A CARDIOACTIVE MATERIAL FROM GAS-PERFUSED CAT HEARTS

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ABSTRACT

A cardioactive substance is removed from gas-perfused cat hearts by intermittent perfusion with Krebs solution. The active material is precipitated from the washings by 25% saturation with ammonium sulphate. This material was called wash-out factor (WOF). WOF increased the contractile force of kitten atria failed by several methods. Myocardial depression caused by pentobarbital resulted in stable, sensitive preparations and this method of inducing failure was used as the standard.

Marked variability in the cardioactivity of samples of WOF obtained from different hearts necessitated treating each sample as a separate entity. Some species sensitivity to WOF was found. Kitten and guinea-pig atria were equally sensitive, those from rats less sensitive and rabbit atria did not respond. The responses to WOF in preparations failed by the standard method were reproducible and showed a dose-response relationship. A positive correlation between the cardiotonic activity and the protein concentration was found. The activity was not due to catecholamines *per se*, but elimination of adrenergic influences decreased the positive inotropic effect of WOF.

The positive inotropic effect of WOF was characterized by studying its effect on the parameters of isometric contraction. The changes induced by WOF were compared with those caused by ouabain and adrenaline. When the concentrations were matched to give similar effects on peak tension (PT) all three agents increased the maximum rate of tension development (dT/dt) to an equivalent degree. WOF and ouabain prolonged time to peak tension (TTP) whereas adrenaline shortened it.

The effects of WOF on PT and $\frac{dT}{dt}$ were decreased approximately

50% by treatment with pronethalol and in muscles from animals pretreated with reserpine. These treatments increased or did not affect the changes in TTP caused by WOF, again indicating that part of the mechanism of action of WOF is due to the release of noradrenaline from the nerve endings. This does not account for all the cardioactivity. Most of this effect was removed by extraction of washings with ether before precipitation of WOF.

When the effects of WOF, adrenaline, ouabain and tyramine on PT were compared at two frequencies of stimulation (0.5 and 1.0 sec^{-1}) WOF and ouabain had the greater positive inotropic effect at the slower frequency. Adrenaline and tyramine had a greater effect at the higher one. All four agents decreased the slope of the frequency reduction test (FRT).

Extraction of heart washings with ether separated WOF into two cardioactive components. The lipid increased the contractile force in isolated atria by releasing endogenous stores of noradrenaline. The positive inotropic effect of the lipid was blocked completely by pronethalol and was absent in atria taken from animals pretreated with reserpine.

The aqueous phase after extraction with ether still contained a cardioactive substance. This material was called extracted WOF. Extracted WOF had about 50% the cardiotonic potency of WOF. The effects of extracted WOF on PT were not blocked significantly by pronethalol or by treatment with reserpine. Extracted WOF prolonged TTP, an action which was increased by pronethalol and reserpine.

WOF, extracted WOF and the lipid, stimulated the transport of 3-MG into rat hemidiaphragm. No correlation between the cardiotonic and the sugar transport stimulating effects of WOF was found. The limited data with the lipid suggests that a positive correlation may exist between the two biological activities.

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INTRODUCTION

CARDIOTONIC SUBSTANCES OF ANIMAL ORIGIN

CARDIOACTIVE MATERIAL IN BLOOD AND SERUM

In 1872 Bowditch demonstrated that the beat of hypodynamic frog hearts was strengthened when serum was added to the perfusate. This was later confirmed by Ringer (1885) and others (Clark, 1913; Hajdu and Szent-Györgyi, 1952a; Nayler and McCulloch, 1960). The stimulant substance was found in alcoholic extracts of serum and was not destroyed by saponification (Clark, 1913). Clark suggested that the active substance was a soap which exerted its action by fixation of calcium on the surface of the muscle cell. Besides failure, prolonged perfusion of cardiac tissues in the absence of serum or plasma results in the loss of the frequency-force relationship (ISR) (Tuttle and Farah, 1962; Hajdu and Leonard, 1961; Barclay et al., 1961) and post-stimulation potentiation (PSP) (Tuttle and Farah, 1962). Addition of serum to the perfusate restores the contractile force (Howell and Cooke, 1893; Hajdu and Szent-Györgyi, 1952a; Nayler and McCulloch, 1960; Clark, 1920), the ISR and the PSP (Hajdu and Szent-Györgyi, 1952a). The positive inotropic action of serum on frog heart cannot be prevented by depletion of cellular potassium by quinidine, depletion of catecholamines by reserpine, or by concentrations of dinitrophenol which prevent the positive inotropic effects of the cardiac glycosides (Nayler and McCulloch, 1960). The addition of serum to the perfusates of hypodynamic hearts also causes an increase in glucose and oxygen utilization (Clark et al., 1938).

The onset of failure both in substrate-free medium and in medium containing glucose is delayed by the incorporation of serum into the perfusate. Cardiac glycosides delay or prevent failure when the

medium contains glucose but have no such activity in substrate-free medium (Zacharia, 1961; Bennett and Chenoweth, 1952). In the rat heart perfused with substrate-free bicarbonate buffer, pyruvate becomes superior to glucose in restoring contractility when serum is present (Zacharia, 1961; Garb et al., 1955), whereas glucose is the superior substrate in the absence of serum (Berman and Saunders, 1955). The action of serum is not species-specific, since bovine serum improves the contractility of cat papillary muscles (Green et al., 1952) and human serum affects the frog heart (Hajdu and Leonard, 1960). Many lipid materials such as the sodium salts of oleic and caprylic acids exert effects on the frog heart preparation similar to the restorative actions of serum (Loewi, 1955; Broadbent, 1963). Like serum, they also antagonize the negative inotropic effects of increasing the potassium ion content of the perfusate. Zacharia (1961) investigated the ability of serum to delay the onset of failure in isolated rat hearts, and reported that heat coagulated serum exerted similar actions. He also reported that a chloroform-methanol extract of serum was as effective as serum itself. These reports, therefore, appear to lend credence to Clark's hypothesis relating a cardioactive lipid substance in serum to the reversal of failure.

Hajdu and coworkers (Titus et al., 1956; Hajdu et al., 1957) isolated a phospholipid, β-palmitoyl lysolecithin from mammalian serum and tissues. Its effects on the hypodynamic frog heart were similar to those of digitalis glycosides (Leonard and Hajdu, 1960). The assay procedure for this cardioactive material was based on the staircase phenomenon of the frog heart (Hajdu, 1957). The pure substance increased the developed isometric tension of squab ventricle strips.

Hajdu et al. (1957) have expressed doubt that this substance has a physiological role in the mammalian organism. Green (1952) reported a positive inotropic effect of a crude liver extract of liver rich in lysolecithin. His findings tend to support Hajdu's results. In 1938, Clark showed that impure lecithins were capable of stimulating the frog heart but that pure lecithins no longer had this action. Perhaps Clark's impure lecithins were contaminated by lysolecithins.

Recently Govier and Boadle (1967) studied the cardiac action of L - α - lysolecithin on isolated preparations of guinea-pig, rat and rabbit heart. The inotropic and chronotropic effects were antagonized by pronethalol and were absent in preparations from animals pretreated with reserpine. They concluded that the effects of lysolecithin on the heart are the result of catecholamine release and are not due to a digitalis-like action.

Experiments with synthetic β-palmitoyl lysolecithin have shown that it bears some resemblance to ouabain. Marro and Capraro (1961) found that the effects of the two agents on potassium fluxes in heart muscle were similar. Although the synthetic β-palmitoyl lecithin increased the tone, it never produced contracture of the frog heart even at high doses, whereas ouabain readily produced contracture (Scarcini et al., 1960). They found that the effects of the synthetic substance were readily removed by washing the heart with fresh Ringer's solution but those of ouabain were more persistent. Cobbin and Thorp (1959) found that synthetic β-palmitoyl lysolecithin had no inotropic action on isolated cat papillary muscles in concentrations up to 5×10^{-5} g/ml. Kahn and Schindler (1962) examined the premise of a digitalis-like action of β-palmitoyl lysolecithin in two mammalian test systems. They

showed that the substance did not inhibit the ATPase of red blood cells, an action typical of digitalis. They also showed that the lipid had only a negative inotropic action on isolated guinea-pig ventricles.

The effect of α - (β-palmitoyl) - lysolecithin and ouabain on action potentials recorded from single fibers of guinea-pig myocardium have been compared (Marro et al., 1961). The most significant effect reported was a delay in the repolarization of the atrial tissue which was interpreted as a potentiation of the sodium pump mechanism, an effect opposite to that shown by high doses of ouabain, but similar to those reported at "therapeutic" concentrations of digitalis glycosides.

A substance provisionally identified as a lysolecithin, produced when blood plasma is incubated at 37°C for 24 hours, was found by Khairallah and Page (1960) to have both vasopressor and oxytocic activity. Similar effects could be obtained with the product of the action of snake venom phospholipase A on lecithin and the authors concluded that a similar enzyme present in the plasma was responsible for the production of the lysolecithin.

The prostaglandins are a group of physiologically active lipids. With the exception of $PGF_{2\alpha}$, the prostaglandins are potent vasodilators. Inconstant effects on the heart have been reported. Lee et al. (1965) found little effect of prostaglandins on the isolated perfused rabbit heart. Berti et al. (1965) found that PGE_1 increased the rate and force in the frog and guinea-pig hearts, but that the cat, rabbit and rat were generally insensitive. On the other hand, Vergroesen et al. (1967) obtained clear cut effects on both the coronary flow and the contractile force of the rat heart, possibly because they used larger doses. In their studies, PGE_1 and PGE_2 increased the coronary flow rate and $PGF_{1\alpha}$

greatly increased the force of contraction. Recently Nakano and McCurdy (1967) studied the cardiovascular effect of PGE_1 in anaesthetized dogs. Many hemodynamic changes were noted including an increase in myocardial contractile force and cardiac output and a decreased arterial pressure. The response was dose related and was not blocked by pronethalol.

However, not all cardioactive materials reported in serum have been lipids. Some substances have been protein and others, low molecular weight materials. None have been isolated in pure form and characterized. Green et al. (1952) found the major component of activity was in the Cohn Fraction IV and V of human and bovine plasma. Activity in the globulin fraction has also been demonstrated by Hajdu and Leonard (1958, 1960, 1965; Leonard and Hajdu, 1960, 1961). They described a protein system present in serum from most species of mammals which increased the contractility of the isolated frog heart. The material consisted of three components (distinguished by fractionation with sodium sulfate) termed cardioglobulins A, B and C. Increased levels were found in sera from patients with essential hypertension or aortic stenosis. They feel that each of the components is a protein. Evidence for this included the observations that the material was heat labile, could be precipitated with acetone, was not extracted by organic solvents, and that ultrafiltration did not affect the activity. The three cardioglobulins function together; there is no cardiotonic activity if any one of the components is absent. Cardioglobulin-C labelled with ^{45}Ca becomes bound to the myocardium only after prior treatment with cardioglobulin-B. Cardioglobulin-A then released the bound cardioglobulin-C into the cell where it exerts an inotropic effect. The cardiotonic activity of the system resembles that of the cardiac glycosides. However, they suggest

that the cellular mechanism of action is probably different. The glycosides require calcium ion for activity whereas the B-component has calcium firmly complexed to the protein. The cardioglobulins are inactivated in the presence of tissue homogenates. The phosphate bond present in cardioglobulin-A becomes hydrolyzed by the tissue enzymes. The bioassay for estimating the plasma concentration of each component is very complex and tedious and is based on the action of the system on the isolated frog heart. No purification was attempted beyond that necessary to establish the existence of the three components. None of the proteins is species specific. This was shown by using combinations of cardioglobulins obtained from various mammals in the assay on the frog heart.

Salter and Taylor (1952) compared serum with the cardiac glycosides and found cardiotonic activity in the albumin fraction. Greater activity was found in serum of healthy young adults than in that from older people. The maximum activity of 100 ml of serum corresponded approximately to that of 30 μg of ouabain. Like ouabain, the albumin fraction shows synergism with calcium ions. The dose-response curve for albumin resembles that for ouabain; and the onset of action is similar. No contracture was observed with the albumin fraction. The activity was expressed in ouabain-equivalent units. The activity decreased 50% after four months in solution at 4^{o}C; lyophilized albumin remains highly active for longer than a year. The activity was not altered by dialysis or treatment with trypsin, although it was destroyed by heat (Green, Giarman and Salter, 1952). The inotropic effect was measured on cat papillary muscles failed by low calcium medium or preparations exhausted after contracting 15 hours.

Nayler and McCulloch (1960) reported that human blood plasma had a positive inotropic effect on the isolated toad heart. Superficially the plasma cardioactivity resembled that of the cardiac glycosides, yet DNP and quinidine sulfate left it unchanged but abolished the response to the glycosides. Curtain and Nayler (1963) extracted from human plasma two cardiotonic substances. One, with a molecular weight exceeding 50,000 was suggested to be identical to the globulin fraction described by Hajdu and Leonard (1958). The other fraction, with molecular weight 4,000 - 10,000, was named Kinekard. It contributed 35% of the total plasma activity. Kinekard occurs in the blood plasma of a number of species (Nayler et al., 1965a). The material was concentrated until 1 μg of the substance had the same effect as 0.3 μg of adrenaline on the isolated toad heart. Nayler and her coworkers have shown that Kinekard has a positive inotropic effect on isolated dog, rabbit, monkey and rat papillary muscles. They reported that purified samples of this material had variable effects on the rate of rise of isometric tension, and that it decreased the time for repolarization of the cardiac action potential. Increases in systolic and diastolic blood pressures as well as increases in cardiac output and ventricular systolic pressures were demonstrated in anesthetized rabbits (Nayler et al., 1965b). Kinekard was inactivated by exposure to prcnase, suggesting that the material is a polypeptide. Its action was not antagonized by the β-adrenergic blocking agents, DCI or pronethalol (Curtain and Nayler, 1963). Its action in vivo was shown to differ from those of bradykinin, angiotensin and vasopressin (Nayler et al., 1965b).

The action of Kinekard on isolated smooth muscle (rabbit thoracic aorta strips, rabbit ileum and guinea-pig uterus) was identical

to that of epinephrine (Dorevitch, Nayler and Lowe, 1967). They concluded that the material had a direct action on smooth muscle and that both α and β receptors may be involved. The action does not depend upon the release of stored catecholamines.

Lowe and Nayler (1965) reported briefly on the plasma Kinekard levels in health and disease. They noted that some patients had plasma concentrations either below or above the "normal" range. Recently Lowe (1967) found some correlation between plasma levels and certain cardiovascular syndromes, such as essential hypertension. The diversity of the actions exerted by Kinekard engender doubt as to any physiological role for this substance.

Yet another substance may be extracted from ox blood. Thorp and Cobbin (1967) fractionated blood into erythrocytes, leucocytes and plasma. The highest concentration of the cardioactive material was found in the erythrocyte fraction. Less activity was found in the plasma. The differences were significant. The leucocyte-rich fraction was devoid of activity. The amount of active substance in red blood cells corresponding to 20 ml of blood almost trebled the force of contraction of isolated papillary muscles, whereas that isolated from the plasma corresponding to 20 ml of whole blood did not quite double the contractile force. They made no speculations as to the chemical nature of the active components.

Naturally occurring polypeptides have been studied extensively for cardiotonic activity. Neither of the octapeptides from the posterior pituitary appears to be concerned with the regulation of cardiac function. Vasopressin causes profound pressor effects and constriction of the coronary arteries in mammals. The latter decreases contractility

(Nakano, 1967). Covino (1963) reported that synthetic oxytocin (15 mU/ml) increases the strength of contraction of isolated cat papillary muscle. Others have disagreed. Nakano and Fisher (1963) described a negative inotropic effect on guinea-pig atria and dog papillary muscle.

The actions of bradykinin upon cardiac tissue are also subject of controversy. General agreement exists that this polypeptide increases cardiac output in anesthetized and conscious animals and in man. There is no agreement as to the mechanism of this increase. In anesthetized dogs, the increase in cardiac output following administration of bradykinin was accompanied by reduced peripheral resistance and reduced stroke volume (Page and Olmstead, 1961; Rowe et al., 1963) suggesting that the effect resulted from an increased venous return. Montague et al. (1963) described increased cardiac output in the rat associated with a small increase in heart rate and a large increase in stroke volume. Also in rats, marked cardiac stimulation to bradykinin was observed when the vasodilatory action was blocked by pentolinium (Rosas et al., 1965). In humans, infusions of bradykinin cause increased cardiac output without significant changes in heart rate or arterial blood pressure (Kontos et al., 1963). Their findings were interpreted as an increase in stroke volume in the presence of decreased peripheral resistance.

