibition of catechol -o- methyl transferase (176) and monoamine oxidase (177, 178), increasing oxygen consumption of a rat liver homogenate system depending on the substrate utilized and adrenochrome concentration used (41), though this is disputed, uncoupling of oxidative phosphorylation by rat brain mitochondria (42) and hamster liver mitochondria (43), and inhibiton of myosin ATPase of rat uterine muscle (50, 51). Pastan et al. (48) reported that catecholamines and adrenochrome increase the oxidation of glucose to carbon dioxide in thyroid tissue, and they thought that the effects of epinephrine may be through its conversion to adrenochrome. Adrenochrome was more potent in this stimulation and the incubated epinephrine was rapidly converted to adrenochrome. Hoffer and Osmond (54) looked at the effects of oxygen toxicity on intact rabbits. They found that substantial amounts of epinephrine were turned to adrenochrome in the brain and adrenal medulla and that this may account for much of the

toxic effects of high oxygen content. To study the psychological effects of adrenochrome Hoffer and Osmond (54) administered small amounts sublingually, intravenously and through an inhalent. A variety of effects were seen including abnormal vision, irregular breathing and visual changes similar to those with LSD administration. Intravenous administration was less effective possibly due to rapid binding by tissues. No other parameters were looked at. Valeri et al. (180) showed that adrenochrome increased the rate of hemolysis of blood from patients with traumatic injuries and the formation of adrenochrome involves the super oxide radical, with superoxide dismutase acting as an inhibitor of this formation (167).

Some recent works have suggested that adrenochrome may have important interactions with the prostaglandins. Gudbjarnason et al. (181) found that arachdionic acid and docahexaenoic acid stimulated microsomal oxidation of epinephrine to adrenochrome. The adrenochrome in turn stimulated peroxydation or oxygenation of the fatty acids to various fatty acid derivatives which they postulated could cause membrane damage and ultrastructural effects. Sun (182) similarly found that prostaglandin synthetase accelerates the rate of epinephrine conversion to adrenochrome in the prescence of arachadionic acid or 8,11,14, - eicosatrienoic acid.

Several independent workers have also recently suggested a role for oxidation products in the genesis of catecholamine induced cardiac necrosis. Severen et al. (15) looked for direct

toxic effects of isoproterenol on cultured heart muscle cells. After incubation with the isoproterenol and observations of toxic effects, they oberved a shift in the absorbtion spectrum to one similar to that of adrenochrome - this observation being similar to those by Yates and Dhalla (14, 33, 39). Carlsten and Poupa (32) found similar results. By using ventricular strips from hearts of frogs injected with isoproterenol. Their results led them to suspect an involvement of oxidation products as oxidized isoproterenol produced greater toxic effects than unoxidized isoproterenol.

Thus, adrenochome has a variety of physiological effects on heart and other tissues which can be detrimental and implicate adrenochrome in catecholamine induced cardiac necrosis.

IV Materials and Methods

For all experiments male Sprague Dawley rats were used with a body weight of between 300 and 375 gm. For heart perfusions the rats were sacrificed by decapitation and the hearts were quickly removed and placed in ice cold perfusion medium. The hearts were then quickly mounted on a cannula and perfused by the conventional Langendorff technique. The perfusion medium was Kreb's Henseleit solution containing NaCl, 120 mM; $NaHCO_3$, 20 mM; KCl, 4.63 mM; Kh_2PO4, 1.17 mM; $CaCl_2$, 1.25 mM; $MgCl_2$, 1.20 mM; and glucose, 8.0 mM. The perfusion medium was always fresh and was continuously gassed with a mixture of 95% O_2 and 5% CO_2. The temperature of the medium was maintained at 37^{o} and the perfusion rate was a constant 7.8 ml/min for all experiments, controlled by using a Harvard peristaltic pump. All the hearts were perfused in an open system and in none of the experiments described was the perfusate recirculated.

Contractile force developed and resting tension were monitered by a steel hook through the apex of the heart, connected via a short silk thread to a Grass force-displacement transducer, model FT 03C. Recordings were made on either a Grass model 7 polygraph or Gilson PR-5 polygraph and a resting tension of 2 gm was applied to the hearts. The recording rate was varied depending on the length of the experiment and the parameter looked at. All hearts were stimulated electrically at 300 beats/min using a Phipps and Bird Square Wave Stimulator to apply pulses of 1 to 3 V of 2 msec duration between two platinum

electrodes. Both atria were removed from the heart and the A-V node was crushed at the beginning of each experiment to facilitate external control of the heart rate. One electrode was placed in the apex of the heart and the other in the intraventricular septum at the base of the heart. This method of heart perfusion is essentially the same as that previously described (183-185).

In some experiments adrenochrome was present in the perfusing buffer (1-50 mg/l, $5.58X10^{-6}$ - $2.79X10^{-4}$ M). The adrenochrome solution was made up immediately before use in pregassed buffer and was always added following a 15 min equalibrium period with control buffer. In other experiments one of corticosterone (10, 30 mg/l; 29, 87 uM), 17-beta-oestradiol (2.72, 10 mg/l; 10, 36.7 uM) and cocaine(10, 34 mg/l; 30, 100 uM) were present in both the 15 min equilibrium period and in the perfusion time with adrenochrome(25 mg/l). It was necessary to dissolve corticosterone and 17-beta-oestradiol in a small volume of ethanol and add this to the perfusion medium. Control hearts with similar volumes of ethanol showed no effects similar to those with ethanol and the drugs. Different perfusing mediums were run through identical columns and hearts were switched from one medium to another through a system of interconnecting valves. Switching from control medium in one column to another had no effect except perhaps a very small transient decrease in contractile force for about 5 seconds. When hearts were perfused with [C^{14}]adrenochrome for later measurement of adrenochrome

content, following the [C^{14}]adrenochrome perfusion they were always perfused with a further 5.8-6.0 ml of cold medium to washout the label from the circulatory vessels.

Control hearts perfused with only Kreb's-Henseleit showed no difference in contractile force and resting tension when these were measured 15 min after the start of perfusion and compared to any point further within 30 min. Thus each heart could be used as its own control and the contractile force and resting tension of experimental hearts was compared to the 15 min control values and expressed as percent change from these 15 min values. Experimentals changes from the 15 min preperfusion values were then compared against controls' changes from their 15 min values.

[C^{14}]adrenochrome was synthesized by two different methods. In one method D,L,-[C^{14}]epinephrine was added with one gram of L-epinephrine (free base) in a total volume of 39 ml of methanol. Ninety percent formic acid was added dropwise to the stirring solution until it was clear. Four gm of freshly prepared silver oxide was then added portionwise over a period of three min while the temperature of the reaction mixture was maintained between 18-23^{o} with an ice bath. The reaction mixture was then filtered under suction through a previously washed Dowex-1-(Cl^{-}, 200-400 mesh) resin bed. The deep red filtrate was collected and allowed to crystallize for about one week at -70 to -80^{o}, in an atmosphere of nitrogen gas. The crystals were obtained by filtering the solution through a porous glass funnel, followed by drying. They were stored at -70 to -80^{o}, in a dessicator.

Dowex-1-(Cl^-) was prepared by washing with 3N HCl and then washing with double distilled water until the pH was neutral. The resin was then washed with methanol and dried. Silver oxide was prepared by washing with distilled water three times (10 vol/gm), then washing three times with acetone followed by two washings with absolute ether. This method of adrenochrome synthesis and preparation of silver oxide and Dowex is essentially the same as that of Heacock *et al.* (186).

The other similar method of adrenochrome preparation was derived from Sobotka and Austin's (187) method and was used when its purity was found to be the same as the previous method's and the yield was found to be higher. One gm of L-epinephrine (free base) was suspended (with labelled epinephrine) in 79 ml of absolute methanol. Hydrochloric acid (specific gravity 1.19) was added dropwise to the stirred solution until it cleared. To this solution 1.04 ml of concentrated (98%) formic acid was added followed by 2.18 gm of anhydrous sodium sulfate. The solution was cooled to 10° and 3.38 gm of silver oxide was added over a period of two min while the solution was stirring vigorously. The now red reaction mixture was filtered through a mat of anhydrous sodium sulfate and freshly prepared Dowex-1-(Cl^-) resin. To the filtrate was added 0.51 ml of concentrated formic acid, this was cooled to 10° and a further 3.38 gm of silver oxide was added over two min as previously described. This was filtered again as above and allowed to crystallize in an atmosphere of nitrogen gas at -70 to -80° for a period of about one week. The crystals were

collected and stored as in the previous manner.