The in vivo experiments are difficult to interpret because it is not possible to distinguish a direct effect from a secondary one due to the increased venous return resulting from the decreased vascular resistance or to the release of adrenaline from the adrenal medulla (Feldberg and Lewis, 1964).

Heeg and Meng (1965) studied the effects of bradykinin on guinea-pig isolated cardiac preparations. They reported a small positive

inotropic effect in perfused hearts, a positive inotropic effect in atria, and no effect on papillary muscle. No significant effect on heart rate was observed.

Angiotensin has a moderate positive inotropic effect in isolated perfused hearts of guinea-pigs (Bianchi et al., 1960), rabbits (Meier et al., 1958), cats (Hill and Andrus, 1941) and in dog heart-lung preparations (Areskog, 1962 ; Fowler and Holmes, 1964). Fowler and Holmes (1964) found that prior treatment with reserpine did not change the pattern of the response to the peptide in the heart-lung preparation. Gross et al. (1965) studied the action of angiotensin in vivo in rats following ganglionic blockade. They reported an increase in cardiac output. Reserpine pre-treatment of the animals eliminated most of the effect. Ross and White (1966) confirmed the indirect action of angiotensin in the cardiovascular responses of the cat. Due to the marked vasoconstrictor properties of angiotensin, in vivo studies are difficult to interpret. (Page and Olmstead, 1961; Downing and Sonnenblick, 1963). Downing and Sonnenblick (1963) reported that angiotensin had no effect on isolated cat papillary muscle. The opposite findings were reported by Fowler and Holmes (1964) using the same preparation. Heeg and Meng (1965) reported a small positive inotropic effect on guinea-pig atrial and papillary muscle preparations. Angiotensin has a positive inotropic effect on isolated cat papillary muscles which persists after pretreatment with either reserpine or nethalide (Koch-Weser, 1965). He concluded that angiotensin did not depend upon intact catecholamine stores for its effect or act directly upon β-adrenergic receptors. His findings are in agreement with results in the dog heart-lung preparation (Fowler and Holmes, 1964).

A number of neutral L-amino acids have been shown to increase arterial blood pressure and cardiac contractile force in dogs (Gatgounis and Hester, 1964). The basic L-arginine had a negative inotropic effect. Garb (1955) demonstrated a positive inotropic effect with cysteine, methionine, and glycine in cat papillary muscles. However Cobbin and Thorp (1967) found no effect with glycine, cysteine and leucine on the same preparation or on isolated guinea-pig atria. Large concentrations of histidine (4×10^{-3} g/ml) and serine (1×10^{-2} g/ml) gave a slight response in papillary muscle preparations. Histidine and proline showed a positive inotropic effect in guinea-pig atria. The basic amino acids arginine and lysine were inactive on the papillary muscle preparation.

Steroids have been examined for cardiotonic properties because of their structural resemblance to the plant glycosides. In general (with the exception of the toad poisons) the naturally occurring steroids have little cardiotonic action in physiological concentration. Bile salts and cholesterol have only slight inotropic effects on perfused frog and rabbit hearts (Loynes and Gowdey, 1952) and do not stimulate cat papillary muscle (Green, 1952). Progesterone abolishes the staircase phenomenon in the frog heart but cortisone, estrone and testosterone modified the staircase only slightly (Hajdu and Szent-Györgyi, 1952b). Emele and Bonnycastle (1956) showed that some but not all "adrenal" steroids exerted a slight positive inotropic effect on cat papillary muscle. Deoxycorticosterone was without effect until concentrations of 0.5% were employed. The high concentrations had a purely depressant effect. Agreement with these findings was reported by Cobbin (1959) using rat heart-lung preparations. These substances

cannot account for the inotropic activity of plasma since the concentrations required to elicit an effect are considerably greater than levels found in plasma under physiological conditions.

Conflicting results have been reported with aldosterone. No effect on cardiac muscle was reported in dog heart-lung preparations (Areskog, 1962), isolated rabbit atria (Levy and Richards, 1962), trabeculae carnae from rat hearts (Ullrick and Hazelwood, 1963) or cat papillary muscles (Thorp and Cobbin, 1967). Other studies report that aldosterone has a positive inotropic action. These include studies using monkey papillary muscle (Nayler, 1965) and cat papillary muscle (Tanz, 1962). The different results reported are probably due to different exposure times to the drug. Nayler, and Tanz exposed their preparations for an hour whereas Thorp and Cobbin exposed their preparations for 5 - 10 min. Lefer and Sayers (1965) found that aldosterone exerted a small positive inotropic effect on cat Langendorff heart preparations failed by pentobarbital and on cat papillary muscle depressed by low calcium. It has a cardiotonic effect on the rat heart-lung preparation (Ballard, Lefer and Sayers, 1960).

Antagonism by aldosterone of the inotropic action of ouabain was suggested by Levy and Richards (1962) who reported antagonism of contracture of atrial muscle. Lefer and Sayers (1965) confirmed this in isolated hypodynamic cat hearts and in isolated papillary muscle. Lefer (1966) evaluated a variety of corticosteroids as ouabain antagonists in the isolated cat papillary muscle preparation in a low calcium buffer. He found that aldosterone, α- fluorocortisol, deoxycorticosterone and corticosterone antagonized the positive inotropic

effect of ouabain. At 27°C aldosterone failed to antagonize ouabain, but at this lower temperature aldosterone exerted a larger positive inotropic effect than that observed at 37°C. In summary, the effects of aldosterone upon cardiac tissues are slight and of doubtful significance. Aldosterone cannot be considered to play an important role in the regulation of cardiac performance.

CARDIOACTIVE MATERIAL FROM MAMMALIAN TISSUES

Some naturally occurring substances in serum posses cardiotonic activity which differs from that of catecholamines and which has some resemblance to the actions of cardiac glycosides on the heart. They may serve a physiological function in supplementing nervous mechanisms in the regulation of cardiac performance under certain conditions. Evidence is accumulating that organs contain substances which may serve as humoral regulators of the heart. Szent-Györgyi (1953) implied that these substances act in a manner similar to the cardiac glycosides. He suggested that the digitalis glycosides are not drugs at all but are "substitutes for the missing screws in our machinery" which had a role in a very basic physiological regulation. The spleen, the liver and the heart itself have been implicated most often as sources of these substances.

The pharmacological action of tissue extracts has been known for many years. In 1929, Major and Weber found that extracts of liver, lung and brain depress the blood pressure of the dog. They believed that this was the result of histamine, choline and a third unidentified substance. Asher and his colleagues observed that fluids perfused through the liver acquired an adrenaline-like property when later tested on isolated amphibian hearts (Asher and Takahashi, 1924; Asher, 1925).

He prepared liver extracts which increased cardiac output and blood pressure in mammals. Similar effects were observed following the administration of cholates. Asher suggested that the liver added cholates to the perfusate which then produced the observed improvement in cardiac function (Asher and Beyeler, 1926).

Zuelzer was interested in the concept of a cardiac hormone. He prepared an extract from liver which was initially tested on frog hearts and heart-lung preparations before clinical trial (Salomon and Zuelzer, 1929). The liver extract was standardized and sold under the name "Eutonon". Zuelzer (1942) proposed Eutonon as a specific cardiac hormone differing from digitalis in possessing a wide margin of safety; it did not accumulate on chronic administration and lacked central or diuretic actions. The extract was alleged to be free of protein, amino acids, adrenaline-like materials, histamine, choline and acetylcholine. However, earlier examinations of Eutonon by Heinsen (1934) revealed the presence of choline and tyramine. Zuelzer (1930) claimed Eutonon arrested failure in the dog heart-lung preparation yet Krayer (1933) found little effect on cardiac output, coronary flow or heart rate in the same preparation.

Early notions of a "cardiac hormone" present in mammalian tissue tended to become centered around substances capable of producing coronary dilatation. In early studies, isolated perfused hearts were used to indicate inotropic activity. The limitations of such preparations for this purpose is well known. The amplitude of contractions are dependent upon other variables such as coronary flow and heart rate.

The early observations of Asher and Takahashi (1924) were confirmed by Roncato (1930) using the Starling heart-lung preparation.

Blood which perfused the liver acquired a cardiac stimulant action which disappeared after traversing the heart and lungs. Roncato concluded from these experiments that the liver added a nutrient or stimulant substance to the blood which was rapidly absorbed by the heart. The effects of hepatic blood were not mimicked by addition of glucose or bile salts (Bassani, 1933). Kiese et al. (1936) suggested that the liver might be contributing small amounts of sympathomimetic amines to the blood perfusing the preparation.

Rein (1942) showed that progressive failure of a heart-lung preparation could be reversed by including the liver in the circuit. Similarly, if the liver was excluded from the circulation of an intact animal, the heart showed signs of progressive deterioration, which could be reversed rapidly upon reintroduction of the liver. This type of cardiac failure was enhanced by hypoxia, and the diminishing performance was shown to be associated with increased oxygen consumption. The failure was not due to a fall in the blood sugar level and the experimental set-up precluded hemodynamic causes. Since small doses of strophanthin exerted similar effects on the failing heart, Rein concluded that the liver might be capable of supplying the heart with a material chemically similar to strophanthin. Pinotti (1942) confirmed Rein's findings. He demonstrated that cardiac metabolism was more efficient when the liver was included in the preparation. Measurement of cardiac work and oxygen consumption showed that the liver factor was labile and had a short duration of action. Althoug the chemical nature of the liver substance was unknown, Pinotti did not believe that it was a sympathomimetic amine or glucose. He agreed with Rein's suggestion that a steroid might be implicated. Kako et al. (1960) have recently

reinvestigated the factors contributing to the failure of heart-lung preparations and have shown that inclusion of the liver and spleen in the preparation delayed both the onset of failure and increase in oxygen utilization of the heart. The inclusion of the liver in the circuit of a failed heart-lung preparation did not reverse the changes in substrate and oxygen utilization but did increase contractile force and therefore the efficiency of cardiac contraction.

Many cardioactive substances have been isolated from liver homogenates. Green (1952) and Green and Nahum (1957) investigated a number of liver fractions and identified tyramine, methionine, menadione and dicumarol(sic) as cardioactive agents. Hajdu et al. (1957) found that the liver contained relatively large amounts of β-palmitoyl lysolecithin which they thought at the time to be the plasma factor increasing contractility.

A "spleen-liver" reaction has been reported by Bücherl and Rein (1949). An unknown substance was released when the splenic nerves were stimulated during partial occlusion of the coronary arteries. The material was transported to the liver where it caused release of a "specific hormonal substance" into the blood which appears to overcome the cardiac hypoxia. The active material resembled strophanthin since it caused greater economy of cardiac work. The substance released from the spleen was called "Hypoxie-lienen." Blood from the splenic veins of donor dogs relieved hypoxia in recipient normal or splenectomized animals when administered transhepatically. These findings have been confirmed in heart-lung preparations to which spleen and liver from donor animals were added to the perfusion circuit (Kako et al., 1960).

Meesman and Schmier (1956) found that during the spleen-liver

reaction the blood flow through the hepatic artery was increased and that through the coronary arteries was decreased. A sustained increase in the aortic blood pressure was observed. Several substances were administered transhepatically in an attempt to identify the humoral factor involved in this reaction. Ferritin, levulose, glucose, lactate, potassium phosphate, kallikrein, adenyl phosphates, serotonin and commercial spleen extract were excluded since they caused coronary vasodilitation rather than coronary constriction characteristic of the spleen-liver reaction. The role of noradrenaline in spontaneous failure is not clear and the question as to whether or not the spleen and liver supply noradrenaline is unresolved. Kako et al. (1960) have suggested that deficiencies of cholinergic and adrenergic substances in the perfusion fluid may be a contributing factor in the decline of the mechanical efficiency in the isolated heart-lung preparation. Meesman and Schmier (1955) noted that although intravenous or transhepatic administration of noradrenaline produced rises in systemic blood pressure and hepatic blood flow, the actions were of short duration and could readily be distinguished from those of splenic nerve stimulation. They concluded that neither noradrenaline nor adrenaline were wholly responsible for the mediation of the spleen-liver reaction. Danforth et al (1960) suggested that the beneficial results of including the spleen and liver in heart-lung preparations may be due to restoration of the depleted supply of catecholamines as it is known that noradrenaline is present in some quantity in these tissues. However, they also showed that administration of dopa to the perfusion fluid resulted in an inotropic action which was due to the conversion of the dopa to dopamine and noradrenaline. When this effect had worn off and the heart was again

in failure, the levels of noradrenaline in the heart remained elevated.

Areskog (1962) found that both hepatic and splenic venous blood contain cardioactive substances which were probably different chemically. Splenic blood differed from liver blood in that it increased coronary blood flow. Hypoxia is not required for the elaboration nor for the action of the active substance in liver venous blood. The addition of liver or splenic venous blood to failing heart-lung preparations restored the mechanical efficiency of the preparation (Schmier, 1958).

Cobbin and Thorp (1957) while studying tissue extracts for cardioactive agents found that the liver and spleen gave the highest yields. The spleen extracts were less contaminated with impurities and were subjected to further study.

Ox spleen contains many pharmacologically active substances but Cobbin and Thorp (1959) have shown that these substances (noradrenaline, adrenaline, serotonin, choline, and histamine) do not appear in acetone extracts of freeze-dried spleen in sufficient quantities to account for the stimulant action of these extracts upon isolated cat papillary muscles. Many separate types of tests were applied to exclude catecholamines as the active substance in the spleen extract. Catecholamines could not be detected in spleen extracts by chromatographic separation; incubation at pH 11 for one hour at 37^{o}C in the presence of manganese dioxide did not destroy the activity; nor were the potency of the extracts changed by DCI in doses sufficient to abolish the inotrophic action of noradrenaline in open-thorax guinea-pigs (Cobbin and Thorp, 1960).

The hypodynamic isolated papillary muscle preparation of

Cattell and Gold (1938) gave the most suitable index of the direct stimulant properties of active spleen factor extracts (Cobbin, 1959). Isolated guinea-pig eudynamic atria also responded to the material in a dose dependent fashion (Loeb, 1965). Activity was the same in muscles contracting isometrically or isotonically and the slope of the dose-response curves was the same. However, muscles contracting isotonically were more sensitive to the material. Adrenaline was used as the reference standard. Loeb concluded that the spleen factor possessed cardioactive properties akin to both adrenaline and ouabain. Neither substance alone satisfies all the properties of the active principle.

Temple et al. (1966) has attempted large-scale extraction and purification of the spleen factor. Several methods of purification were used. Each step in the process was monitored by bioassay, and all inactive by-products were discarded. The bioassays were done on isolated cat papillary muscles and guinea-pig atria. They also determined the partition coefficient of the active material between common immiscible solvents and water. Methylisobutyl ketone gave the most satisfactory purification; all the cardioactive material remained in the aqueous phase. The cardiotonic activity was correlated with an ultraviolet absorption maximum near 260 mμ. The molecular weight of the cardioactive spleen factor lies between 200 and 300.

The cardiotonic substance in spleen resembles the cardiac glycosides in some of its properties. The onset of its inotropic effect on papillary muscles is gradual (5 to 10 min to maximum effect) and is sustained for a long period dependent on the dose. The spleen substance is readily washed out of the bath which suggests that the substance is not very firmly bound to cardiac tissue. The positive inotropic action

is also seen in perfused kitten hearts where it is accompanied by a moderate increase in coronary flow and a slight increase in heart rate. In vivo experiments show that the time course and the nature of the inotropic effects resemble the action of small doses of cardiac glycosides. The action of the spleen extract on the staircase phenomenon in cat papillary muscle does not resemble that of the cardiac glycosides. Doses capable of producing a good inotropic response fail to modify the pattern of staircase. Small doses of crude spleen extract or cardiac glycoside potentiated and prolonged adenosine induced heart block in guinea-pig left atrium. The spleen substance was not destroyed by chymotrypsin, trypsin or pronase. The resistance to destruction by pronase suggests strongly that the substance in spleen extracts differs from the polypeptide substance (Kinekard) with inotropic properties found in normal human plasma (Curtain and Nayler, 1963).

CARDIOACTIVE MATERIAL FROM HEART

Haberlandt (1929) prepared a "heart hormone" from beef heart, alleged to be free of protein, lipid, adrenaline and histamine, which was capable of arresting atrial fibrillation in mammalian heart and which augmented the rate and amplitude of the frog heart. He obtained positive effects in only about 50% of his experiments. He did no control experiments with extracts of tissues other than the heart to support his contention that this was a specific hormone.