D,L,-[7-^{14}C] Epinephrine (0.35 mg) was obtained from New England Nuclear with a specific activity of about 52.3 mCi/mmole. This was in the form of a bitartate in 1.0 ml of in 0.15 N L-tartaric acid. It was necessary to remove the epinephrine from this solution as we found the presence of bitartate greatly inhibited adrenochrome formation. This was accomplished by passing the mixture through a Dowex-1-(Cl^-) column (prepared by washing similar to the previous manner) in a 25 ml syringe. The procedure was carried out at 4^o and the column contained double distilled deionized water. One ml fractions were collected, sampled for radioactivity and immediately frozen at -70 to -80^o. The few fractions with [C^{14}]epinephrine were then freeze dried and used for [C^{14}]adrenochrome synthesis. Changes in pH showed that the epinephrine seperated well from the tartaric acid, with the acid appearing much later off the column.

The purity of synthesized adrenochrome was checked routinely by thin layer chromatography and infra-red spectrophotometry. The chromatography was carried out on Schleicher and Schuell silica gel sheets (F 1500/LS254) obtained through Sigma. Sheets were either 20 X 20 cm or approximately 10 X 6 cm and chromatography was ascending with a solvent containing 7 parts acetone to 3 methanol. It was done in an atmosphere of nitrogen gas to reduce any possible oxygenation of adrenochrome or other substances tested. After chromatography for some experiments involving radioisotopes, 0.5 cm strips of the gels were removed ascending

along the sheets and they were counted for radioactivity.

Infra-red spectrophotometry was done on a double beam Perkin-Elmer infra-red spectrophotometer using an alkali halide pellet (188). Samples were ground in a potassium bromide pellet, and pressed at high pressure into a disc. The infra-red absorbtion spectra of Sigma's and the synthesized adrenochrome were compared.

After heart perfusion many hearts and subcellular fractions were isolated by various means. Hearts were removed from the cannula and immediately placed in ice cold perfusing buffer. Any non ventricular tissue was removed from the hearts and hearts were homogenized with a Waring Blender for 45 sec in a medium containing 0.25 M sucrose, 20 mM Tris-HCl(pH 7.0), 1 mM EDTA. The homogenate was filtered through four layers of gauze and centrifuged at 1,000g for 20 min to remove nuclei, myofibrils sarcolemma and other cell debris. The supernatant was spun at 10,000g for 20 min to remove mitochondria and any other left over contaminants. The new supernatant was spun at 40,000g for 45 min. The pellet obtained was rinsed three times and suspended in 0.6 M KCl containing 20 mM Tris-HCl at pH 6.8 and then centrifuged at 40,000g for 45 min. This final pellet was the heavy microsome fraction and its isolation was essentially the same as that described by Harigaya and Schwartz(189). The supernatant of the first 40,000g spin was centrifuged at 100,000g for one hour; the pellet collected from this centrifugation is called the light microsome fraction.

To obtain the mitochondrial fraction from the same hearts or from different ones, the pellet of the first 10,000g centrifugation described above was washed, resuspended in homogenizing medium, spun at 1,000g for 10 min and the residue discarded. The supernatant was further centrifuged at 8,000g for 10 min to obtain the final mitochondrial fraction. In both mitochondria and SR preparations all pellets which were further processed were rinsed three times with the next homogenizing medium. The procedure for isolating mitochondria and SR from the same tissue is similar to the isolation methods of Harigaya and Schwartz (189) and Sordahl and Schwartz (190). It has been described in detail by Muir et al. (191) and Sulakhe and Dhalla (192) and fractions well characterized. The final suspension medium for the mitochondria and SR fractions was 50 mM KCl, 20 mM Tris-HCl at pH 6.8.

Sarcolemma or myofibrils were isolated either from the first 1,000g fraction of the previously described isolations, or from fresh heart tissue alone. In the former case, for sarcolemma, the pellet was resuspended in 10 vol/gm homogenizing medium (10 mM Tris pH 7.0, 1 mM EDTA) and isolation begun. In the latter case for sarcolemma, the hearts were homogenized and washed in 10 vol/gm of the same medium then filtered through four layers of gause. The rest of the isolation was as with the method of McNamara et al. (193). The homogenate was centrifuged at 1,000g for 10 min followed by washings of the pellet. Washings consisted of a homogenization with a glass to glass homogenizer, then

suspending in 10 vol/gm of 10 mM Tris-HCl (pH 7.4) followed by a 15 min stirring and a 1,000g X 10 min centrifugation. The wash was repeated again but using a Tris buffer of pH 8.0. This was followed by a centrifugation at 1,000g X 10 min. The sediment was then homogenized and suspended and washed in a washing buffer also containing 0.4 M LiBr and 0.4 mM EDTA. This was stirred for 30 min and followed by another normal wash in pH 7.4 buffer and further 1,000g X 10 min centrifugation. The next sediment was homogenized and resuspended and washed in 0.6 M KCl and 10 mM Tris-HCl (pH 7.4), followed by a 10 min X 1,000g centrifugation. The pellet was then washed with normal buffer containing 1 mM EDTA and spun at 1,000g X 10 min to obtain the final pellet.

As with sarcolemma, myofibrils were isolated from either the first 1,000g fraction of the SR prepartion or from whole heart tissue itself. In the fromer case the pellet was suspended in 10 vol/gm 0.3 M sucrose, 10 mM imidazole (pH 7.0). In the latter case, hearts were trimmed, cut up and homogenized in isotonic saline medium using a Waring Blender for 45 seconds. This fraction was then filtered through 4 layers of gauze. The rest of the isolation was the same and followed the method of Solaro et al. (194). The homogenates were centrifuged at 17,300g for 20 min. This pellet was suspended in 10 vol/gm of 60 mM KCl-30 mM Imidazole (pH 7.0) and 2 mM $MgCl_2$ using a glass-Teflon homogenizer and the suspension was centrifuged at 750g for 15 min. This procedure was repeated four more times. The fifth pellet was then suspended in the standard buffer containing 2 mM

EGTA and centrifuged at 750g X 15 min This pellet was suspended and homogenized in the standard buffer containing 1% Triton X-100 and was again centrifuged at 750g X 15 min. This was repeated once more and was followed by two more suspensions and recentrifugations in the standard buffer of 60 mM KCl-30 mM Imidazole (pH 7.0) to obtain the final pellet.

In one series of experiments (n=4) mitochondria from one heart was isolated from a heart perfused with [C^{14}]adrenochrome (50 mg/l X 30 min) and was mixed with sarcolemma from a control heart. The sarcolemma was then reisolated from the mixture and the amount of adrenochrome present was measured.

All of the above procedures for isolating subcellular fractions were carried out at 0-4^{o}. Neither the sarcolemma nor the myofibril fractions showed any differences in adrenochrome content which depended on whether they were isolated from the heart tissue or from the first 1,000g fraction of tissue used for other isolations.

Protein concentration was determined by the Lowry technique (195) and [C^{14}]adrenochrome content of various fractions or homogenates was determined by liquied scintillation counting. Measured amounts of fractions were added to Beckman Ready-Solve GP to make 10 ml of fluid. Any corrections necessary for quenching were made and quench curves were generated with similar and larger amounts of protein in the same final volume of scintillation fluid. The counts of the fractions were compared against standard curves generated by using the synthesized

[C^{14}]adrenochrome. The small amounts of adrenochrome used to generate the standard curves and present in the samples had no quenching effects. In some cases only the final fractions were sampled, in others the first 1,000g, 10,000g, 40,000g, 100,000g and supernatant of the 100,000g fractions were sampled. In other cases the final product of the purified fractions were sampled and the sarcolemma fraction was sampled after the KCL step and after lithium bromide treatment (crude sarcolemma). The supernatants of many of the purified fractions were tested for adrenochrome content and very small amounts were found except in the supernatant of the 100,000g fraction.

For estimation of Na^+-K^+ ATPase activity fractions were incubated in a total volume of 1 ml containing 50 mM Tris-HCl (pH 7.4), 1.0 mM EDTA, 4.0 mM $MgCl_2$, 100 mM NaCl 10 mM KCl and 0.03-0.06 mg protein in the presence or absence of ouabain (2 mM). After 3 min of pre-incubation at 37^o the reaction was started by addition of ATP (4 mM final concentration) and the reaction was incubated for 10 more min. The reaction was stopped by addition of 1 ml of ice-cold 12% trichloroacetic acid. This was centrifuged for 10 min at 1,000g and the phosphate released determined by the method of Taussky and Shorr (196). The difference of the activity in the presence and abscence of ouabain is refered to as Na^+-K^+ stimulated, Mg^+ dependent, ouabain sensitive ATPase (Na^+-K^+ ATPase).

Myofibrillar ATPase was determined at 37^o in the presence of 50 mM KCl, 20 mM imidazole (pH 6.8), 2 mM $MgCl_2$ and 2 mM ATP.

Total activity in the presence of 100 uM $CaCl_2$ was subtracted from basal activity, (in the presence of 1.6 mM EGTA and no calcium). The incubation time was 2 min and final protein concentration was 0.8-1.0 mg/ml. The reaction was stopped by addition of 1 ml of ice-cold 12% trichloroacetic acid (194).