Oppenheimer (1929) repeated these experiments and was unable to confirm the presence of a specific heart hormone but was of the opinion that extracts from several tissues (heart, skeletal and smooth muscle, liver and lung) possessed, in adequate concentrations, and inotropic action on the heart. Drury and Szent-Györgyi (1929) analyzed the

effect of heart, brain, kidney and spleen extracts on the mammalian heart and believed that the pharmacologic action of these extracts was due to adenylic acid. Hajdu et al. (1957) found that the heart muscle contained large amounts of β-palmitoyl lysolecithin which they thought to be the plasma factor increasing contractility.

The occurrence of adenosine, adenylic acid, inosine and hypoxanthine in the perfusate from isolated hearts have been reported (Khairallah and Mommaerts, 1953a, 1953b; Jacob and Berne, 1960). The enzyme adenosine deaminase has also been found in the perfusate (Jacob and Berne, 1960). ADP and ATP have not been reported to be removed from the heart. However, the presence of nucleoside phosphorylase (Jacob and Berne, 1960) indicates the possibility that they were decomposed and determined as adenylic acid. The adenylates are lost into the perfusate in increased amounts during period of hypoxia (Berne, 1963) and they are potent relaxants of coronary arterial smooth muscle (Berne, 1963; Winbury et al., 1953). Buckley and coworkers (Buckley et al., 1959, 1961) investigated the cardioactivity of several nucleosides and their corresponding purine and pyrimidine bases on failing and nonfailing isolated dog and rabbit hearts. Positive inotropic activity was demonstrated for inosine, guanosine, thymidine, uridine and related bases: a negative inotropic effect was reported with adenosine and cytidine. They concluded that cardioactivity depended on the substituent on the number 6 pyrimidine ring-carbon. Cobbin and Thorp (1959) found cardiotonic activity only with uridine and deoxyguanosine when these compounds were tested on cat papillary muscle and guinea-pig atrial preparations. Rosenblum and Stein (1964) showed that guanosine has no direct effect upon the heart but that its action is mediated by the release of nor-

adrenaline from the nerve endings. Other investigators have reported contrary observations. Hollander and Webb (1957) reported that both adenosine and inosine reduced contractility in rat atria while guanosine had essentially no effect on contractile force. Angelakos and Glassman (1961) reported no effect from guanosine on contractile force in dogs as measured with a strain gauge arch sutured to the left ventricle. The conflicting results were probably due to the doses used. Rosenblum and Stein (1964) showed that both positive and negative inotropic effects could be produced with guanosine in isolated rat atria. Low concentrations of the nucleoside produced only the negative effect, high concentrations produced only the positive effect.

Isolated hearts perfused with substrate-free solutions fail rapidly (Clark, 1913; Garb et al., 1955; Robb, 1953; and Zachariah, 1961). Clark et al. (1938) observed that recirculation of a liquid perfusion medium lengthened the period of adequate contractility of frog hearts and indicated that failure might be due to the loss of an active substance from these preparations. Recently Gabel, Bihler and Dresel (1966) reported that isolated cat hearts perfused with 95% O_2 - 5% CO_2 beat more strongly and fail more slowly than do hearts perfused with substrate-free Krebs-Henseleit solution. The prolonged viability of gas-perfused hearts could not be explained on the basis of any physiological or biochemical differences. They concluded that the gas perfusate failed to remove a factor from the hearts which was necessary for the maintenance of optimum contractile force. They suggested that the factor would accumulate in the extracellular space of gas-perfused hearts and might be obtained in concentrated form by perfusion with small volumes of liquid perfusate. They reported (1967) that intermittent perfusion with

small volumes of Krebs (5.0 ml) at thirty-minute intervals caused rapid failure of gas-perfused hearts. The "washings" were found to exert a slight positive inotropic action on failed isolated atria. Treatment of the washings to 25% saturation with ammonium sulphate caused the precipitation of a material which was strongly inotropic to the test preparation. They found that pretreatment of gas-perfused hearts with very low concentrations of ouabain (1×10^{-10} to 1×10^{-9} g/ml) decreased in a dose-related manner both the quantity and the specific activity of the recovered material. They concluded that the perfusate removed an inotropic factor from the gas-perfused hearts and that the cardiac glycosides interfere with this removal. Cardiolipin pretreatment of the gas-perfused hearts also protected the preparations against wash-out failure (Howell and Dresel, unpublished).

Since the active component could be precipitated with ammonium sulphate they concluded that the substance was either one of several proteins in the precipitate or a nonprotein material coprecipitated under their conditions. Only extremely small amounts of the material were obtained and great variability of the activity of preparations obtained from different hearts was observed. The reconstituted material was thermolabile and activity was retained for longer periods of time by the addition of glutathione (GSH), 1 mg/ml, as an antioxidant and by keeping the solution in an icebath during testing. Preliminary experiments showed that failed preparations were more responsive to this material than were unfailed preparations. Therefore, their material was assayed for cardiac inotropic activity on isolated left atria from kittens failed by prolonged electrical driving. They found it difficult to maintain the contractile force of the driven atria at the desired constant level of approximately 30% that of

the control level. The effects of the heart material on increasing the contractile force was expressed as percent of the initial strength of contraction. The maximal response obtained from the material in the assay system was a return to 75-85% of the contractile force measured before failure was induced by driving.

It was the purpose of the present work to delineate further the pharmacology of this material and to attempt preliminary steps in its purification.

METHODS

METHODS

I PHYSIOLOGICAL

Gas-perfused Kitten Hearts

Kittens of either sex weighing1.0 - 2.4 Kg were fed a diet of meat and fish for 2 - 3 days before use. The animals were stunned by a blow on the head. The hearts were quickly removed and placed at once in a beaker containing previously aerated (95% O_2 - 5% CO_2) Krebs-Henseleit bicarbonate buffer (Krebs solution - see p 34) at room temperature. The hearts were cleaned of extraneous tissue. The aortae were then cannulated and the hearts connected to the perfusion apparatus. A small slit was made in the wall of each ventricle, care being taken to avoid the coronary vasculature.

The technique of gas perfusion was the same as that of Gabel (1965). Briefly, the method involved perfusion of the heart through the aorta with substrate-free Krebs solution for 5 min. The perfusate was quickly switched to the gas mixture (95% O_2 - 5% CO_2) saturated with water vapor. The temperature of both the perfusates was regulated at $37.5 \pm 0.4^{o}C$. The perfusion pressure was maintained at 60 mm Hg for the liquid and the gas-perfusion. The heart rate was kept constant at 168 beats/min by suprathreshold electrical stimulation from a Grass SD5 or S6 stimulator. Small clip electrodes were placed on the right atrial appendage and the ventricular apex. Rectangular pulses of 5 msec duration (5 - 10V) were used. A stainless steel hook pierced the apex of the perfused hearts and a string attached to the hook was led around a pulley to a Grass FT-03 transducer. The resting tension remained at 10 g throughout each experiment.

The gas-perfusion apparatus of Gabel (1965) was slightly

modified in order to accomodate 2 heart preparations with independent controls for liquid or gas-perfusion.

Cardiac Muscle Preparations

Left atria of cats, guinea-pigs, rats and rabbits were used. The weight ranges were:

Kittens	0.45 - 2.0 Kg
Guinea-pigs	250 - 390 g
Rats	200 - 340 g
Rabbits	2.5 - 4.0 Kg

The hearts were removed from the stunned animals. They were placed on an inverted petri dish covered with a sheet of filter paper moistened with Krebs solution. The left atria were quickly excised. Whole atria were used from rats, guinea-pigs and small kittens (< 0.8 Kg). The atria from the other animals were divided into halves by cutting either one side off and using the outer surface or by a single vertical cut to make 2 cup shaped preparations. A loop was made with terylene thread at the septal end of the atrial tissue and a thread tied at the opposite end for attachment to the hook of the strain gauge. Atria were suspended in Krebs solution containing 2 g/ml of glucose. The bath volume was 10 ml, the temperature 37 $\pm$ 0.5^{o}C. Contractions were obtained by electrical stimulation through large platinum electrodes placed on both sides of the atria. Suprathreshold rectangular pulses (5 msec; 5 - 12V : 1.5 times threshold) were supplied by a Grass SD5 or S6 stimulator. Preparations used for interval-strength relationship studies were stimulated by pulses of voltage 1.1 - 1.2 times that of the threshold. The frequency of stimulation was 1.0 sec^{-1} unless otherwise stated.

The resting tension was set at approximately one-half the tension which resulted in maximum contractility. The resting tensions for the preparations from various species were:

Kitten	1.75 - 2.25 g
Guinea-pig	0.7 - 1.0 g
Rat	1.0 - 1.25 g
Rabbit	2.0 - 3.0 g

During the first few minutes after mounting, small adjustments in length were made to keep resting tension constant. This was not necessary during the actual course of the experiment. The vast majority of the left atrial preparations were quiescent. A sixty minute period was allowed to elapse before the addition of any drug. The initial value of contractile force was then taken. The bathing solution was changed every 10 to 15 min during this period.

Right atria from small kittens and guinea-pigs were used. The method was the same as that used for left atria except electrical stimulation was omitted. Heart rate was counted from the recording of contractile force.

Papillary muscles from the right ventricles of cats weighing 1.5 - 2.2 Kg were used. An opening was quickly cut through the right ventricular wall and the wall carefully removed. The chordae tendinae were tied *in situ* with a long thread for future attachment to the transducer. The chordae end was freed and the papillary muscle was gently raised and a second thread was tied with a small loop, as close as possible to the mural end of the muscle. The papillary muscle plus a small piece of ventricular wall were then dissected free. The muscle was suspended in the bath under the same conditions as for left atria.

Resting tension was 1.0 g, the temperature 37 ± 0.5°C. The frequency of stimulation was 0.5 sec^{-1} except when interval-strength relationships were investigated. Suitable muscles ranged in weight from 0.2 - 14.0 mg and in length from 3.0 - 9.5 mm. None exceeded 1.3 mm^2 in cross-sectional area.

Smooth Muscle Preparations

Guinea-pig ileum

Guinea-pigs of either sex weighing 250 - 390 g were fasted overnight and killed by a blow on the head. Both terminal and non-terminal ileum were used. Terminal ileum is defined as the first 5 - 8 cm of ileum adjacent to the ileo-caecal junction; non-terminal as that more than 15 cm from the ileo-caecal junction.

Sections 2.5 - 3.0 cm long were suspended in a 10 ml bath containing well aerated Krebs solution. The temperature was 37 ± 0.5°C, resting tension was 1.0 g.

Before starting the actual experiment, the effect of 10^{-6} g/ml noradrenaline was determined in each preparation. Typically, terminal ileum responds by contraction whereas non-terminal ileum relaxes. For the purposes of this dissertation, this pharmacological differentiation was considered more important than the anatomical.

Rabbit aorta strips

Spiral strips of thoracic aorta, approximately 2.3 cm in length and 2.5 mm in width were cut for mounting (Furchgott and Bhadrakom, 1953). The strips were bathed in Krebs solution. Resting tension was 2.0 g.

Rat uterus

Virgin female rats weighing 200 - 250 g, not pretreated with estrogen, were killed by a blow on the head. The uteri were removed and placed

in de Jalon's solution (Holton, 1948). A strip of uterus approximately 1.2 cm in length was excised from the central portion of the uterine horn. The uterine muscle was suspended in a 5 ml bath at 30°C. Acetylcholine (1×10^{-7} g/ml) was sometimes used to induce contractions. With all smooth muscle preparations, a 1 - 2 hour period was allowed to elapse before the addition of any drugs. The bathing fluid was changed every 10 - 15 min during this period.

Recording

Isometric contractile force was measured with Grass FT-03 force displacement transducers and recorded on a Grass polygraph.

The course of isometric contraction was recorded using a Tektronic oscilloscope Type 502. Two methods of amplifying the signal from the strain gauge were used. In one method, the signal was amplified by means of an operation amplifier designed in this laboratory. In the second method, the signal was amplified by a Grass polygraph and then coupled to the oscilloscope input by a Grass model R5 D.C. A reverter. 35 mm photographs were taken with a Shackman oscilloscope camera.

Isotonic contractions of all smooth muscle preparations were recorded on a slowly moving (1.4 mm/min) kymograph paper with a 7 or 10 fold magnification.

II PREPARATION OF WOF

Routine washing of the gas-perfused heart was the same as that reported by Gabel et al. (1967). Their method involved four intermittent washes with 5 ml of Krebs solution each: the first wash one hour after the start of gas-perfusion, the subsequent three following at 30 min intervals.

Protocol for washing of the hearts for centrifugation and

sonication studies, and ether extraction varied from the routine method. These hearts were washed once an hour, 3 or 4 times starting 60 min after the start of gas-perfusion.

In most experiments, the four washes from one heart were saturated to 25% with ammonium sulphate and allowed to stand for 30 min in an ice-bath. The precipitated material was collected on an 8.0 μ and a 0.8 μ Millipore filter in the cold. Details of filtering and storage have been described by Gabel et al. (1967).

The dried, frozen precipitate (0.8 + 8.0 μ filter) from one heart was reconstituted in 5 ml of either 0.9% NaCl or in non-aerated Krebs solution to which 1 mg/ml of reduced glutathione (GSH) had been added as antioxidant. The solution was prepared immediately prior to use and kept in an ice-bath.

III SEPARATION TECHNIQUES

Centrifugation Studies

Single 5 ml washes from 2 hearts were pooled and the solution was centrifuged for 10 min at 8000 g in a Sorvall refrigerated centrifuge. Any sediment was discarded. The supernatant was then centrifuged at 100,000 g for 60 min in an International Model B-60 centrifuge using an A-211 head. The supernatant was carefully decanted. The sediment was resuspended in the original volume of Krebs solution. Ammonium sulphate, to 25% saturation, was added to the suspension and the supernatant and the precipitates collected separately on 0.8 μ Millipore filters. The material was redissolved on the same day, in 2.5 ml of 0.9% NaCl (with GSH added) and tested.

Disintigration with Ultrasound

The washing procedure was the same as for the centrifugation

study. The washes from each heart were treated separately. Glutathione was added and the wash after mixing divided into two 2.5 ml aliquots. One aliquot was transferred to an 8 ml heat resistant flat-bottom tube and placed in an ethanol-dry ice bath maintained at 0 to + 2°C. A titanium probe 3/8 inch in diameter was immersed 1 - 2 mm below the surface of the solution. Sound frequencies of approximately 20 Kilocycles/sec were generated in the specimens by means of a Bronwill Biosonik disintegrator (Bronwill Scientific, Rochester, New York). To prevent heating, the disintegrator was turned on for only 30 sec/min for 4 minutes. The two aliquots were then saturated to 25% with ammonium sulphate, filtered and the material reconstituted(1.1 ml of 0.9% NaCl with GSH added). The cardiotonic activity was assayed immediately.

Extraction with Organic Solvents

Flow sheets for the extraction of heart washings with ether or iso-amyl alcohol are given in the appropriate section of Results.

Reconstitution of the Solvent Extracted Material

The material extracted into the solvent had very limited solubility in water. The material was transferred by 3 small unmeasured volumes of ether into a small test-tube. The ether was then evaporated off under N_2 with the test-tube placed in an ice-bath. A known volume of acetone (0.4 - 0.8 ml) was added to the pooled material transferred with ether. The resultant solution was cloudy.

The rocking dialysis method of Fleischer and Klouwen (1961) was used to attempt to solubilize the lipid extract. A butanol-cholate mixture of material extracted with ether was dialyzed against saline for 7 days in the cold-room. The saline was changed every 24 h. The ether extract from one heart was dissolved in 1.0 ml of the alcohol-cholate

solution.

IV BIOCHEMICAL

Protein

The method of Lowry et al. (1951) was used, with bovine serum albumin as the standard. Glutathione blanks were included.

Catecholamines

The catecholamines were determined by a modification of the fluorimetric method of von Euler and Lishajko (1961) in common use in this laboratory. The amines were oxidized to lutines and the solutions stabilized by the addition of ethylenediamine to the alkaline-ascorbate solution. Fluorescence was read in a Farrand fluorimeter.

Assay of Effect of WOF on Sugar Transport

Young male hooded rats weighing 100 - 200 g, bred in this laboratory were used. They were killed by a blow on the neck and "intact" hemidiaphragms were prepared as described by Kono and Colowick (1961).