Calcium binding by the mitochondria and SR was determined at 25° by the method of Sulakhe and Dhalla (192) in a medium containing 100 mM KCl, 20 mM Tris-HCl (pH 6.8), 10 mM $MgCl_2$ and 4 mM ATP, 4 mM ATP in the presence or abscence of 5 mM sodium azide. Protein (0.2-0.3 mg/ml final concentration) was added before a three min preincubation and followed by addition of $Ca^{45}Cl_2$ (0.1 mM final concentration). The reaction was incubated for 5 min and terminated by Millipore filtration. Total Ca^{+2} stimulated ATPase activity of the microsomal fraction was determined at 37° in the same medium by the same procedure, and the phosphate released was determined after the reaction was terminated by additon of 1 ml of ice-cold 12% trichloroacetic acid.

The reagents propranolol, iproniazid, epinephrine, ATP, corticosterone, 17-beta-oestradiol and Dowex-1-(Cl^-) were all obtained from Sigma Chemical Company. Silver oxide was obtained from Fisher and anhydrous sodium sulfate from J. T. Baker Chemical Company. Cocaine was supplied through Dr. Davidson of the department of Biochemistry, University of Manitoba.

The results obtained are expressed as mean plus or minus standard error. Control values only, were compared against

experimentals using the Students t test or when comparing controls to more than one experimental analysis of varience followed by Duncan's Multiple Range test was used. The null hypothesis was that there was no difference between the control and experimental means.

V Results

The two procedures used to synthesize adrenochrome gave a pure labelled product. The method of Heacock et al. (186) was first used and resulted in a yield averaging only 80 mg/gm of starting epinephrine, while the method of Sobotka and Austin (187) yielded about 200 mg/gm of starting epinephrine. The purity was equivalent in both methods of synthesis. Infra-red spectrophotometry (Fig. 1) positively established the identity of the purified product and indicated that the synthesized adrenochrome was purer than that obtained comercially. Thin layer chromatography confirmed these results (Fig. 2). The R_f of adrenochrome averaged 0.47 and that of epinephrine averaged 0.20. Further oxidation products of adrenochrome and epinephrine such as adrenolutin and melanin had R_f values of 0.60 and 0.85 respectively.

Perfusion of hearts with 50 mg/l adrenochrome resulted in a continual decrease in contractile force over 30 min to a value 30% of control (Fig. 3), though contractile force was increased slightly in the first min. The resting tension of these hearts increased steadily throughout the perfusion period and reached a final mean of 4.4 gm, which was 220% greater than the initial value (Fig. 3).

Hearts perfused with [C^{14}]adrenochrome (50 mg/l) for 5, 10 and 30 min were analysed for total content (ug/gm tissue) and specific activity (ug/mg protein) of adrenochrome in the various

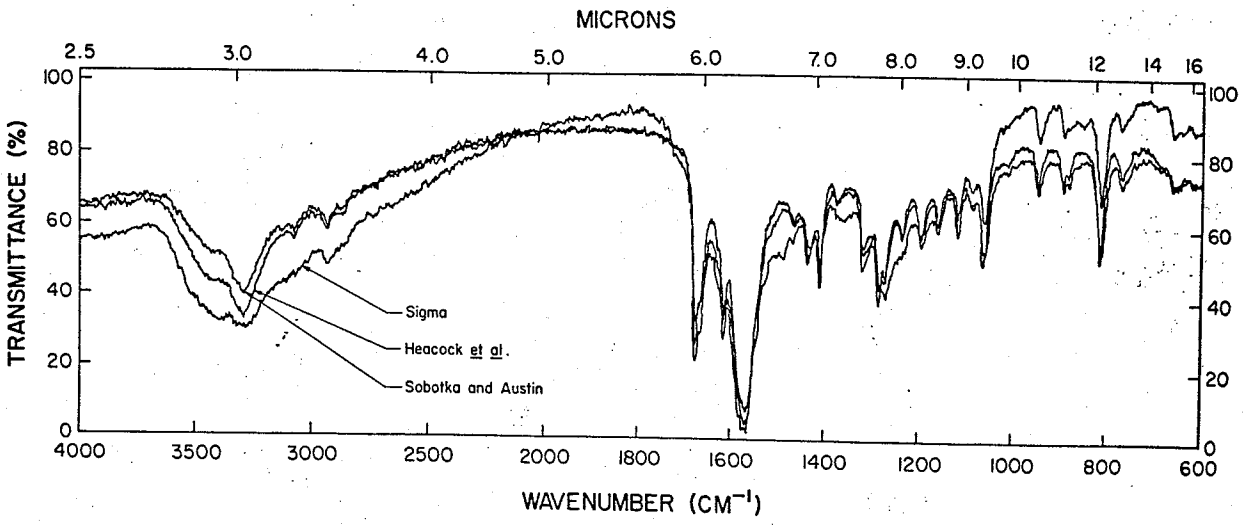

Fig. 1. Comparison of infra - red absorbtion spectra of comercially obtained adrenochrome and adrenochrome synthesized by the methods of Heacock et al. (162) and Sobotka and Austin (187).

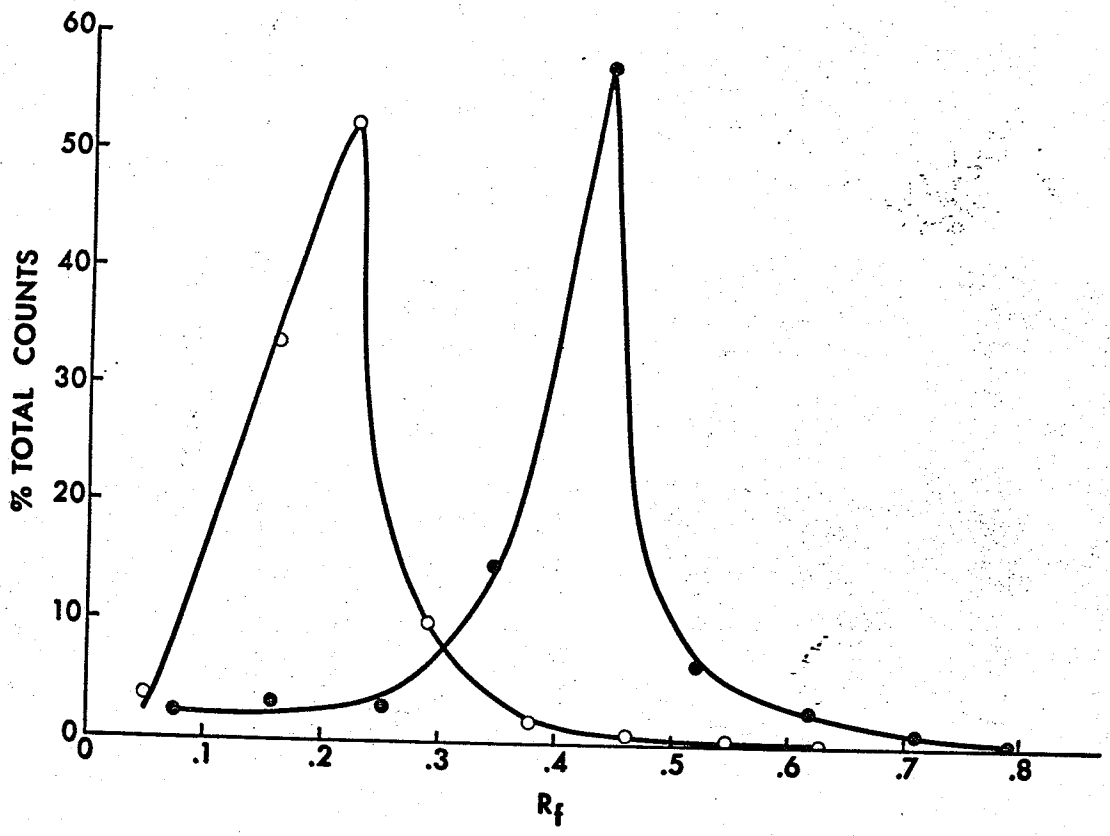

Fig. 2. Thin layer chromatography of [C^{14}]adrenochrome ●-----● and [C^{14}]epinephrine ○-----○ . Each point is the mean of 4 experiments.

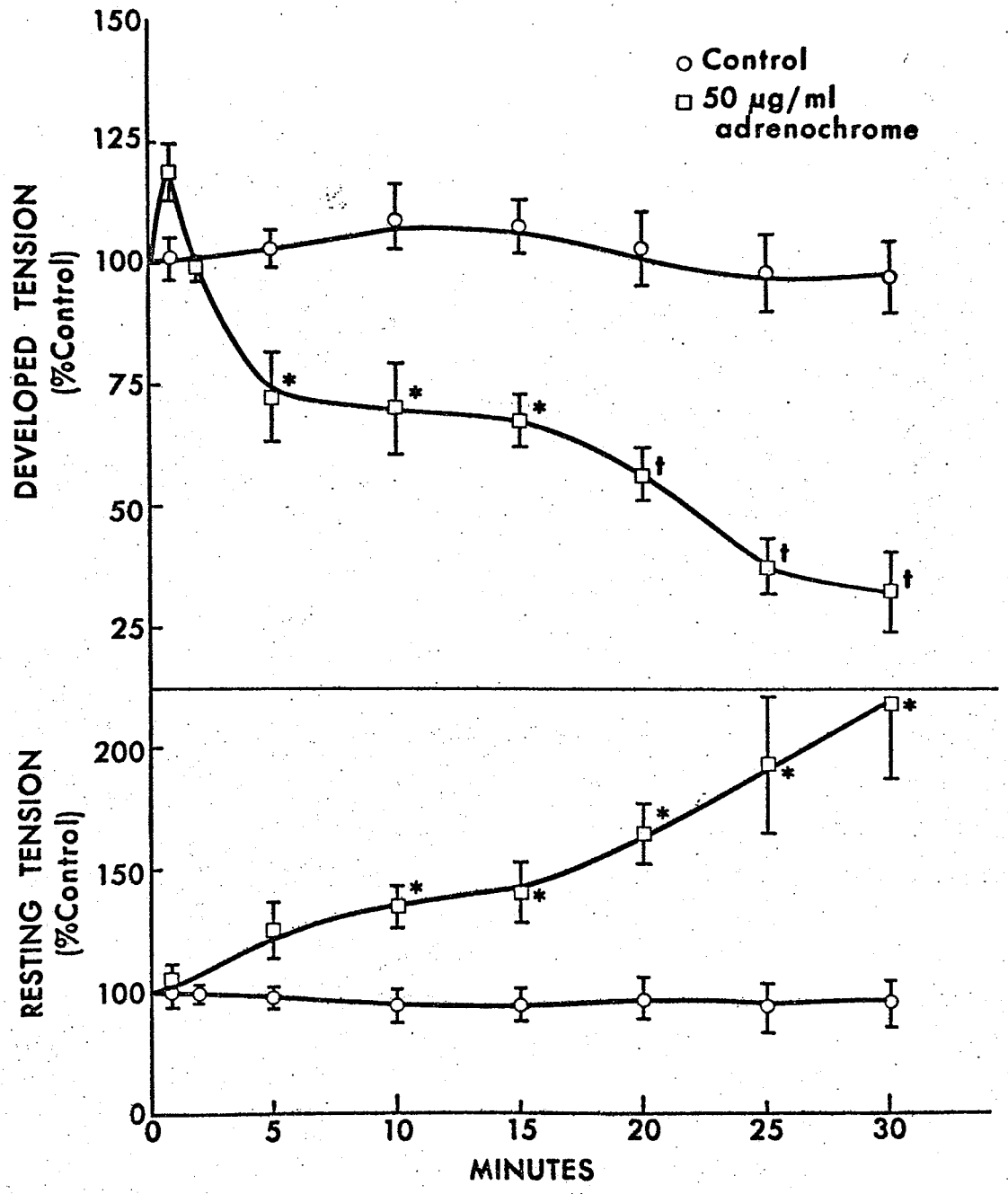

Fig. 3. Contractile force and resting tension of hearts perfused with 50 mg/l adrenochrome for 30 minutes. Each value is the mean ± S.E. of 5 experiments. * Signifigantly different from control value (*0.05 > P, †0.01 > P).

subcellular fractions. The adrenochrome content increased quite linearly with time (Table 1). After 30 min the heart accumulated 108 $\pm$ 30 ug/gm wet weight ventricular tissue. The crude fraction with the greatest total of adrenochrome was consistantly the 1,000g fraction which contains the sarcolemma and myofibrils. The final supernatant contained a high total amount of adrenochrome, but a low specific activity. The specific activity of the 40,000g fraction, which contains the SR, was high, but the total activity was low due to the small yield of this fraction. After purification of the different fractions by the various means, it was found that the sarcolemma had the greatest specific activity followed by the microsomes; both the myofibrils and the mitochondria had much lower activities (Fig. 4).

The ouabain sensitive Na^+-K^+ ATPase activity of the sarcolemma was 11.8 $\pm$ 1.0 umoles Pi/mg/hr, that of the mitochondria, SR, and myofibrils was negligable. Calcium stimulated ATPase was 221 $\pm$ 19 nmoles Pi/mg/min in the myofibrillar fraction and was 2.38 $\pm$ 0.21 umoles Pi/mg/min in the microsomal fraction. Calcium binding by the microsome fraction was 32.8 $\pm$ 2.3 nmoles/mg/5 min and was not inhibited by sodium azide, calcium binding by the mitochondria was 40.7 $\pm$ 2.0 nmoles/mg/5 min and was inhibited 100% in the prescence of 5 mM sodium azide. The sarcolemmal fraction did not show ATP dependent calcium binding or calcium stimulated magnesium dependent ATPase activity.

Perfusion of hearts with various concentrations of

Table 1. Adrenochrome content of various fractions of the heart after 5, 10 and 30 minutes of perfusion.

	5 minute			10 minute			30 minute		
	ug A/ mg pt	mg pt/ gm ti	ug A/ gm ti	ug A/ mg pt	mg pt/ gm ti	ug A/ gm ti	ug A/ mg pt	mg pt/ gm ti	ug A/ gm ti
Heart Homogenate	0.12 ± 0.04	141 ± 7	17.2 ± 5.13	0.28 ± 0.04	115 ± 6	33.6 ± 4.35	0.91 ± 0.27	123 ± 5	108 ± 30
1,000g X 20 min	0.08 ± 0.03	81.6 ± 1.3	7.62 ± 1.6	0.24 ± 0.04	71.9 ± 2.66	17.2 ± 3.66	1.03 ± 0.3	78.9 ± 6.0	81.4 ± 27.0
10,000g X 20 min	0.04 ± 0.03	7.68 ± 1.12	0.03 ± 0.02	0.32 ± 0.11	5.65 ± 0.1	1.88 ± 0.6	0.49 ± 0.14	5.13 ± 0.4	2.55 ± 1.3
40,000g X 45 min	0.58 ± 0.09	1.97 ± 0.2	1.10 ± 0.1	2.78 ± 0.2	1.40 ± 0.1	3.85 ± 0.1	2.61 ± 0.1	1.44 ± 0.2	3.74 ± 1.1
100,000g X 60 min	0.27 ± 0.08	0.65 ± 0.06	0.13 ± 0.06	1.07 ± 0.3	0.63 ± 0.04	0.69 ± 0.21	1.06 ± 0.5	0.36 ± 0.11	0.34 ± 0.06
Supernatant of 100,000g	0.30 ± 0.08	24.4 ± 0.8	7.44 ± 1.75	0.38 ± 0.06	24.79 ± 1.71	9.56 ± 1.81	0.77 ± 0.36	23.0 ± 1.3	17.8 ± 1.7

[A=adrenochrome, pt=protein, ti=tissue]
[Mean ± S.E. of 4-5 experiments.]