Each hemidiaphragm was incubated for 30 min with gentle shaking at 37°C in 2 ml of Krebs solution. The following substances were added to the buffer: 0.8% bovine serum albumin (to prevent adsorption of insulin and perhaps of WOF to glass), 5 mM mixture of ^{14}C-labelled and unlabelled 3 - O - methyl-D-glucose (3-MG) serving as test sugar, tracer amounts of ^{3}H-mannitol serving as extracellular marker, and when indicated insulin or WOF. In experiments with the lipid extracts of heart washings reconstituted in acetone, the same volume of the solvent was added to the control hemidiaphragm. In separate experiments it was found that this concentration of acetone (5 - 25 μl/ml) had no effect on sugar transport.

After incubation, the muscles were rinsed briefly (about 1 sec)

in ice-cold buffer and blotted gently. The diaphragm was trimmed of extraneous tissue, weighed and homogenized. Samples of the incubation media and of the tissue homogenate were deproteinized by the method of Somogyi (1945) using $Ba(OH)_2$ - $ZnSO_4$ and centrifuged. The medium was diluted 1:5 and aliquots of 0.3 ml of the diluted medium and the deproteinized tissue homogenate were added to 10 ml volumes of modified Bray's liquid scintillation mixture (Adamic and Bihler, 1967). The radioactivity of the supernatants was determined by double label liquid scintillation spectrometry. The Packard Tri-Carb, Model 3003 Scintillation Spectrometer was set so that only ^{14}C was counted in one channel. All ^{3}H counts and also some ^{14}C counts were registered in the other channel. The settings were 8% gain and a "window" of 125 - 1000 for the ^{14}C channel and 45% gain and a "window" of 50 - 250 for the ^{3}H channel. Under these conditions the ^{14}C counts in the ^{3}H channel were approximately 16.5% of the ^{14}C counts in the ^{14}C channel. This ratio was checked with the appropriate standard in every experiment and was used to calculate the ^{3}H counts in the sample.

Calculation of results

1) The tissue water content was determined by drying the tissue samples in vacuum to constant weight. It was 80.0% and this figure was used in subsequent calculations.

2) The distribution of the radioactive marker between medium and the tissue water was calculated as follows:

$$M = \text{cpm/ml of medium} = \frac{\text{cpm/vial x dilution factor}}{\text{aliquot/vial}}$$

$$T = \text{cpm/ml total tissue water} = \frac{\text{cpm/vial x vol. of homogenate}}{\text{aliquot/vial x vol. of } H_2O \text{ in tissue sample}}$$

Vol. of H_2O in tissue sample = wet weight x 0.8

Vol. of homogenate = H_2O in tissue sample + vol. of addition for homogenization and deproteinization.

3) The ratio T/M was then calculated for ^{14}C and ^{3}H. Assuming that T/M for ^{3}H, that is, the distribution space for mannitol indicates extracellular space, the distribution value for ^{14}C (i.e. the sugar) was corrected to express T/M for sugar in the intracellular water only.

$$T/M \text{ (intracellular } H_2O) = \frac{T/M \text{ (total cell water)} - \text{mannitol space}}{(1 - \text{mannitol space})}$$

4) The T/M (intracellular) was compared for the paired control and treated hemidiaphragms and the difference expressed as per cent increase or decrease in sugar transport, compared to the control.

V DRUGS USED

Bathing media

The liquid perfusate used in the gas-perfused heart experiments was substrate-free Krebs-Henseleit bicarbonate buffer of the following composition:

	g/l	mM
NaCl	6.90	118.0
KCl	0.35	4.7
KH_2PO_4	0.16	1.2
$CaCl_2 \cdot 2H_2O$	0.37	2.5
$MgSO_4$	0.29	2.4
$NaHCO_3$	2.20	26.0

The solution was saturated with 95% O_2 - 5% CO_2 before use. The pH was 7.4.

Glucose 2.0 g/l (11 mM) was added to the above medium of all other preparations.

The following drugs were used:

Acetylcholine chloride	Calbiochem Corp.
Adrenaline bitartrate	Sterling-Winthrop Inc.
Aerosol OT	Fisher Scientific Co.
Atropine sulphate	British Drug Houses
Cetyltrimethyl ammonium bromide	Eastman Kodak Co.
Glutathione (reduced)	Calbiochem Corp.
Histamine diphosphate	Nutritional Biochemical Corp.
5-Hydroxytryptamine creatine phosphate	Calbiochem Corp.
Insulin-Toronto	Connaught Medical Research
Isoproterenol hydrochloride	Sterling-Winthrop Inc.
^{3}H-D-mannitol	Radiochemical Center, Amersham.
3-O-methyl-D-glucose	Ayerst, McKenna and Harrison Ltd.
3-O-^{14}C methyl-D-glucose	New England Nuclear Corp.
Noradrenaline bitartrate	Calbiochem Corp.
Ouabain	Nutritional Biochemical Co.
Pentobarbital sodium	British Drug Houses
Phenoxybenzamine hydrochloride	Smith, Kline and French Corp.
Pronethalol hydrochloride	Ayerst, McKenna and Harrison Ltd.
Reserpine	Ciba Co. Ltd.
Tyramine hydrochloride	Calbiochem Corp.
Zinc chloride	Fisher Scientific Co.

Stock solutions were prepared as follows:

Acetylcholine chloride, histamine diphosphate, reduced glutathione and Aerosol OT, 10 mg/ml were made in deionized water. Pentobarbital sodium (5 mg/ml), cetyltrimethylammonium bromide (2.5 mg/ml),

pronethalol hydrochloride and ouabain (1 mg/ml) were made in deionized water.

The bitartrates of noradrenaline and adrenaline and the hydrochlorides of tyramine and isoproterenol were dissolved in 0.1 N HCl to give 10 mg/ml of the base.

Phenoxybenzamine hydrochloride, 10 mg/ml was dissolved in 25 ml propylene glycol acidified with a few drops of 5N HCl.

Reserpine, 5 mg/ml was prepared by dissolving 100 mg in 2.0 ml glacial acetic acid, 2.5 ml propylene glycol and 2.5 ml 95% ethanol and adding sufficient distilled water to make the final volume 20 ml.

All stock solutions were stored at $4^{o}C$. Dilutions were made in 0.9% NaCl on the day of the experiment and were placed in an ice-bath. In the case of catecholamines, dilutions were acidified by adding HCl, 0.01N to prevent rapid oxidation.

Additions were made to the muscle chamber with micro-pipettes. The drugs were rapidly equilibrated with the bath fluid by the aeration stream. The total volume of drug solutions added was rarely over 0.3 ml and was usually 0.1 ml or less.

Drug concentrations are expressed as final concentrations in the muscle bath in g/ml; ion concentrations are expressed as mM.

Washout of a drug from the muscle chamber, unless otherwise specified, was accomplished by an initial 3 changes of the bath fluid, followed by one change every 5 minutes until contractility became stabilized.

Pretreatment of animals with reserpine

Reserpine was given to cats (1.0 mg/Kg), guinea-pigs and rabbits (2 mg/Kg) by intraperitoneal injection 18 - 24 hours prior to

experiment.

Depletion of tissue noradrenaline results in loss of response to moderate doses of tyramine (Burn & Rand, 1958). In most experiments using heart muscle obtained from reserpine pretreated animals, depletion was tested for, by observing the response to tyramine. In the case of gas-perfused hearts removed from kittens pretreated with reserpine, the spleen strips from the same animals were tested for depletion by adding 10^{-5} g/ml tyramine to the bath.

Statistics

Mean values were compared by Student's t test for paired data (Goldstein, 1964) except where the t test for unpaired data is specifically indicated. A probability of 5% or less was considered significant.

Means are reported $\pm$ their standard error.

RESULTS

RESULTS

I THE POSITIVE INOTROPIC EFFECT OF WOF IN KITTEN ATRIA FAILED BY DIFFERENT METHODS

Gabel, Bihler and Dresel (1967) reported that a cardioactive material was washed out of gas-perfused hearts by intermittent liquid perfusion. The washings exerted a slight positive inotropic effect on isolated atria failed by prolonged stimulation. Treatment of the washings to 25% saturation with ammonium sulphate caused the precipitation of a material with clear-cut cardiotonic activity. Throughout this dissertation this material will be called wash-out factor (WOF). WOF had more activity in failed than in normal atria. Gabel (1965) used kitten preparations which had been failed by prolonged driving at high rates to assay the cardioactivity. Several hours were required to fail the preparations by his method and the failed atria were unstable. The degree of failure was difficult to regulate. Many preparations either recovered or showed further declines in the strength of contraction after being returned to the slower rate.

It appeared necessary to find a stable and reliable preparation which could be prepared easily and on which the effects of the material could be studied in detail. This part of the study might also serve to characterize the inotropic effect in a manner similar to the work of Broadbent (1963) who showed that the effect of digitalis in frog hearts failed with acetylcholine was less than in hearts failed by narcotic agents.

Left atria isolated from kittens were used throughout. They were stimulated at a frequency of 1.0 sec^{-1}. Regardless of the method

used to induce failure, the composition of the bathing medium was changed so as to reduce the strength of contraction by 50%. In all cases, the positive inotropic effect of WOF was expressed as per cent increase in contractile force from the failed baseline. A constant volume of 0.4 ml (1/12.5 of the total amount of WOF obtained from one heart) of sample was added to the 10 ml bath.

Atria were driven electrically at a rate of 5.0 sec^{-1} for several 30 min periods between which 5 min periods of stimulation at 1.0 sec^{-1} were interposed. Many hours (4-10) of this treatment were required to reduce the contractility by 50%. Gabel (1965) had reported that the contractile force of driven atria reached 30 - 35% of the initial within 2-3 hours. The differences in the two studies were probably due to variation in the size of the kittens from which the atria were removed. He used small kittens (approx. 0.4 Kg) whereas here the weight range was 1.0 - 1.5 Kg.

The initial concentration of pentobarbital sodium was 5×10^{-5} g/ml. Contractile force decreased to a new level within 5 min. Dependent on the degree of depression developed, further additions of the drug were made in increments of 2.5 or 5.0×10^{-5} g/ml. Either 2 or 3 additions of the agent were required to reduce the strength of contraction to 50% of the initial. The range of concentrations of pentobarbital required to depress contractility by 50% in kitten left atria was 5×10^{-5} - 1.5×10^{-4} g/ml. Failure could be reversed by washing the preparations. It was thus possible to adjust the concentration downward if the failure was too great. The preparations were very stable and responded well to known cardioactive agents. Responses to WOF were consistent and were dose-related (see page 57).

The increase in the strength of contraction caused by 6 samples of WOF were compared in 9 pairs of atria failed either by prolonged driving or by the addition of pentobarbital to the bath. The results summarized in Table I, series 1, indicate that the two groups were equally sensitive to the cardiotonic effect of WOF. All other comparisons of the positive inotropic effect of WOF were made using atria failed by pentobarbital as reference preparations. Additions of pentobarbital will be referred to as the "standard method" and atria failed in this manner as the "standard preparation".

The contractility of 10 atria was reduced to 50% by decreasing the calcium ion concentration in the Krebs solution supporting the muscle. The concentration of calcium was reduced from 2.5 mM to 1.25 - 0.625 mM. The preparations were set up in standard Krebs solution and contractility allowed to stabilize. The initial strength of contraction was noted and then the bathing medium was changed to the low calcium solution. Myocardial depression was rapid (10-20 min) and the preparations were stable at the decreased contractility.

The positive inotropic effect of 6 samples of WOF in these atria was compared with their effect in the standard preparation. The results are shown in Table I, series 2. The mean response of the low calcium group was 19.2 $\pm$ 4.8 and that of the pentobarbital group was 24.8 $\pm$ 4.8. The results indicate that the sensitivity of the atria failed by low calcium was less than that of the standard preparations.

Zinc ion has been shown to depress the contractility of isolated heart muscle from several species (Nayler and Anderson, 1965; Ciofalo and Thomas, 1965). The effect of WOF was studied in 6 atria treated with zinc ion. $ZnCl_2$ was added to the bath to make a 0.05 mM solution. An

Table I

The positive inotropic effect of WOF in kitten atria failed by four different methods.

Series	Failure	No. Atria	% Increase in Contractility Mean ± S.E.
1	Prolonged stimulation	9	24.1 ± 2.8
	Pentobarbital	9	27.3 ± 5.8
2	Pentobarbital	10	24.8 ± 4.8
	Low Ca^{++}	10	19.2 ± 4.8
3	Pentobarbital	6	56.8 ± 17.3
	$ZnCl_2$	6	56.5 ± 15.8

initial positive inotropic effect was observed in all atria. The duration of the increase in the strength of contraction was 20-40 min. The concentration of the ion in the bathing medium was increased by 0.025 mM increments at 20 min intervals until the contractility was decreased by 50%. The range of final concentration of Zn ion required was 0.05 - 0.10 mM. Sixty to 90 min were required to decrease contractility by 50%. This is 2 - 3 times longer than reported by Ciofalo and Thomas (1965). Furthermore, only depression was observed in their studies and in those of Nayler and Anderson (1965). However both groups of workers used perfusion media low in calcium ion which may account for the differences found.

The effects of 6 samples of WOF on contractility in the preparations failed with Zn ion were compared with those in atria failed by the standard method. The results, presented in Table I, series 3, show that the mean responses of the two groups to the cardioactive material were the same. However, responses to WOF in $ZnCl_2$ - failed atria were not as reproducible as those in the standard preparations.

Gabel (1965) had indicated that WOF might be lipoprotein in nature and might therefore be part of a membrane structure. It would be attractive to decrease contractility with a surfactant agent and to test the possibility that WOF might be specific for this type of failure. In 1964, Webb studied the effects of several ionic and nonionic surfactants on the twitch tension of frog sartorius muscle. In general, he found that the ionic surfactants in high concentrations produced an irreversible decrease in twitch response, whereas nonionic agents had little effect. With low concentrations (2.5×10^{-5} M) he observed that cetyltrimethyl ammonium bromide (CTAB - a cationic agent) produced a

reversible decrease in twitch tension and that Aerosol- OT (AOT - an anionic agent) caused an increase in twitch response.

The effect of either type of detergent on cardiac muscle had not previously been tested. It was therefore necessary to observe the effects of a number of procedures on atria treated with these substances. The effects of CTAB and of AOT were investigated to determine their effects on isolated atria and their interaction with WOF.

The initial concentration of CTAB in the bath fluid was 2.5×10^{-4} g/ml. If contractility was depressed only minimally (< 20%) after 10 min, the concentration was increased by an increment of 1.25×10^{-4} g/ml. The range of concentrations of CTAB required to depress contractility by 50% was $2.5 - 5 \times 10^{-4}$ g/ml. Failure was fairly rapid (20-30 min). The degree of failure was difficult to regulate because the depression was irreversible. The effect of atropine (5×10^{-7} g/ml) on 2 kitten atria failed by this agent was tested. The preparations were depressed to 35 and 38% of the initial contractile force. After the addition of atropine contractility progressively increased for 2-3 min until 50 and 57% of the initial strength of contraction was attained. When the effect of atropine is expressed as per cent increase from the failed baseline, the results were 44 and 50%. These findings indicate that the failure induced by this agent is in part due to a cholinergic action.

WOF was added to 4 atria failed with CTAB. The responses were very variable, reproducibility was poor and no dose-response relationship was seen.

Preparations treated with CTAB responded to increases in calcium ion concentration (to 5 mM) with only minimal increases in the strength

of contraction (20%). Normal preparations respond by increases of 100-200%. The mean per cent increase in the strength of contraction when adrenaline (3×10^{-8} g/ml) was added to the bath was 146% (3 atria). This response is within the range of those observed with normal atria. The inotropic effect of adrenaline was blocked 80% by pronethalol (4×10^{-7} g/ml) in these preparations.

A marked effect of stimulus voltage was observed in preparations failed with CTAB: small increases in voltage resulted in increases in contractility. This effect is seen in Figure 1 which is typical of the response to increased voltage in all 4 atria studied. An increase in voltage resulted in an abrupt increase in contractile force. Progressive step-wise increases in voltage caused progressive increases in contractile force (see Figure 1). Sudden decreases in stimulus voltage resulted in abrupt decreases in contractility. With very high voltages, the strength of contraction became greater than that seen initially. In 1 of 4 preparations partial contracture developed at very high voltage. If the increases in contractile force observed were due to the release of endogenous stores of noradrenaline one would expect the responses to changes in voltage to be gradual rather than abrupt. The effects of increasing voltage were not changed by β-adrenergic blockade thus confirming that the responses were not due to the release of noradrenaline. One explanation for these results is that CTAB may have destroyed conduction in the heart muscle and that the all-or-none response was lost. If this is the case, each cell would now have its own voltage threshold and as the voltage is increased more cells would fire.