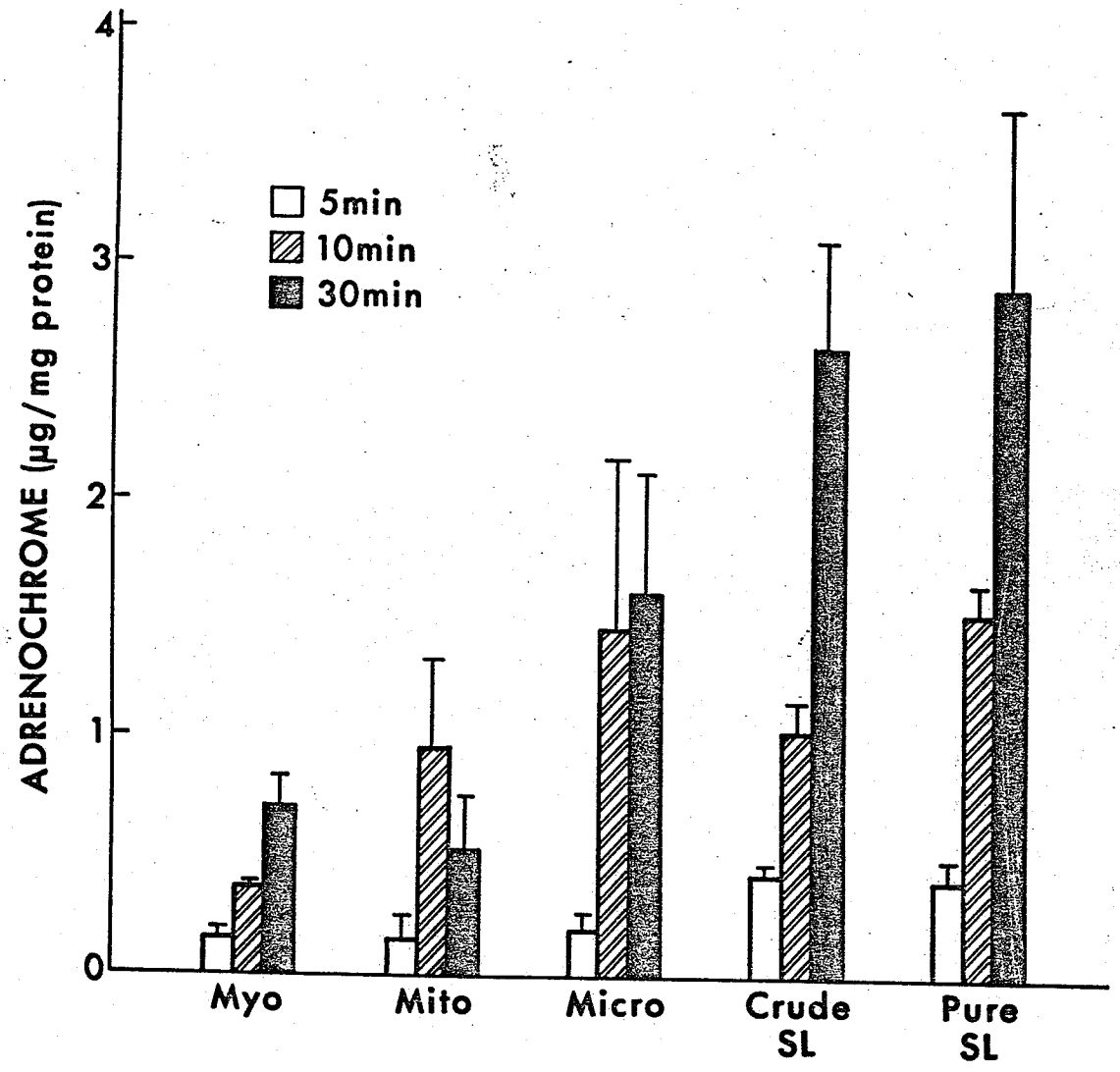

Fig. 4. Adrenochrome content of various subcellular fractions after perfusion of isolated rat hearts for 5, 10 or 30 minutes.

adrenochrome resulted in a decrease in the contractile force and an increase in the resting tension (Table 2). The changes were significant in hearts perfused with 12, 25 and 50 mg/1. The adrenochrome content increased with the higher perfusion concentrations though it was not closely correlated with the changes in developed and resting tension. In contrast to the linearity of uptake during perfusion with 50 mg/1 over 30 min, the rate of uptake was relatively slow with 10 min perfusions at high concentrations in comparison to lower ones. The Eadie and Hofstee plots according to Iversen (141), yielded a Km of 258 X 10^{-6} M and a Vmax of 54.6 ug/min/gm (Fig.5).

The adrenochrome content, contractile force and resting tension of hearts perfused for 10 min with 25 mg/1 followed by a 10 or 20 min washout was also examined. After 10 and 20 min of washout the adrenochrome content was significantly reduced to 96.7 $\pm$ 19.4 and 74.5 $\pm$ 11.6 from a control value of 200 $\pm$ 15 ng/mg protein. After a 10 min adrenochrome perfusion the contractile force was decreased about 20% and did not recover with a 10 to 20 min washout. On the other hand the resting tension was increased about 16% by a 10 min adrenochrome perfusion and kept on increasing for 5 min after washout began and then declined gradually to a value only slightly higher than controls (Table 3).

When mitochondria with adrenochrome were mixed with uncontaminated sarcolemma and the sarcolemma was reisolated, little cross contamination of adrenochrome was found. Though the

Table 2. Adrenochrome content, contractile force and resting tension of hearts perfused with varying concentrations of adrenochrome for ten minutes.

	Adrenochrome Concentration mg/l			
	50	25	12	1
Adrenochrome (ng/mg protein)	272 ± 56	197 ± 25	80 ± 15	19.7 ± 2.5
Developed Tension††	61* ± 10.0	60* ± 9.0	69* ± 7.0	80 ± 10.0
Resting Tension**	140* ± 10	133* ± 8	125 ± 6	120 ± 6

†† Percent of control value before adrenochrome perfusion. Absolute value was 8.6 ±0.9 gm/gm wet tissue.

** Resting tension was adjusted to 2.0 gm before adrenochrome perfusion. Values given are percent change of this value.

*, †, Significantly different from the control hearts, *0.05 > P., † 0.01 > P.

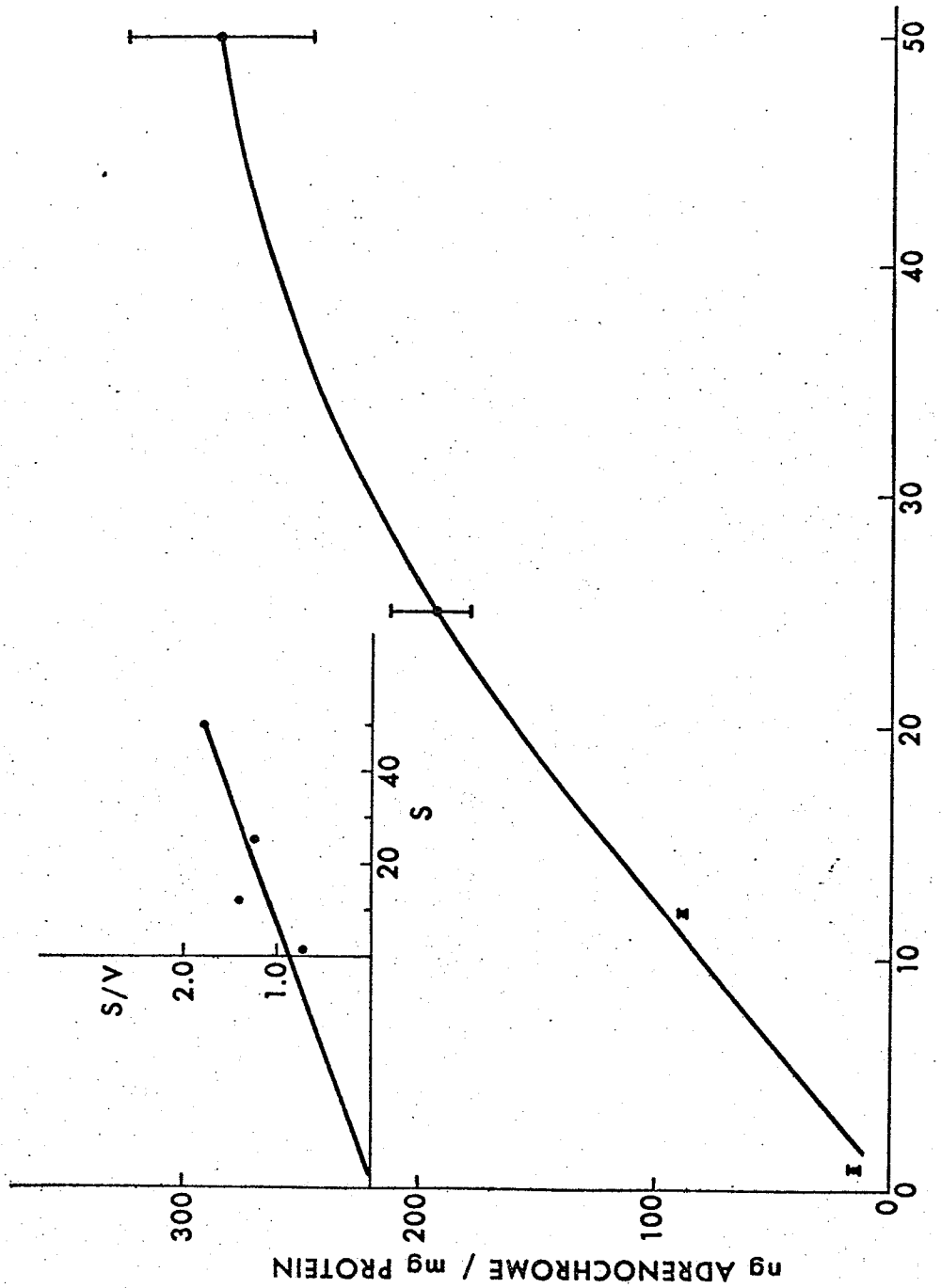

Fig. 5. Uptake of [C^{14}]adrenochrome after 10 minutes of perfusion of isolated rat hearts with 1, 12, 25 and 50 mg/l adrenochrome, and Michaelis - Menton kinetics.

Table 3. Adrenochrome content, contractile force and resting tension of hearts perfused with [C^{14}]adrenochrome (25 mg/l) for 10 minutes followed by 10 or 20 minutes of washout with control medium.

	Time after adrenochrome perfusion (min)		
	0.0	10.0	20.0
Adrenochrome ng/mg protein	200 ± 15	96 ± 19 †	74 ± 12 †
Developed Tension ‡‡	82 ± 2.5 †	80 ± 2.8 †	84 ± 1.4 †
Resting Tension**	119 ± 4 †	127 ± 5 †	107 ± 8 †

**, †, See Table 2 for legend.

‡‡ Percent of control value before adrenochrome perfusion. Absolute value was 9.5 ± 0.3 gm/gm wet tissue.

yield of sarcolemma decreased, making exact quantification difficult, only a very small amount of radioactivity could be detected in the reisolated sarcolemma, even on a ug/mg protein basis.

Cocaine, (10.2, 34 mg/l) the neuronal inhibitor of catecholamine uptake, resulted in 35% and 41% decreases in adrenochrome (25 mg/l) uptake (Table 4). The extraneuronal inhibitors of catecholamine uptake corticosterone and 17-beta-oestradiol, inhibited adrenochrome uptake 33% to 35%. Cocaine itself caused transient changes in contractile force and resting tension including some apparent arrhythmias in control hearts, and thus its effects on these in combination with adrenochrome were not measured. Similar effects are noted by some other authors (145). Corticosterone and 17-beta-oestradiol showed no such effects on control hearts and in combination with adrenochrome they showed dramatic effects. Both slightly augmented the decrease in contractile force caused by a 10 min 25 mg/l adrenochrome perfusion (Fig. 6). Similarly, they both also greatly augmented the increase in resting tension caused by adrenochrome. After 10 min of 25 mg/l adrenochrome alone, resting tension increased only about 20% while in the presence of the various inhibitors, it increased dramtically 50% to 390% (Fig. 6.).

Propranolol and iproniazid, the two agents which inhibit adrenochrome induced cardiac necrosis (35, 39) inhibited adrenochrome uptake up to 57% (Table 2). Very different effects

Table 4. Effect of inhibitors of adrenochrome-induced cardiotoxicity and catecholamine uptake inhibitors on [C^{14}]adrenochrome uptake.

	[C^{14}]Adrenochrome ng/mg protein
Control	201 ± 11.6
Inhibitors of Cell Necrosis	
Propranolol	
1 mg/l	88 ± 4.6†
3 mg/l	86 ± 3.5†
Iproniazid	
10 mg/l	97 ± 5.9†
25 mg/l	93 ± 4.2†
Catecholamine Uptake Inhibitor	
Corticosterone	
10 mg/l	135 ± 9†
30 mg/l	130 ± 7†
17-beta-Oestradiol	
2.7 mg/l	135 ± 19*
10 mg/l	134 ± 12†
Cocaine	
10.2 mg/l	131 ± 13†
34 mg/l	118 ± 11†

Each value represents the mean ± S.E. of 4-6 experiments. *, †, See Table 2 for legend.

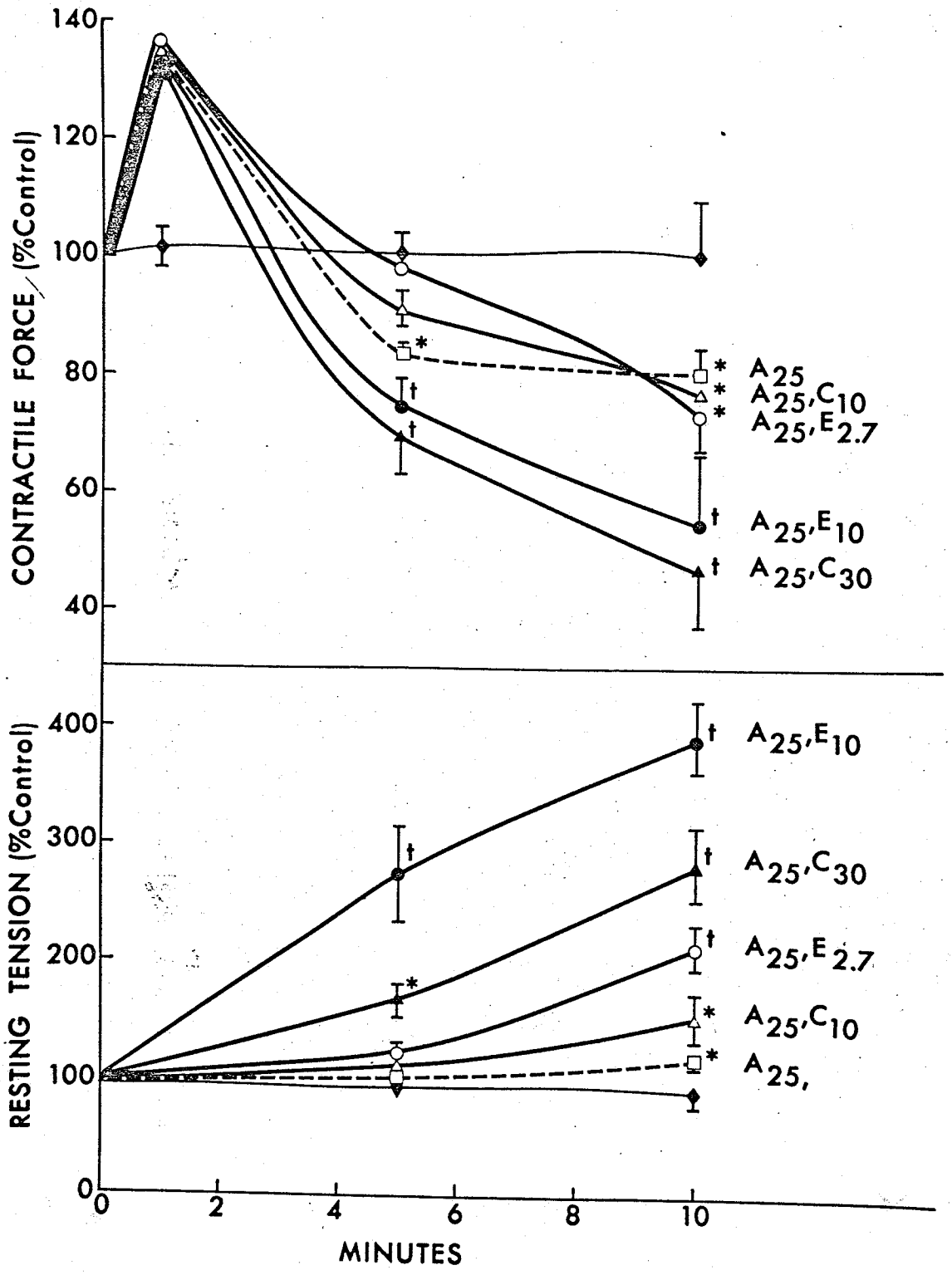

Fig. 6. Effects of inhibitors of extraneuronal uptake in combination with adrenochrome (25 mg/l), on heart contractile force and resting tension. A=adrenochrome, C=corticosterone, E=17-beta-oestradiol, subscripts represent concentrations in mg/l. Each point is the mean ± S.E. of 4-6 experiments.*, †, See Fig. 3 for legend

were seen on the contractile force (Fig. 7). The initial positive inotropic effect seen for about one min in hearts with 25 mg/l adrenochrome alone, appeared to be exaggerated and took more time to peak. This was followed by a gradual decline in developed tension, although after 10 min the developed tension was still greater than that of controls and much greater than that of hearts perfused with adrenochrome alone. Perfusion with 3 and 1 mg/l of propranolol alone, after a 15 min equilibration period, resulted in a mean depression of mean contractile force of 28% and 5% to 10% respectively, and remained at these levels for over 15 min. Perfusion with iproniazid (25 or 10 mg/l) alone, after a 15 min equilibration period, resulted in a slight initial increase in contractile force (5-10%), after which hearts returned to control values.