The initial concentration of AOT in the bath was 2.5×10^{-4} g/ml. A positive inotropic effect (10-50% increase in contractility) was

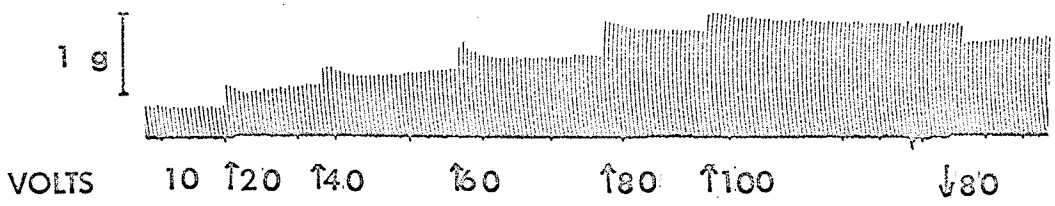

Figure 1. The effect of increases in stimulus voltage on the contractile force of kitten left atria failed by CTAB, 3.75×10^{-4} g/ml. Time, 40 min after addition of agent. Frequency of stimulation, 1.0 sec^{-1}. Arrows indicate voltage changes.

observed in all 9 preparations after the addition of AOT. The duration of the increase in the strength of contraction was 30-60 min. Twenty minutes after the initial addition of AOT, the concentration of the anionic surfactant in the bath was increased by increments of 1.25×10^{-4} g/ml until the contractility was decreased by 50%. The range of final concentrations of AOT required was $2.5 - 5 \times 10^{-4}$ g/ml. The effect of atropine (5×10^{-7} g/ml) was tested in one muscle failed with AOT. The contractility was increased 30% suggesting that myocardial depression with this agent was also due partly to a cholinergic action.

Although similar concentrations of AOT were used and the atria were exposed to the agent for the same time period, two types of failure were encountered. In 5 of 9 preparations, the response to calcium ion (5 mM) was marked, the responses to WOF and adrenaline (3×10^{-8} g/ml) were moderate and a negative inotropic response to ouabain was observed. In the other 4 preparations, the response to calcium was negligible or absent and marked positive inotropic effects were observed with WOF, adrenaline and ouabain.

The inotropic effects of adrenaline (3×10^{-8} g/ml) and increased calcium ion concentration (5 mM) were tested in 4 atria, twice before and twice after treatment with AOT. With adrenaline, the mean per cent increase in the strength of contraction was 50.8 ± 7.2 and 113.5 ± 20.3 before and after treatment respectively. These results suggest that AOT treatment increased the stimulant effects of this agent. With increased calcium ion concentration the responses were varied. In 2 of the atria, no response or a negative inotropic effect was observed. In the other preparations the response was equal to that observed before treatment. The former preparations responded to ouabain (5×10^{-7} g/ml)

with a positive inotropic effect: the latter ones responded with a negative inotropic effect.

The effects of graded amounts of WOF on the contractility in preparations failed with AOT were compared with those in the standard preparation. The amounts added varied in the 4 atria. The responses are shown in Table II. The response elicited by WOF was greater in 7 of 9 cases in the atria failed by the standard method than in those failed with AOT.

In four experiments where the atria were stimulated with punctate electrodes, a marked increase in threshold voltage was required to initiate a mechanical response after treatment with AOT. In these preparations the threshold voltage had to be increased 20-90 fold to elicit contractions. The threshold voltage also increased when field electrodes were used to stimulate atria failed with either CTAB or AOT but the changes in threshold were only 3 - 6 fold.

Although treatment of the atria with AOT always resulted in preparations which were sensitive to changes in stimulus voltage, two types of responses were observed. The first type of response occurred in 4 of 9 atria and resembled that seen with muscle failed with CTAB (see Figure 1). Here increases in voltage resulted in abrupt, rapid increases in the strength of contraction. This effect of changes in voltage remained unchanged following pronethalol treatment. The second type of response was seen in 5 of 9 atria. An example is seen in Figure 2, upper record. Here an increase in the voltage caused a progressive increase in the contractile force. When the voltage was abruptly decreased, the contractility declined but less rapidly than that in the atria failed with CTAB. This effect is in part due to the release of

Table II

The effect of different doses of WOF on the contractile force of atria failed by the standard method compared to that in preparations failed with Aerosol-OT.

WOF Vol. of Sample (ml)	% Increase in Contractile Force Pentobarbital	Aerosol-OT
0.25	62	30
0.35	14	69
0.40	20	60
0.40	10	22
0.50	117	0
0.60	93	49
1.00	47	25

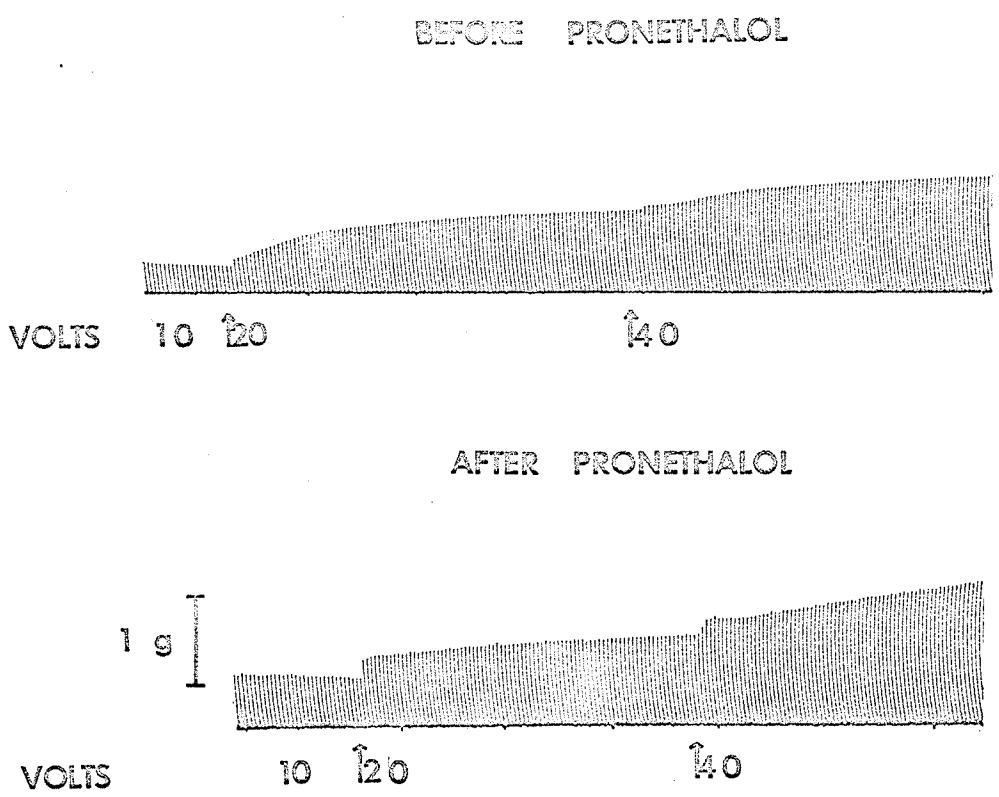

Figure 2. The effect of increases in stimulus voltage on the contractile force of kitten left atria failed by AOT, 2.5×10^{-4} g/ml. Time, 60 min after addition of agent. Frequency of stimulation, 1.0 sec^{-1}. Arrows indicate voltage increases. Bottom record shows the response after pronethalol, 4×10^{-7} g/ml.

endogenous stores of noradrenaline since the response to increasing voltage was decreased slightly by the addition of pronethalol (4×10^{-7} g/ml) to the bath. The effect of β-adrenergic blockade is shown in Figure 2, lower record. An increase in voltage after pronethalol treatment resulted in a larger abrupt increase in the strength of contraction than was seen in the same atria before blockade. The same changes in contractile force occurred when the voltage was held constant and the duration of the stimulus pulse was prolonged. Partial contracture developed in response to very high stimulus voltage in 3 of 9 atria exposed to AOT.

In general, surfactant treatment of the atria resulted in certain complications. Both the cationic and anionic agents caused extensive frothing and bubbling which resulted in irregularities in the base-line of the mechanical response. The preparations were very subject to anoxia. This could readily be seen by increasing the previously adequate gas flow through the medium. This procedure resulted in increases in the strength of contraction; such responses were not seen in other (normal or failed) preparations.

Cattell and Gold (1938) using isolated cat papillary muscles suspended in phosphate buffer found that the preparations became hypodynamic. These muscles were responsive to cardiac glycosides. The effects of WOF on contractility of 4 atria failed by the use of phosphate buffer (Creese, 1949) as the perfusion media were investigated. The atria were set up in normal Krebs-Henseleit bicarbonate buffer. The initial strength of contraction was noted when the contractility of the preparations had stabilized. The bicarbonate buffer was changed to phosphate buffer gassed with 100% O_2. The myocardial depression was slow, 60 - 120 min elapsed before the strength of contraction decreased by 50%. The failure produced

was readily reversed by replacing this medium with bicarbonate buffer.

The effects of 3 samples of WOF on contractility were compared in the atria failed with phosphate buffer and those failed by the standard method. The mean per cent increase in the atria failed with phosphate buffer was 21.9 $\pm$ 4.7 compared to 29.2 $\pm$ 3.4 in the standard preparations.

The studies showed that WOF, the cardioactive material removed from gas-perfused hearts, increased the strength of contraction in kitten left atria depressed by 6 methods. Pentobarbital failure of the preparations was rapid and reversible. The degree of myocardial depression produced was regulated easily. These atria were as sensitive to the cardiotonic effects of WOF as the atria failed by any of the other methods. The responses to WOF in the heart muscle treated with pentobarbital were reproducible (see page 57). This agent was therefore used to induce failure in isolated heart muscle as a routine procedure.

Control experiments were carried out to determine whether ammonium sulphate treatment might cause the positive inotropic effect observed with WOF. Millipore filters were washed with the same volume of Krebs solution 25% saturated with ammonium sulphate, as were the heart sample filters. The control filters which had been treated with ammonium sulphate were dried, frozen, reconstituted and tested for cardiotonic activity in the same way as the samples. The control filters when suspended in Krebs solution resulted in a small increase in contractile force in 50% of the tests in the standard preparations. The mean positive inotropic effect of the 10 active tests was 7.8 $\pm$ 2.1%. In the test preparation of Gabel, Bihler & Dresel (1967), small amounts of ammonium sulphate dried on Millipore filters had no effect on contractility. Although ammonium sulphate had a small effect in these experiments, the

results clearly indicate that the increase in contractile force caused by WOF is not due to the precipitation procedure used.

The effects of glutathione on the strength of contraction in 4 standard preparations was also investigated. Concentrations of GSH up to 5 times that usually added to the bath when samples of WOF were tested did not effect contractility. These findings agree with those reported by Gabel, Bihler and Dresel (1967).

II CARDIAC EFFECTS OF WOF UNDER VARIOUS CONDITIONS

All the studies carried out with WOF were severely hampered by the extremely limited amount of material available and the instability of the active component. Marked variation in the amount of cardioactive material obtained from different hearts was found. The mean response to different samples of WOF in the standard preparation varied from 24.8 $\pm$ 4.8 to 56.8 $\pm$ 17.3 in the 3 series shown in Table I (page 41). The large S.E. indicate that similar variability existed in the activity of individual samples. This marked variation in the positive inotropic effect resulting from the addition of 0.4 ml of WOF obtained from different hearts is summarized in Figure 3. The magnitude of the per cent increase in contractile force is divided into several ranges and the frequency distribution of the responses in each range is shown. The distribution of the inotropic responses in 62 left atria from kittens failed with pentobarbital are seen on the left. Thirty-two samples of WOF were used and 73 additions to the preparations were made. The greatest frequency, 27 of 73, was observed in the range of 20 - 29% increase in contractile force. Fifteen responses were less than 20% and 31 were equal or greater than 30%. Of the latter group, 12 of 31 additions resulted in responses

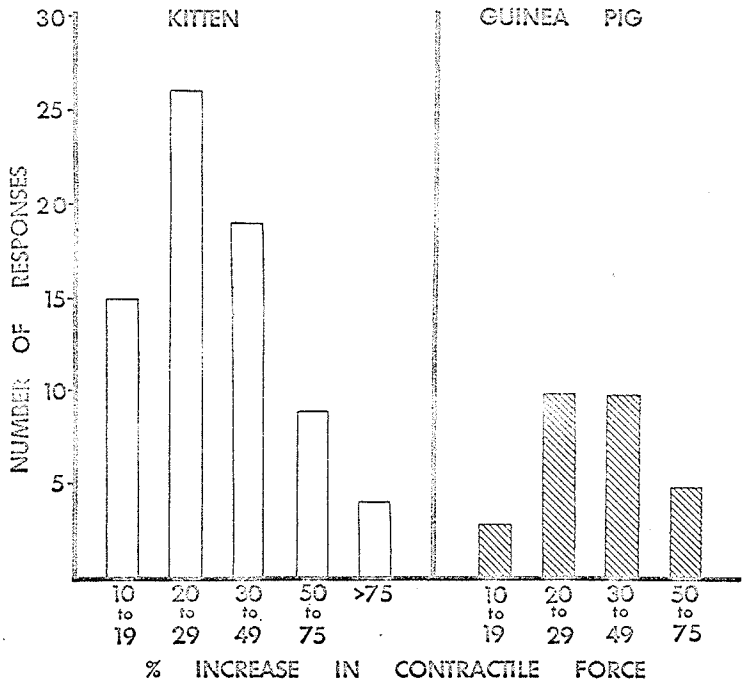

Figure 3. The frequency distribution of the responses caused by a constant volume of WOF obtained from different gas-perfused hearts. Per cent increase in contractile force divided into 4 or 5 ranges. Ordinate shows the number of responses in each range. Open bars represent responses in kitten atria; hatched bars those in guinea-pig atria.

greater than 50% increase in contractility and in 4 of these, the contractile force reached levels greater than the control before pentobarbital failure. Gabel, Bihler and Dresel (1967) found that the maximal response obtained from this material in their assay system (kitten atria failed by prolonged electrical driving) was a return to 75 - 85% of the contractile force measured before failure was induced which corresponds to an increase of 50 - 70% as expressed in the present work.

The Effects of WOF in Right Atria

The effects of WOF on the contractile force and the rate in right atria were investigated. It is well known that the strength of contraction increases with increases in rate within the physiological range. Four samples of WOF were tested in 3 unfailed preparations from kittens. Both changes were expressed as per cent increase from the control. The mean increase in rate was 24.5 $\pm$ 3.7 and for contractility 35.6 $\pm$ 7.8. Similar changes were observed when adrenaline (5×10^{-9} - 1×10^{-8} g/ml) was tested and when the calcium concentration was increased to 3.5 mM. Ouabain (2×10^{-7} g/ml) increased the contractile force a similar degree but had a negative chronotropic effect. These results indicate that WOF increased both rate and force in right atria. However the observed increases in the strength of contraction were in part due to the changes in rate.

The Effect of WOF in Papillary Muscle

The effects of WOF on the contractility of isolated papillary muscle were compared with those on left atria. Both preparations were failed with pentobarbital and were stimulated at a frequency of 1.0 sec^{-1}. Seven samples of WOF were used and a constant volume of the material was added to the bath (0.4 ml). The mean per cent increase in the strength

of contraction ± SE was 31.4 ± 7.7 for the atria and 15.5 ± 4.8 for papillary muscle. The response in the two types of heart muscle from kittens could be matched by doubling the volume of WOF added to the papillary muscles. Thus, when 0.8 ml of sample was added to papillary muscle the mean responses (n=5) were 26.6 ± 5.5 and 26.8 ± 5.6 for atria and papillary muscle respectively.

The Effect of WOF in Left Atria from Different Species

It is well known that there is great species variability in the responses of cardiac muscle to cardiac glycosides (Straub, 1955). Resistant vertebrate species include the toad and rat, while the cat, dog, rabbit, guinea-pig, pigeon and frog are sensitive. Species differences have also been observed in the activity of several naturally occurring substances which might be considered candidates for WOF. Govier and Boadle (1967) studied the cardiac action of lysolecithin on heart muscle obtained from three species and found the sensitivity to be in the order of guinea-pig > rat > rabbit. Species specificity of prostaglandin E_1 effects on the isolated heart have also been reported: no effect was observed in cat and rabbit hearts, modest effects were seen in rat heart whereas guinea-pig and frog hearts were very sensitive to this lipid (Berti et al., 1965).