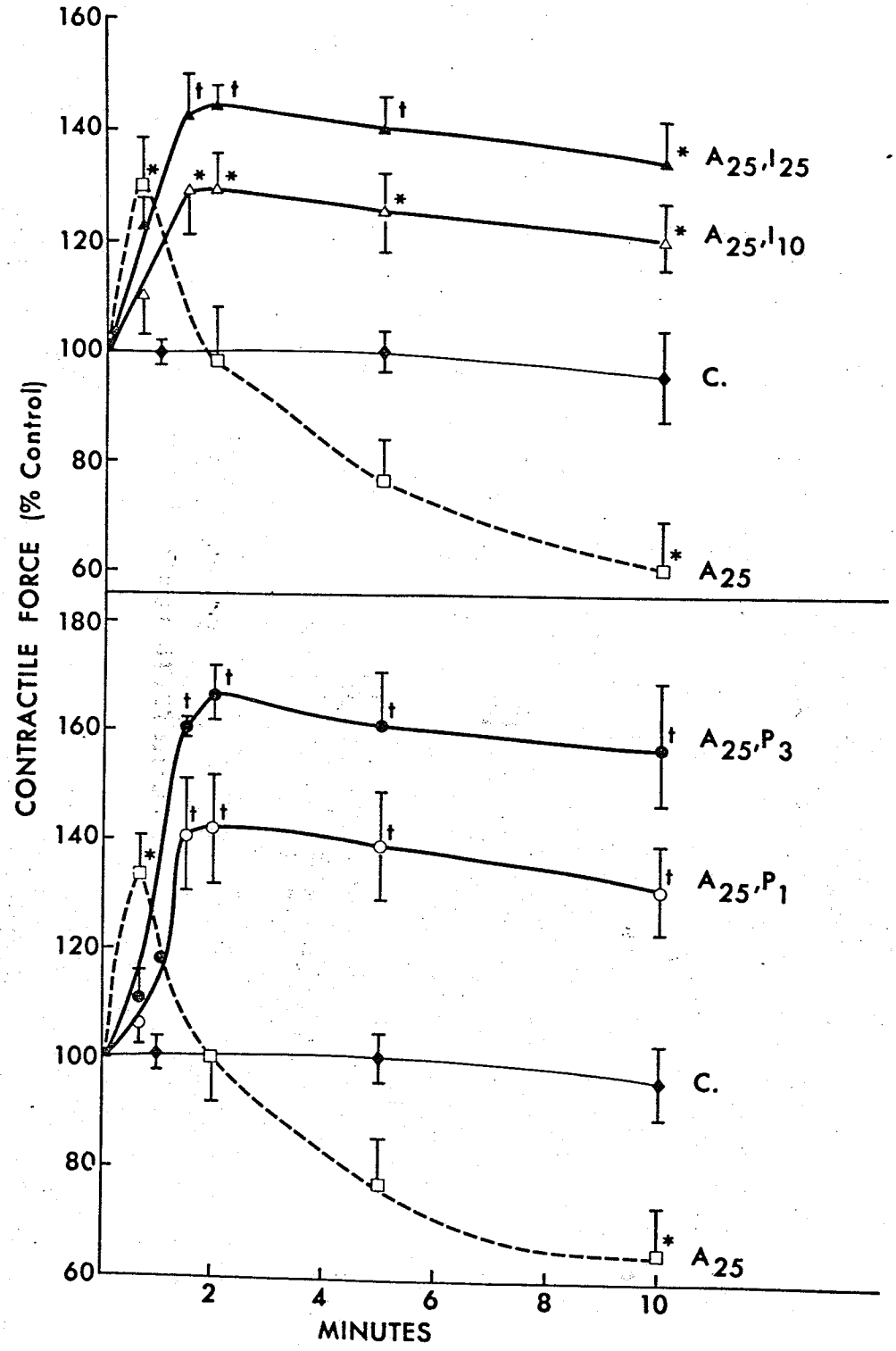

Fig. 7. Effects of Propranolol and Iproniazid with adrenochrome (25 mg/l), on heart contractile force. A=adrenochrome, I=iproniazid, P=propranolol. Subscripts represent concentrations in mg/l. Each point is the mean ± S.E. of 5 experiments. *, †, See Fig. 3 for legend.

VI Discussion

The two methods used to synthesize [C^{14}]adrenochrome yielded a pure product with the method of Sobotka and Austin's (187) having the higher of the two yields. This is probably due to the anhydrous conditions present in this reaction mixture as adrenochrome is very soluble in water. The main problem in the synthesis of adrenochrome is that left over catalyst, such as silver oxide, can cause further oxidation of adrenochrome to other products over a period of time (186). The method of Heacock et al. (186) is the most recent and well developed of the procedures using an anion exchanger to remove any silver oxide left in the medium before crystallization. However, in their paper they state that the method of Sobotka and Austion (187) yielded a pure product also, one free of silver oxide. The sodium sulphate the final reaction mixture was filtered through had acted as an anion - exchange resin and removed silver ions from the solution (186). In this study when the method of Sobotka and Austin's was used, we included dried Dowex -1- (Cl^-) as an anion exchange resin in with the final sodium sulphate the reaction mixture was filtered through. Thus, little if any silver would remain in the filtrate and the purity would be ensured.

The results of the 30 min 50 mg/l perfusion with adrenchrome verified previous findings (14, 33, 39). The perfusions with adrenochrome markedly decreased contractile force in the hearts with a simultaneous increase in resting tension. The content of adrenochrome also rose over the 30 min period in the whole heart

and in almost all the crude or pure fractions. Only in the mitochondrial fraction did an earlier value exceed a later one (Fig. 3), but the error involved was large. This raises the possibility that the increased adrenochrome content over time directly causes the decreases in contractile force and increases in resting tension. Later data though, suggests a very close simple relationship is not the case. The steroids corticosterone and 17-beta-oestradiol very greatly augmented the rise in resting tension in combination with adrenochrome, but decreased heart adrenochrome content (Table 4) and did not greatly augment the decrease in contractility casued by adrenochrome (Fig. 6). Also, perfusion with varying concentrations of adrenochrome resulted in great changes in the adrenochrome content of the tissue (Table 2) but the changes in contractility and resting tension did not vary nearly as greatly from one concentration to another. In experiments with perfusion of adrenochrome followed by adrenochrome free perfusion, a dissociation was also seen between changes in contractile force, resting tension and adrenochrome content. Adrenochrome content decreased about 50% after 10 min of perfusion with adrenochrome free solution and after 10 more min a further 10% decline was noted. In contrast, developed tension did not markedly improve after either 10 or 20 min of perfusion with adrenochrome free solution. Resting tension continued rising for 10 min and then declined to near normal levels. It therefore appears that after perfusion with adrenochrome, hearts took time to return to their preset resting tension, while the effects on

contractile force could not be reversed by decreasing adrenochrome content by perfusion with adrenochrome free buffer. It is likely some ultrastructural and biochemical damage had occured which could not be very quickly reversed, such as an influx of calcium ions (14, 33, 39).

The natural catecholamines and isoproterenol do not bind irreversibly to the heart. Iversen (141) perfused hearts for ten min with catecholamines containing solution, followed by 20 min of catecholamine free perfusion. This procedure removed roughly 85% of the tissue catecholamines. The present study found that slightly under 40% of the adrenochrome remained after 20 min of post perfusion, indicating a stronger binding. Callingham and Burgen (151) found isoproterenol, similar to the natural catecholamines, was removed rapidly by post perfusions. Also, isoproterenol did not bind strongly to subcellular fractions. Much more isoproterenol was lost to supernatants of fractions than was retained in the pellets of fractions. This was not the case with adrenochrome which was not so easily washed from the hearts and remained strongly bound to the pellets. Thus, the binding of adrenochrome was stronger, being less reversible.

The linearity and the apparent ease with which adrenochrome entered and bound to the cells suggests that some strong uptake mechansim is involved. A single experiment involving intravenous injection of labelled $[C^{14}]$adrenochrome in rats confirmed that this uptake is very active and occurs in whole animals. Thus, adrenochrome binding and uptake observed in this study may

reflect processes occuring naturally in the heart (unpublished observations*). Further work is necessary to clarify and quantify this point.

The nature of the uptake of adrenochrome is still not known. Though Michaelis - Menton kinetics were found an active transport system or facilitated diffusion both could result in such kinetics (150). In extraneuronal catecholamine uptake isoproterenol and norepinephrine do not bind strongly intracellularly (150, 151) and are highly concentrated making active transport more likely than facilitated diffusion (150). In this case, intracellular binding was strong suggesting either possibility, though it is still premature to speculate on this point.

Recent work in our lab has suggested that adrenochrome has an important role in vivo (Dhillon, K.S., In preparation). Intravenous injection of adrenochrome (10 mg/kg - 50 mg/kg) in male Sprague Dawley rats, immediately caused changes in blood pressure and various types of arrhythmias. The severity of the

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*After an injection of 20 mg/kg of [C^{14}]adrenochrome with a 5 min equalibrium period, the hearts contained 65 ug adrenochrome/gm tissue. This is well above the amount of adrenochrome present in the extracellular space of the heart, assuming adrenochrome distributes homogeneously in a vascular interstitial space of 24% and that the extracellular space of the myocardium is 19% of the myocardial volume.

effects depended on the concentration and sometimes resulted in the death on the animals.

The great affinity of adrenochrome for the sarcolemma and the sarcolemma rich 1,000g fraction, was not surprising. Adrenochrome has been shown to strongly inhibit sarcolemmal Na^+ - K^+ ATPase *in vitro* or in perfused hearts. It has also been indirectly suggested to bind to this fraction in previous work (38). Part of the mechanism by which adrenochrome inhibits this enzyme could thus be direct, by an interaction with the enzyme, or some other related components of the sarcolemma.

Adrenochrome also was bound in relatively high amounts to the SR, although this makes up a much smaller total amount of adrenochrome, due to the lower yield of SR. Since adrenochrome has been shown to affect SR calcium accumulation when added to isolated SR (37), it may be that this action is also exerted by binding to the SR.

The binding to both myofibrils and mitochondria was relatively low. The marker enzymes indicated that these fractions were quite pure, thus the adrenochrome found there was probably not from other fractions. Myofibrils isolated from hearts perfused with adrenochrome, or exposed to adrenochrome directly, are not affected (38). Therefore it is not surprising that little adrenochrome is bound there. In contrast, mitochondria have decreased ability to accumulate calcium in the presence of adrenochrome (37). Also the mitochondria of hearts perfused with adrenochrome or oxidized isoproterenol have impaired oxidative

phosphorylation and adrenochrome can impair oxidative phosphorylation when added to isolated mitochondria (33, 42, 43). Thus, the small amounts bound by the mitochondria are enough to impair them, or the binding itself is not resposible for impairing mitochondrial function. Other factors such as calcium may be involved. Calcium has been implicated in adrenochrome induced cardiotoxicity (36, 37, 39) and can impair mitochondrial oxidative phosphorylation (81, 99-101).

The great affinity of adrenochrome for some fractions as opposed to others, and the specificity of adrenochrome in its actions on some enzymes as opposed to others (38) indicates some possible specific sites and modes of action, despite the large amounts bound.

Though it is possible that in the homogenization and isolation procedures, some adrenochrome was lost or rearranged where it was bound, this is unlikely for several reasons. Firstly, the binding of adrenochrome to its fractions was very strong. In isolation procedures only one fraction, the supernatant of the 100,000g fraction contained any signifigant amount of adrenochrome. This fraction would contain soluable proteins and thus probably did not represent freely dissociated adrenochrome. Other fractions showed no appreciable loss of adrenochrome, even after such drastic treatments as hypotonic shock and treatments with high ionic strength salts such as lithium bromide and potassium chloride. The recovery of adrenochrome from homogenate to crude fractions was very great,

between 96% and 99%, indicating the little loss of these fractions to their supernatants. Also, all of the isolations were done at 0-4° so that diffusion would be minimal and after perfusion the hearts were immediately placed in ice cold buffer to help prevent any rearrangement of adrenochrome.

Little cross contamination was shown when mitochondria from hearts perfused with [C^{14}]adrenochrome were mixed with sarcolemma isolated from control hearts, at 0-4°. Sarcolemma which was then soon isolated again contained very little of the radioisotope. This suggests that little cross contamination occurs under these conditions, even from a fraction binding little adrenochrome to one binding larger amounts.

Propanolol and iproniazid both inhibit adrenochrome induced cardiotoxicity (35). Both of these reduced adrenochrome uptake between 48% and 43% (Table 4). Since neither iproniazid nor propranolol can maintain both contractile force and prevent ultrastructural damage over longer times (35), some adrenochrome-induced alterations must remain and these remaining alterations may be what is reflected in the adrenochrome still found in the myocardium. In this study the addition of adrenochrome in the presence of either drug, prolonged and pronounced the increase in initial contractility caused by adrenochrome alone. This occured in a dose dependent fashion (Fig. 7) and was followed by a gradual decline in developed tension. The signifigance of these effects are not yet clear. Propranolol itself was observed to be cardiodepressent -

inhibiting developed tension up to 28% - an observation reported by other authors and one possibly related to actions on calcium movements (197, 198). Relief of this inhibition may be responsible for some of the observed effects. Iproniazid had no such cardiodepressent effects and its effects on developed tension, although similar, were somewhat smaller.

Iproniazid has been used successfully in the past to treat patients with angina (199-201) and inhibition of monoamine oxidase with iproniazid or other inhibitors has been reported to reduce myocardial damage by isoproterenol (202-205). This may be the result of decreased accumulation of catecholamines or of an oxygen sparing effect (205). In this study iproniazid acted to decrease adrenochrome accumulation, possibly protecting the myocardium in this manner. Propranolol has also been shown to reduce catecholamine induced cardiac necrosis (69, 74, 83, 88, 92, 93,) in addition to its well known use in the treatment of patients with arrhythmias (206, 207). In catecholamine-induced cardiac necrosis propranolol can prevent the increases in calcium due to isoproterenol and delays or reduces the ultrastructural damage (69, 74, 83, 88, 92, 93). Since adrenochrome has been shown not to affect adenylate cyclase (38) it is unlikely that the protective effect of propranolol against adrenochrome toxicity acts through beta blockade. Eisenfeld et al. (208) found that some adrenergic blocking agents, including beta blockers, reduced the extraneuronal accumulation of catecholamines. Thus propranolol may act through this mechanism, though this is

probably not the major mechanism of its actions since propranolol reduced adrenochrome content to a greater degree than specific inhibitors of catecholamine uptake.

The uptake of adrenochrome exhibited some similarities and some disimilarities to that of catecholamine uptake. The affinity for adrenochrome was low but the capacity was high, similar to extraneuronal uptake. The Km and Vmax values for adrenochrome uptake were 258 X 10^{-6} M and 54.6 ug/min/gm, which is very similar to the Km Iversen (141) reported for extraneuronal norepinephrine uptake (252 X 10^{-6} M), though the maximum velocity of adrenochrome uptake was greater than what Iversen reported for norepinephrine (17 ug/min/gm) or epinephrine (11.8 ug/min/gm). The uptake of adrenochrome detected here was operating at the range of extraneuronal uptake. This does not exclude the possibility that the uptake also occurs in the same ranges that neuronal catecholamine uptake occurs.

Both the neuronal and extraneuronal catecholamine uptake inhibitors resulted in partial decreases in adrenochrome uptake. Since these are quite specific in their action, especially cocaine and corticosterone (137, 140-143, 145, 150, 154, 155), if the uptake of adrenochrome involved either neuronal or extraneuronal catecholamine uptake mechanisms alone, then 100% of the uptake should have been inhibited in only one case. As it is, partial involvements of both neuronal and extraneuronal uptake mechanisms are implicated or a completely different mechanism must be postulated. Similar to the early experiments of Iversen's

(141) and others', any adrenochrome taken up from the perfusate and then released (possibly being o-methylated) would not be detected in these experiments. Thus the estimation of adrenochrome uptake could still be an underestimation, though this error would probably be relatively small because of the small contribution this uptake makes in catecholamine uptake (150).

The potentiation by the steroids of the effects of adrenchrome on the resting tension of the heart is unexplained. Though such data for cocaine was not analysed due to the instability induced by cocaine, no such rise in resting tension was apparent. Steroids and in particular corticosteroids, greatly potentiate the damage that catecholamines do to the heart (30, 115-122). Fleckenstein (81, 87) showed these agents may act by greatly augmenting calcium accumulation in the myocardium. Since an involvement of calcium accumulation has also been suggested in adrenochrome induced cardiotoxicity (36, 37, 39) it is possible these effects on resting tension are calcium mediated. Large increases in intracellular calcium through a variety of mechanisms, are known to cause contracture which would be reflected as an increase in resting tension in this system (209-211).

Thus, it can be concluded that adrenochrome can be taken up by the myocardium in a perfused heart and the possibility of this uptake occuring <u>in vivo</u> is implicated. The uptake of adrenochrome is of low affinity and high capacity and has similarities and

dissimilarities to catecholamine uptake mechanisms. Further work on adrenochrome is suggested, including work with labelled adrenochrome *in vivo*. Some such experiments are already planned for this laboratory. Work with various calcium antagonists might also clarify some of the effects of adrenochrome with respect to the action of steroids.

VII SUMMARY

1. Perfusion of hearts with adrenochrome caused marked decreases in developed tension and increases in resting tension as previously reported.

2. Adrenochrome content of hearts increased with time of perfusion reaching high values and was dependent on the concentration of adrenochrome the heart was perfused with. A high capacity low affinity system was implicated with the Km and Vmax values of adrenochrome uptake 258 X 10^{-6} M and 54.6 ug/min/gm, respectively.

3: Various subcellular fractions bound varying amounts of adrenochrome with the sarcolemma having the highest specific and total activities. The amounts bound increased with the time of perfusion in most fractions.

4. The binding of adrenochrome was quite strong. It was not readily reversible by adrenochrome free perfusions and fractions containing adrenochrome did not readily lose it. The post perfusions of hearts with Kreb's Henseleit after perfusion with adrenochrome, resulted in an eventual return to normal of resting tension, but contractility did not recover greatly.

5. Both propranolol and iproniazid, inhibitors of adrenochrome-induced cardiotoxicity, prevented adrenochrome uptake greatly.

6. Both the neuronal and extraneuronal inhibitors of catecholamine uptake, depressed the uptake of adrenochrome somewhat. Corticosterone and 17-beta-oestradiol in combination with adrenochrome, caused large increases in the resting tension of the isolated perfused hearts.

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