To determine whether species variability in the response to WOF might exist, the material was tested on failed atria from guinea-pigs, rabbits and rats. The concentration of pentobarbital required to depress contractility of the atria to 50% was 2.5 - 7.5 x 10^{-5} g/ml for guinea-pigs, 4 - 6 x 10^{-5} g/ml for rabbits and 1 - 2 x 10^{-4} g/ml for rats.

The distribution of the inotropic responses in 24 left atria from guinea-pigs is seen in the right hand portion of Figure 3. Thirteen samples of WOF were used and 28 additions to preparations failed by the

standard procedure were made. Ten of the responses fell within the 20 - 29% range and 10 were within the 30 - 49% one. This marked variability in the cardioactive potency of different samples of WOF in both kitten and guinea-pig atria made it necessary to treat each sample as a separate entity. The mean per cent increase in the strength of contraction resulting from WOF was 35.9 $\pm$ 3.0 and 35.1 $\pm$ 2.8 in the atria from kitten and guinea-pig respectively. Although different samples of WOF were tested in the atria from the 2 species, the equal mean responses suggest that the two preparations were equally sensitive to WOF. Figure 4 shows a direct comparison of the effect of 4 samples of WOF in 8 atria from each species. A constant volume of sample (0.4 ml) was added. Equal increases in contractility of each pair of kitten atria were caused by 3 of 4 samples (open bars). Only 1 of 4 samples gave equal responses in the pairs of guinea-pig atria (hatched bars). However, in these experiments the inotropic effects were greater in 6 of 8 atria from guinea-pigs than in those from kittens.

Variable results were obtained when the inotropic effects of 4 samples of WOF were studied in 4 atria from rats. Ten additions were made. No effect on contractility was observed in 5 of the tests. The remaining additions resulted in a mean increase in contractile force of 6.9 $\pm$ 3.1%. The same samples tested in kitten atria caused increases of 18.9 $\pm$ 3.1%.

No response to 5 samples of WOF was observed in 8 rabbit atria although these samples were active in kitten preparations (27.4 $\pm$ 4.1%).

The results indicate that the species sensitivity to WOF was in the order guinea-pig $\geq$ kitten > rat > rabbit. This order of sensitivity resembles that reported with lysolecithin in heart muscle (Govier and Boadle, 1967).

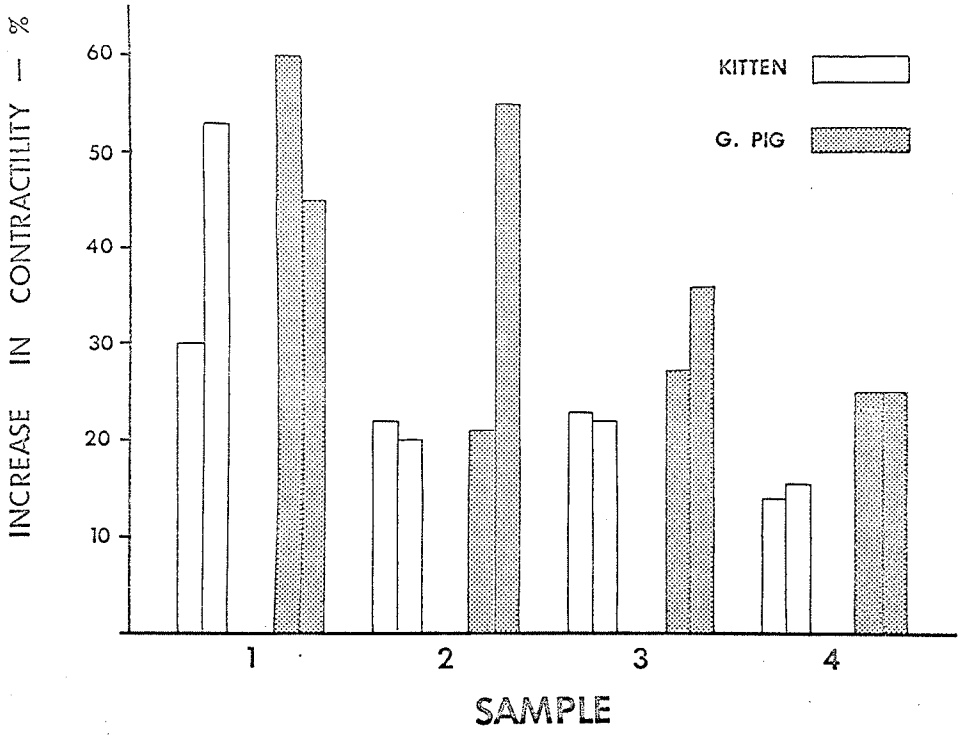

Figure 4. Individual responses to WOF in left atria from kittens and guinea-pigs. Each sample tested in 2 preparations from each species.

Reproducibility of the Inotropic Effects of WOF

The reproducibility of the positive inotropic response to 0.4 ml of WOF was studied in paired standard preparations. No significant difference ($P > 0.3$) was found. The mean per cent increase in contractile force was 47.8 ± 12.7 in one group and 51.2 ± 18.2 in the other.

Dose-dependence of the Response to WOF

The effect of increasing doses of the same sample of WOF on the contractile force of the standard preparation was investigated. The WOF was removed from the bath before a new higher concentration was tested. Figure 5 shows the result of one such experiment. The upper left record shows the initial contractile force present before failure was induced. Following the addition of pentobarbital (1×10^{-4} g/ml) and prior to the addition of WOF, the failure ranged from 50 - 60% of the initial. The responses shown in this record were of considerable magnitude suggesting that this preparation was very sensitive to the material and that this sample of WOF possessed considerable activity. The minimum dose tested resulted in a 100% increase in the contractile force. Increasing the dose resulted in larger responses. At 1.0 ml/10 ml (1/5 the total sample) the response was a 175% increase from the control baseline and was considerably larger than the contractility present before failure was induced (138% of initial).

Figure 6 shows the dose-response curve to increases in WOF obtained in 5 kitten (solid line) and in 4 guinea-pig atria (broken line), all failed by the standard method. The positive inotropic effect is expressed as per cent increase in contractility from the failed baseline. The means of the responses are shown. The bars indicate the standard errors.

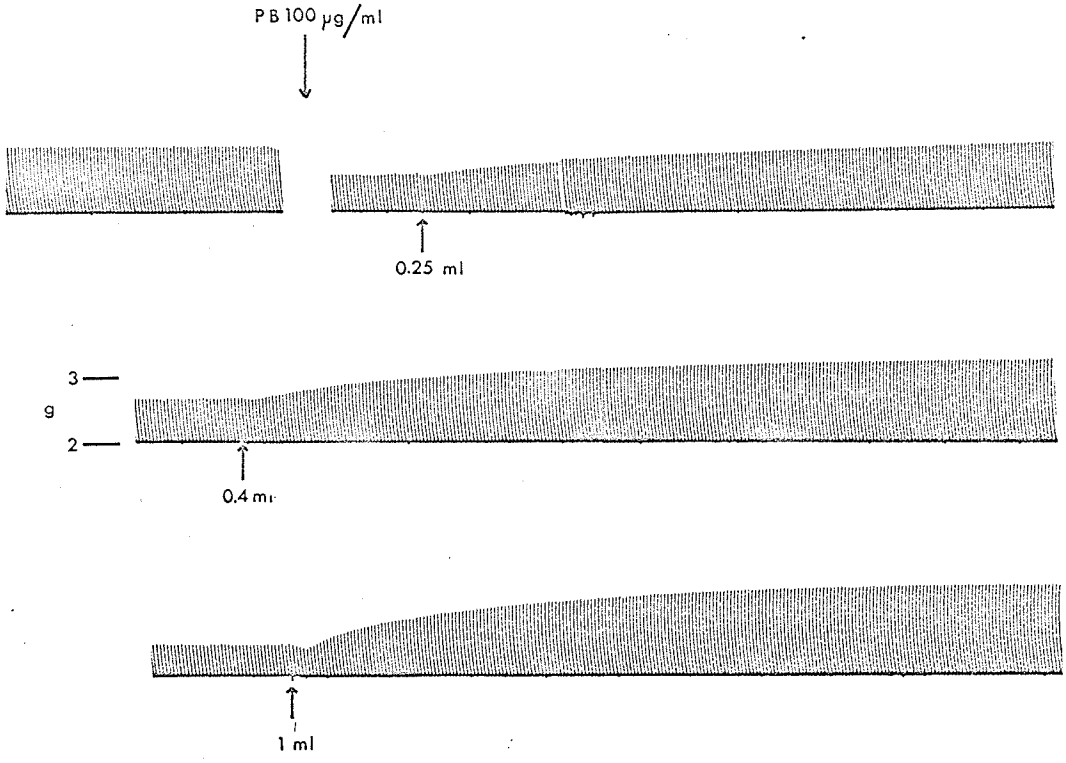

Figure 5. Record showing the typical response of the standard preparation to increasing doses of WOF. 3 separate additions. Control level before induction of failure, upper left hand corner. Pentobarbital concentration, 1×10^{-4} g/ml. Contraction rate, 1.0 sec^{-1}.

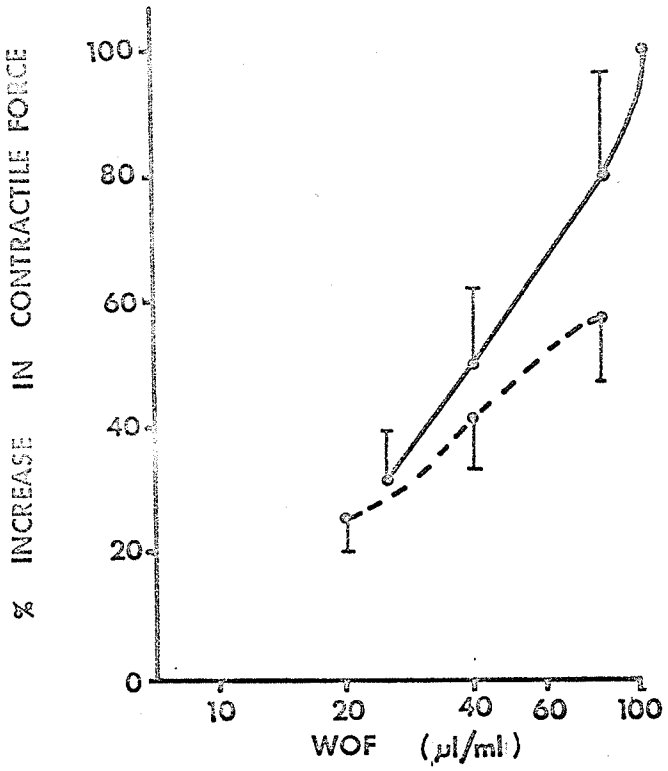

Figure 6. The effects of increasing doses of WOF on the response of kitten and guinea-pig atria. Mean per cent increase in contractile force. Bars indicate the S.E. Kitten responses represented by the solid line; guinea-pig ones by the broken line.

Correlation Between the Inotropic Effects and Protein Concentration

An attempt was made to find a correlation between the positive inotropic effect of WOF and the concentration of protein in the bath. A constant volume of WOF (0.4 ml) was added. The protein concentrations varied from 1 - 15 μg/ml. In this series of experiments, 6 of 38 additions of WOF resulted in a negative inotropic effect. Out of hundreds of tests with WOF these six were the only additions causing this response. On this basis, these samples were omitted from the results presented in Figure 7. Some correlation between the increase in contractile force resulting from 32 additions of WOF to the bath and the resulting protein concentration in the bathing medium was found. The association found between the two variables was greater than that which could occur by chance ($P < 0.05$).

The Effect of Temperature on the Inotropic Effect of WOF

Cardioactive agents have quantitatively different effects on the strength of contraction of isolated heart muscle at low temperatures. The positive inotropic effects of the cardiac glycosides were found to be decreased by cooling (Blinks and Koch-Weser, 1963). The effect of WOF on the contractile force of the standard preparation maintained at 3 bath temperatures was investigated. The same samples were tested in the same atria at 2 temperatures, 37°C and either 45° or 29°C. The mean per cent increases in contractile force were 96.3 $\pm$ 28.1 and 31.5 $\pm$ 12.1 when the bath temperatures were 45° and 37° respectively. The responses to other samples of WOF were compared when the preparations were maintained at 37°C and when the temperature was lowered to 29°C. The mean per cent increasesin contractile force were 44.5 $\pm$ 3.2 and 13.3 $\pm$ 2.2 respectively. In agreement with reports in the literature, the strength of contraction

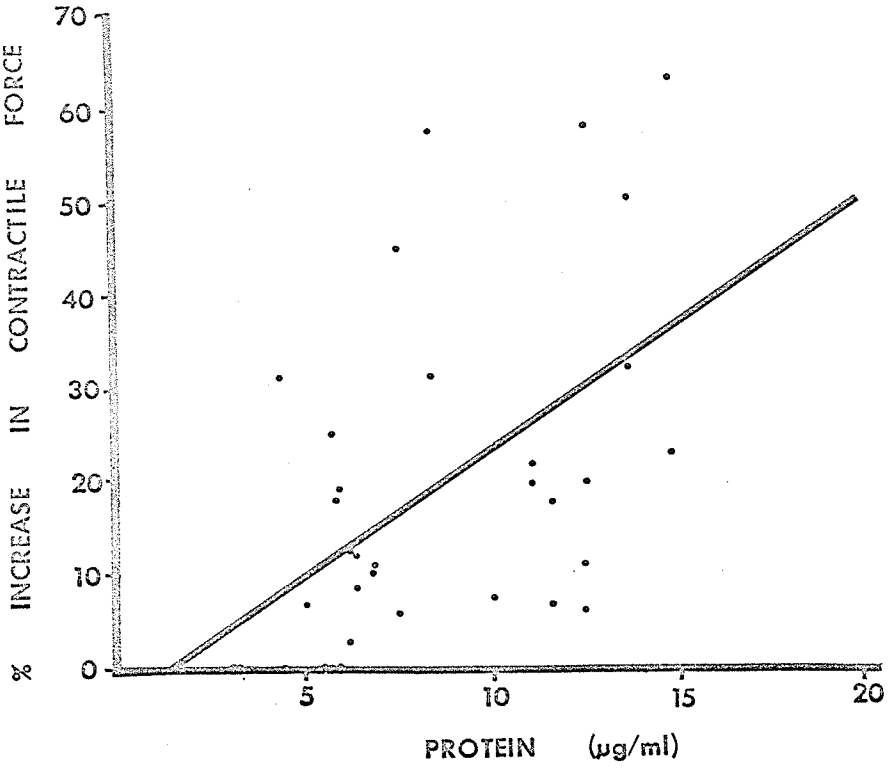

Figure 7. The positive correlation between the inotropic effect of WOF and the protein concentration. Total number of comparisons, 32. r value = 0.521. $P < 0.05$.

of untreated atria was greater at the lower temperature (29°C). These results indicate that the positive inotropic effect of WOF varied quantitatively with the temperature of the bathing media. An increase in the temperature resulted in an increase in the positive inotropic effect of the heart material. In this respect, WOF resembled the cardiac glycosides, adrenaline and noradrenaline.

Effect of WOF on the Adrenaline Threshold Concentration

Bohr and Johansson (1966) studied the contraction of vascular smooth muscle in response to plasma. Concentrations of plasma too small to produce contraction potentiated the tension developed in response to adrenaline. It was possible that minimal doses of WOF might change the threshold concentration of adrenaline. To test this hypothesis the threshold concentration of adrenaline was found in 3 atria and 5 papillary muscles from kittens. In papillary muscle the threshold concentration ranged from $1 - 6 \times 10^{-9}$ g/ml, similar to that found in atria. A small volume of WOF (0.25 ml) was added to the bath and at the peak of the response (2 - 3 min) a subthreshold dose of adrenaline was added to the bath. No change in the threshold concentration was found in atria. In all 5 papillary muscles, however, the concentration of adrenaline required for further increase in contractile force was reduced by $2 - 3 \times 10^{-9}$ g/ml. The results indicate that WOF changed the concentration of adrenaline required to elicit an inotropic effect in papillary muscle but was ineffective in kitten atria.

The Role of Catecholamines in the Inotropic Effect of WOF

Catecholamine Content of WOF

To test whether the increase in the strength of contraction induced by WOF was due to catecholamines present in the heart washings,

the catecholamine content of the material from gas-perfused hearts was determined. The individual washes from 2 gas-perfused hearts were combined. The resulting 4 solutions were analyzed for catecholamine content by fluorimetric assay. The heart washings did not contain noradrenaline in amounts detectable by this method of assay.

If catecholamines were involved in the positive inotropic effect of WOF, pretreatment of the kittens from which the hearts for gas-perfusion were taken with reserpine, should result in a decrease in the cardiotonic activity of the WOF. The effects of WOF from 4 hearts taken from normal animals was compared with that from 4 hearts taken from kittens pretreated with reserpine. Twelve additions of each type of WOF were made and the effects on the contractile force in guinea-pig atria failed by the standard method were compared. The mean per cent increase in the strength of contraction was the same whether the WOF came from the hearts of normal kittens or from the hearts of animals pretreated with reserpine. The mean responses were 34.8 ± 5.8 and 31.9 ± 8.3 respectively. These results indicate that reserpine pretreatment of the kittens does not change the amount of cardiotonic material washed from the gas-perfused hearts. The cardioactivity of WOF is not due to the catecholamine content of the heart washes.

Release of Catecholamine by WOF

To test the hypothesis that WOF might cause a positive inotropic effect by releasing noradrenaline from the nerve endings of the heart muscle, the effect of pronethalol (3×10^{-7} g/ml) on the cardiotonic response of WOF was studied. A constant volume of 0.4 ml of sample was added to the bath. In all cases, the pronethalol was added and 20 min allowed to elapse before any agonist or WOF was tested. The mean positive

inotropic effect of adrenaline (3×10^{-8} g/ml) was 146.0 ± 19.8 and 100.4 ± 17.7 in atria taken from kittens and guinea-pigs respectively. In papillary muscles, the mean increase in contractile force when adrenaline (4×10^{-8} g/ml) was added to the bath was 82%. Following pronethalol treatment, the adrenaline responses were blocked 83% in atria taken from kittens (n=25), 79% in those from guinea-pigs (n=20) and 68% in papillary muscles from kittens (n=4).

Six samples of WOF were tested before and after β-adrenergic blockade in 11 standard preparations. The mean per cent increase in contractile force was 26.3 ± 5.4 and 19.9 ± 2.4 respectively. However the effect of only 6 of 15 additions were decreased by pronethalol. In the other 9, the responses were the same or greater after β-adrenergic blockade. The results suggest that in some instances the positive inotropic effect of WOF in kitten atria may be due in part to the release of endogenous stores of noradrenaline.

The effect of 8 samples of WOF on the contractility of 14 guinea-pig atria failed with pentobarbital was investigated before and after pronethalol treatment. The mean per cent increases in contractile force were 25.0 ± 4.4 and 19.2 ± 2.3 respectively. However only half of the responses (11 of 22) were decreased by pronethalol. The remaining responses were the same or greater than those observed before treatment. These results suggest that the positive inotropic effect caused by WOF in guinea-pig, as in kitten atria, is due in part to the release of endogenous stores of noradrenaline.

The effects of 2 samples of WOF were tested in papillary muscles failed with pentobarbital, before and after pronethalol treatment. The mean per cent increases in contractility were 15.0 ± 2.3 and 10.4 ± 2.0

respectively. The positive inotropic response to WOF was decreased by β-adrenergic blockade in 3 of 4 cases. The results suggest that the effect of WOF in papillary muscle resembles that in atria and is due in part to the release of endogenous stores of noradrenaline.

If part of the increase in contractile force caused by WOF is due to noradrenaline release, the effects of this material should be less in atria taken from animals pretreated with reserpine and pronethalol should not affect the responses in such preparations. Table III suggests that in general these suppositions are true. The mean per cent increases in the contractile force resulting from the addition of the same samples to atria from normal animals and to those from kittens pretreated with reserpine were 26.8 ± 7.8 and 21.0 ± 3.2 respectively. The differences however were not significant ($P > 0.05$ by unpaired data analysis). Pronethalol (4×10^{-7} g/ml) did not significantly decrease the responses in either group.

Responses to WOF were compared in atria obtained from normal guinea-pigs and from those pretreated with reserpine. The results are presented in Figure 8. Four samples of WOF were used. The responses were not decreased in the atria taken from animals pretreated with reserpine (hatched bars). Sometimes these atria were more sensitive to the cardiotonic effects of WOF than were those from normal animals (open bars). The findings suggest that noradrenaline release plays only a minor role in the inotropic effect of WOF in guinea-pig atria.

The responses of the atria from animals pretreated with reserpine were significantly more sensitive to β-adrenergic blockade than were those from normal animals. When 8 samples of WOF were tested in

Table III

Comparison of the positive inotropic effect of WOF in atria taken from normal kittens and those from animals pretreated with reserpine.

Atria from Normal Kittens		
Before Pronethalol	After Pronethalol	P value
26.8 ± 7.8	20.3 ± 3.3	> 0.3
Atria from Kittens Pretreated with Reserpine		
Before Pronethalol	After Pronethalol	P value
21.0 ± 3.2	21.8 ± 2.4	> 0.8

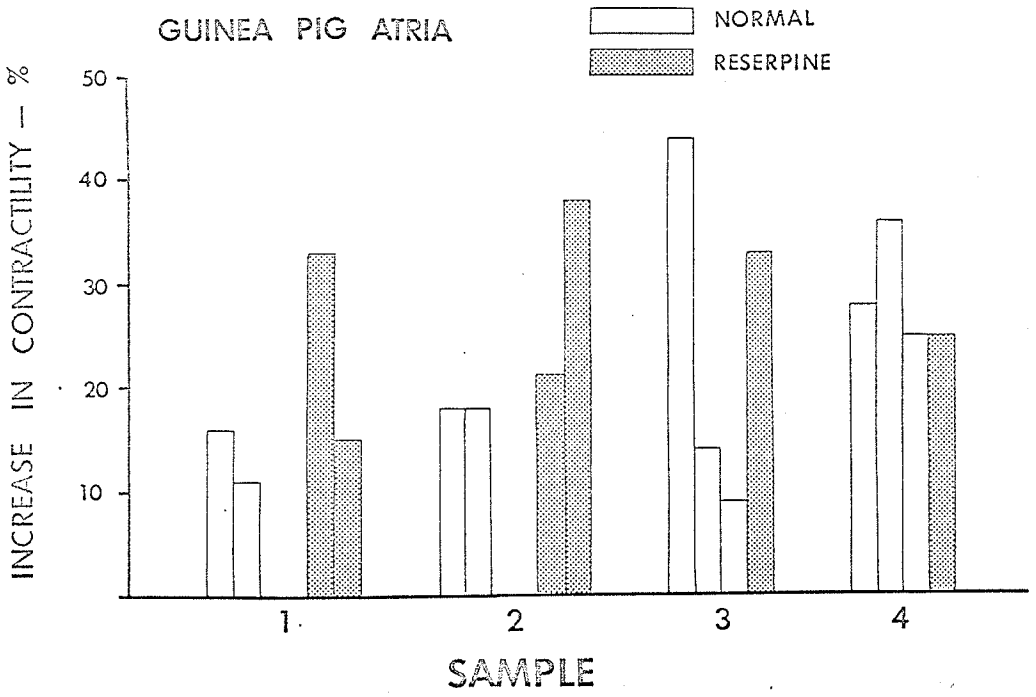

Figure 8. The effect of WOF on the contractile force of atria from normal guinea-pigs and those from animals pretreated with reserpine. Increase in contractile force expressed as per cent.

14 atria of each group, the mean per cent block of the effect was 22% in the atria from normal animals and 65% in those from guinea-pigs pre-treated with reserpine. This sensitization by reserpine to pronethalol blockade of the responses caused by WOF cannot be explained at present. It was not seen in kitten atria.

The results presented here are inconclusive as to the role of the release of endogenous stores of noradrenaline in the inotropic effect of WOF. With kitten atria, it is possible that such a factor may play a small role. With guinea-pig preparations however it is more doubtful whether noradrenaline release plays any role in the positive inotropic effect of WOF.

III SEPARATION TECHNIQUES APPLIED TO THE HEART WASHINGS

Determination of the Physical Nature

Gabel (1965) found that WOF was precipitated from the washings of gas-perfused hearts by 25% saturation with ammonium sulphate. He found that further salting out with increased saturations of 50, 75 and 100% did not result in the collection of active precipitates when the inotropic activity was assayed in kitten atria failed by prolonged driving. He adopted saturation to 25% as the standard procedure. The precipitated material gave positive tests for protein and lipid ester bonds (hydroxylamine method). Tests for phosphate were positive but cannot be considered reliable because of the possibility of residual contamination from inorganic phosphate present in the Krebs solution from which the material had been precipitated.

Centrifugation Studies

The active substance may be one of several proteins in the

precipitate or a nonprotein material coprecipitated under these conditions. WOF may be a lipoprotein which was part of the membrane structure of the cardiac muscle cell. The isolation of a purified membrane fraction by differential centrifugation methods has proved successful. Centrifugation studies were carried out to determine whether WOF was particulate. The washings from 4 gas-perfused hearts were centrifuged at 1000 g for 10 min. The supernatant was recentrifuged at 100,000 g for 60 min. The supernate was removed and the pellet resuspended in the same volume of Krebs solution as the volume of supernate. Both the particulate (resuspended pellet) and the soluble fractions were 25% saturated with ammonium sulphate. The precipitates were collected and reconstituted in 2.5 ml of Krebs solution. The cardiotonic activity was tested immediately on 4 atria taken from guinea-pigs. The preparations were failed by the standard method. The mean $\pm$ S.E. of the responses of the particulate fraction was 22.9 $\pm$ 4.0 and that of the soluble fraction was 23.5 $\pm$ 2.2. Each mean represents the effects of 14 additions. The cardioactive material was therefore equally distributed between the two fractions ($P > 0.8$). The results suggested that the active principle might be released into the supernatant from the particulate material.

Disintegration with Ultrasound

Searcy and Bergquist (1965) have reported changes in serum lipoprotein structure following ultrasonic treatment. They also found that protein is dissociated from lipid when purified lipoprotein preparations are exposed to sonic forces. The effect of sonication on the cardioactivity of WOF was studied. Equal volumes of the same

washes were not subjected to this treatment. Washings from 4 gas-perfused hearts were used. The inotropic activity of 15 aliquots of the sonicated material was compared with the untreated material in 3 atria from guinea-pigs. The preparations were failed by the standard method. No difference ($P > 0.9$) between the positive inotropic effects of the 2 groups of samples was found. The mean per cent increase in the contractile force with the treated material was 24.1 ± 2.7 and that of the untreated WOF was 24.3 ± 2.7. Ultrasonic treatment of the washings therefore did not change the cardiotonic effect of WOF subsequently precipitated from them.

Solvent Extraction of Heart Washings

Several naturally occurring lipids have been shown to possess cardiotonic activity. The heart washes were therefore extracted with ether to determine whether the cardioactivity of WOF was due to a lipid. The material extracted with ether was found to be sparingly soluble in water, soluble in ethanol and in acetone. These solutions were cloudy.

Solubilization of the Material Extracted from the Washings with Solvents

The method of solubilizing lipids greatly affects their biological activity. Ethanol, used to dissolve steroids, has been shown to prevent the action of some of these substances on cardiac muscle (Lefer, 1967). An attempt was made to solubilize the lipid extracted from the washings by the dialysis method of Fleischer and Klouwen (1961). The lipid was dissolved into butanol-sodium cholate solution and dialyzed against appropriate buffers for several days. Small volumes of the lipid treated in this manner were inactive: large volumes were found to depress contractility. Dilution of the retentate occurred (3.6 times) and perhaps

this contributed to the lack of activity. Several days' dialysis were required to eliminate all traces of butanol; WOF is labile and may have lost the cardiotonic activity during this time interval. The depressant effect on contractility seen with large volumes of the retentate may have been caused by small residual amounts of butanol. The threshold for myocardial depression by this alcohol is very low (1×10^{-7} g/ml).

Study of the activity of the lipid had to be preceded by determination of the effects of small volumes of water miscible organic solvents on the test systems. The threshold for a negative inotropic effect of 4 solvents was determined in at least 4 kitten atria failed by the standard method. Ethanol and methanol, in agreement with the literature, depressed myocardial contractility at a bath concentration of 1 μl/ml. The mean per cent decrease in contractile force of the atria was 9% for ethanol and 6% for methanol. The threshold concentration for chloroform was lower (0.5 μl/ml) and the mean negative inotropic effect was 21%. The threshold concentration of acetone was 1-2 μl/ml but the mean decrease in contractile force (3%) was much less than that observed with the alcohols. The dose-response curve to acetone was less steep than that observed with the other solvents tested. Also the reproducibility of the myocardial depressant effect with acetone was better than that due to methanol or ethanol. Acetone in bath concentrations of 5-25 μl/ml had no effect on the transport of 3-methyl glucose into rat skeletal muscle and did not affect smooth muscle (guinea-pig ileum). Acetone was therefore used as the final solvent for the material extracted from the washings by both ether and

amyl alcohol. However, the solvent could be expected to have significant cardiac effects in the concentrations to be used in testing the material. The effects of acetone on isolated hearts have not been described and were therefore investigated in detail.

The Inotropic Effects of Acetone in Isolated Atria taken from Kittens

The effects of acetone on contractility in isolated atria from kittens are presented in Figure 9. They are expressed as per cent change in contractility from the control baseline. The effects of the drug were evaluated in unfailed and standard preparations and no significant difference in the responses was observed; the data from both preparations were therefore combined. Low concentrations of acetone resulted in either a positive or a negative inotropic effect. Thirty tests were done with a concentration of 5 μl/ml. Fourteen atria responded with an increase in contractile force which reached a plateau in 4 - 5 min. The remaining 16 preparations responded with a decrease in contractile force at this concentration of solvent. The depressant effect was rapid in onset and the depression was greater at 3 min than at 5 min. All inotropic effects of the solvent were measured at 5 min. The two types of responses were evaluated separately, the height of the hatched bars in Figure 9 representing the means of the preparations responding with an increase, the open bars representing the means of those atria giving a decrease in the strength of contraction. The S.E. are indicated. The same distribution of responses was observed at 10 μl/ml. The negative inotropic effect became dominant when higher concentrations of acetone were used. A decrease in contractile force was observed in 11 of 11 preparations when the acetone concentration in the bath was raised to 20 μl/ml.

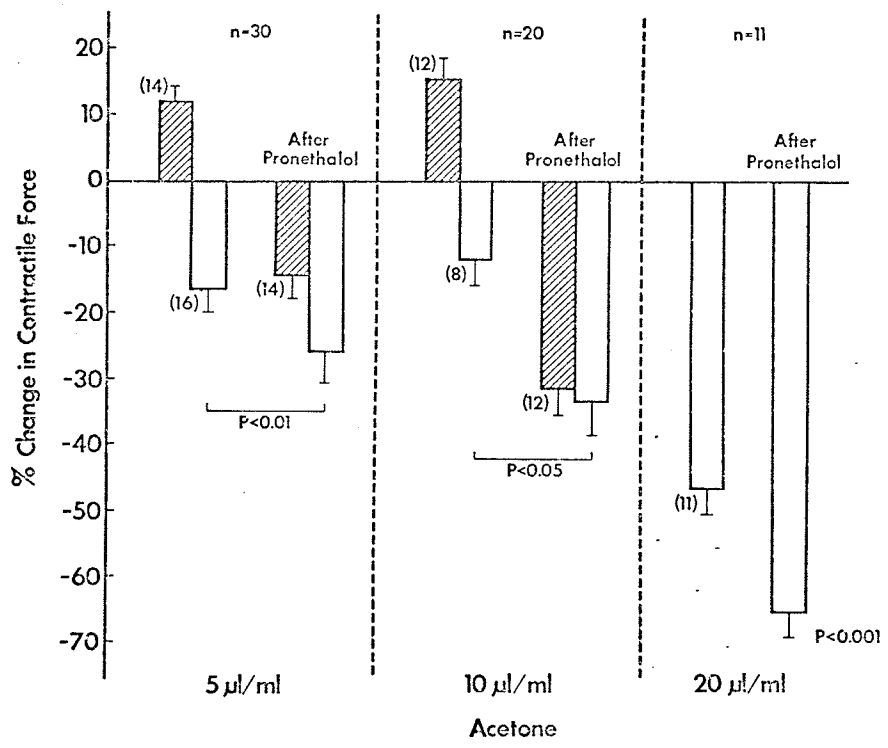

Figure 9. The effects of three concentrations of acetone on the contractile force of kitten atria. Hatched bars represent the mean responses of preparations showing increased contractility; open bars those with decreased contractility as the first response. S.E. are indicated. Pronethalol concentration, 4×10^{-7} g/ml.

The effect of increasing doses of acetone was investigated further in 2 groups of preparations. The cumulative dose-response curve as determined in 4 atria is shown as the solid line in Figure 10. The means of the responses of all the preparations are shown although some responded with increases in the force of contraction at the lower concentrations of the solvent. The potency of acetone to cause decreases in the strength of contraction was lessened when the drug was removed from the bath before a new higher concentration was tested. This is shown in the broken line in Figure 10. The difference in potency is abolished if the atria were obtained from animals pretreated with reserpine.

To test the hypothesis that acetone might have its positive inotropic effect by releasing noradrenaline from the nerve endings of the atria, the effect of pronethalol (4×10^{-7} g/ml) on the responses to acetone was studied. The 26 preparations which before pronethalol treatment had responded with an increase in the strength of contraction (see Figure 9, hatched bars) all showed a decrease in contractile force after treatment. The atria which before β-adrenergic blockade had responded with a decrease in contractility (see Figure 9, open bars) showed a further decrease. There was a significant difference between the negative inotropic responses before and after pronethalol treatment at each acetone concentration tested. The response rapidly reached a plateau and did not change between 3 and 5 min. These findings indicate that the positive inotropic effect of acetone is due to the release of endogenous stores of noradrenaline. In all cases the responses of the atria to acetone was the summation of the direct depressant effect and

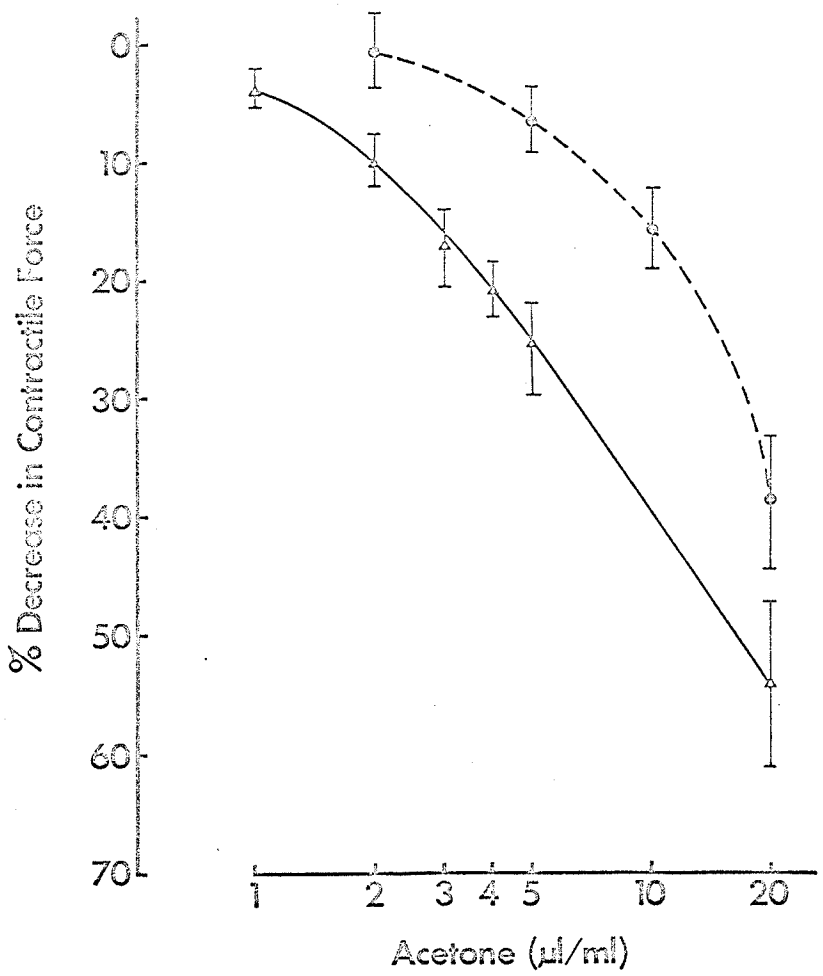

Figure 10. The effects of increasing doses of acetone on the contractile force of isolated left atria from kittens. The solid line represents the cumulative dose-response curve; the broken line the responses to single, increasing doses. The bars indicate the S.E.

the noradrenaline releasing effect. In Figure 10, the dose required for an equal myocardial depression in contractility with single additions was greater than that with the cumulative dose. A single dose resulting in a bath concentration of 10 µl/ml decreased the strength of contraction a similar degree to that of 3 µl/ml given stepwise. This suggested that single additions of solvent release more noradrenaline from the nerve endings.

A significant increase in the strength of contraction above control was observed when larger, depressant doses of acetone were removed from the bath. The inotropic effects of 3 concentrations of acetone followed by the effect on contractility of removal of the drug by washing are shown in Figure 11. The lowest concentration of drug (5 µl/ml) caused no significant change from the control baseline when the solvent was removed. Contractile force became greater than control when concentrations of 10 µl/ml or greater were removed from the bath. This positive inotropic effect following removal of the acetone was sustained for several minutes; contractility then returned to the control level. It was blocked by low concentrations of pronethalol (Figure 11) and was absent in atria taken from animals pretreated with reserpine.

The inotropic effects of acetone before and after pronethalol were similar in both left and right atria. The chronotropic effects of two doses of acetone were studied in 4 isolated right atria from kittens. Figure 12 shows the rate expressed as per cent change from the control. Both 10 and 20 µl/ml concentrations increased the rate of all atria (open bars). A further positive chronotropic response was seen when

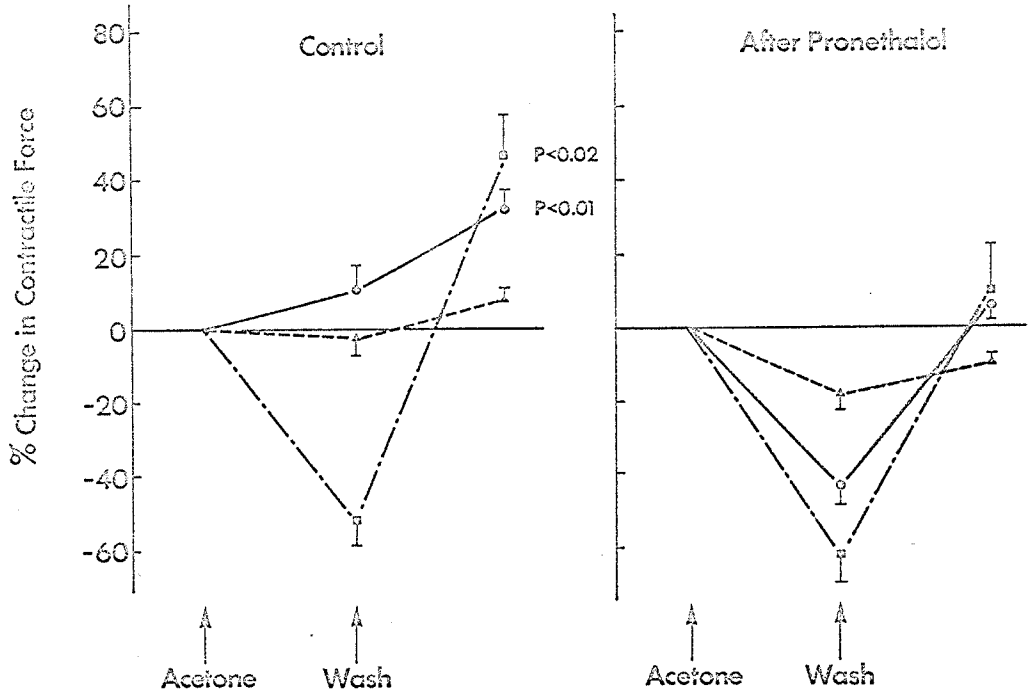

Figure 11. The effects of 3 concentrations of acetone on the contractile force of isolated left atria from kittens. The effects are shown during acetone treatment and immediately after removing the solvent from the bath. The initial contractility of the preparations is indicated at the point where acetone is added. The preparations were washed at 5 min. The broken dashed line represents the effects of 5 µl/ml; the solid line those to 10 µl/ml; and the broken dot-dashed line the effects of 20 µl/ml concentration of acetone. Pronethalol concentration, 4×10^{-7} g/ml.

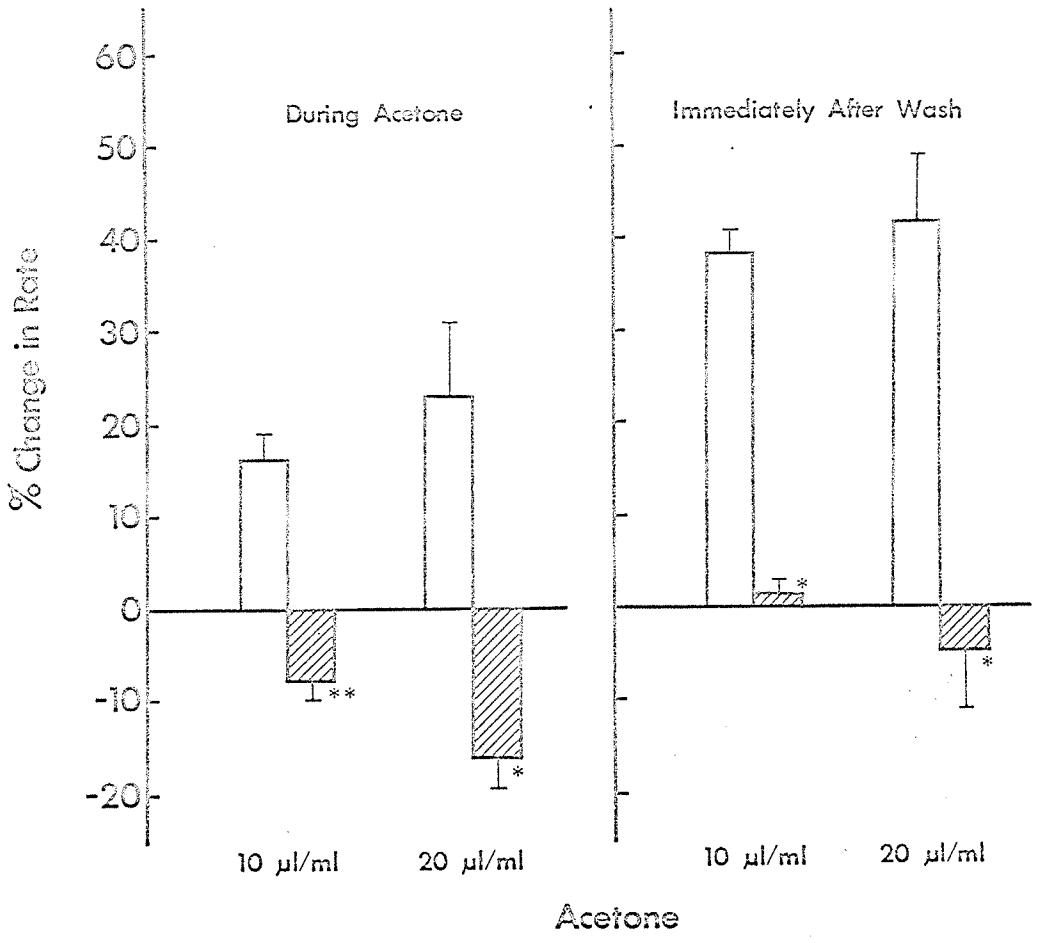

Figure 12. The effects of 2 concentrations of acetone on the heart rate of isolated right atria from kittens. The effects on rate are shown during treatment and immediately after removal of the agent. The open bars represent the mean per cent increase in rate; the hatched bars the effect after pronethalol. Pronethalol concentration, 4×10^{-7} g/ml. The S.E. are indicated. * $P < 0.05$ and ** $P < 0.01$ by paired data analyses.

these doses of acetone were removed. Pronethalol treatment reversed the drug effects on rate (hatched bars). The chronotropic effects of acetone therefore also appear to be the summation of the direct depressant effect and the noradrenaline releasing effect.

In summary, kitten atria may respond to low concentrations of acetone with either a negative or a positive inotropic effect. When the concentration of the solvent is increased the depressant effect becomes dominant. Two concentrations of the drug increased the rate in isolated right atria. Pronethalol reversed the positive inotropic and chronotropic responses. Preparations which initially responded with a decrease in contractile force showed a further significant decrease after β-adrenergic blockade. Only depressant effects on the strength of contraction and on the rate were observed in atria taken from kittens pretreated with reserpine. These results indicate that the inotropic and chronotropic effects are dose-dependent and are the summation of two opposing effects, a direct depressant one and an indirect stimulant one, due to the release of noradrenaline from the nerve endings in the atria.

The Inotropic Effect of Acetone in Atria from Guinea-Pigs

The effects of acetone on contractility in isolated left atria from guinea-pigs are presented in Figure 13. They are expressed as per cent change in contractility from the control baseline. The open bars indicate the means of the responses of all the preparations. Low concentrations of acetone (5 µl/ml) had a negative inotropic effect in all 8 preparations studied. When the dose of acetone was doubled (10 µl/ml), 8 of 11 atria showed a decrease in the strength of contraction.

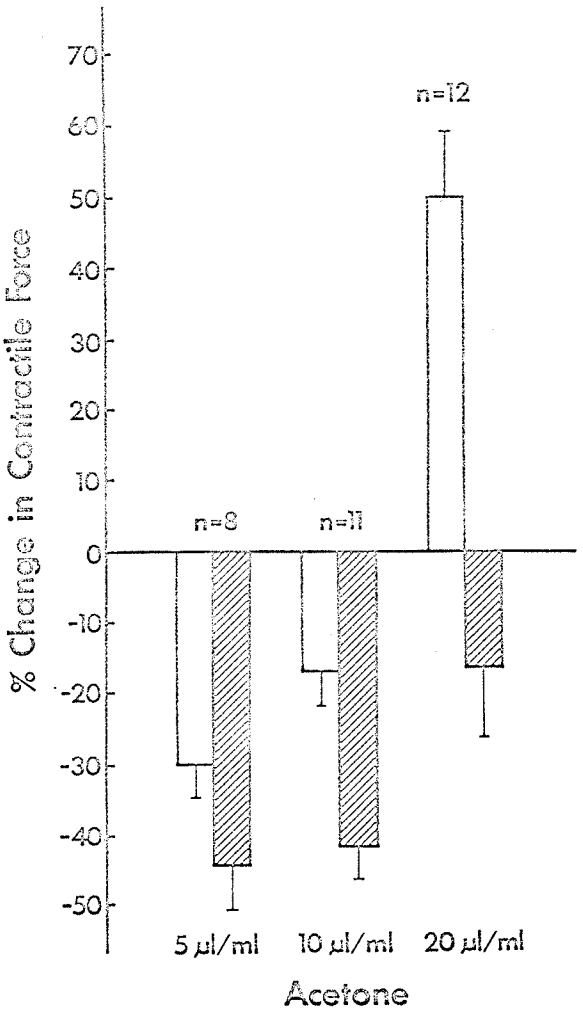

Figure 13. The effects of 3 concentrations of acetone on the contractile force of isolated left atria from guinea-pigs. The open bars represent the mean response of all the preparations. The hatched bars show the responses after pronethalol, 4×10^{-7} g/ml. The S.E. are indicated.

The remaining 3 preparations responded with an increase in contractility. With an acetone concentration of 20 µl/ml, 10 of 12 atria responded initially with an immediate depression in contractility which was followed by an increase.

The effect of increasing doses of acetone was investigated further in 2 groups of 4 preparations each. Figure 14 shows the dose-response curve obtained with stepwise increases of the concentration (solid line in the Figure). The response to single additions of acetone are shown with the broken dashed line. All 8 atria responded with a negative inotropic effect to doses $\leq$ 10 µl/ml regardless of the method of administration of the drug. The mean responses of all the preparations are shown although at concentrations higher than 10 µl/ml the response was either an increase or a decrease in contractile force. The potency of 20 µl/ml of acetone to cause a positive inotropic effect was increased when the drug was removed from the bath before this highest concentration was tested. Under these conditions the strength of contraction was increased 46% above control levels. When the concentration of acetone was increased gradually to 20 µl/ml, contractility reached control levels or slightly higher. However, the differences observed were not significant due to the marked variability in the responses seen and the limited number of tests done. Surprisingly, acetone had the same biphasic effect in 4 atria taken from animals pretreated with reserpine in that contractility at the highest dose rose to levels comparable to those observed on cumulative addition in normal tissue. These findings suggest that more noradrenaline was released when large single doses of acetone were applied then when the dose was progressively increased.

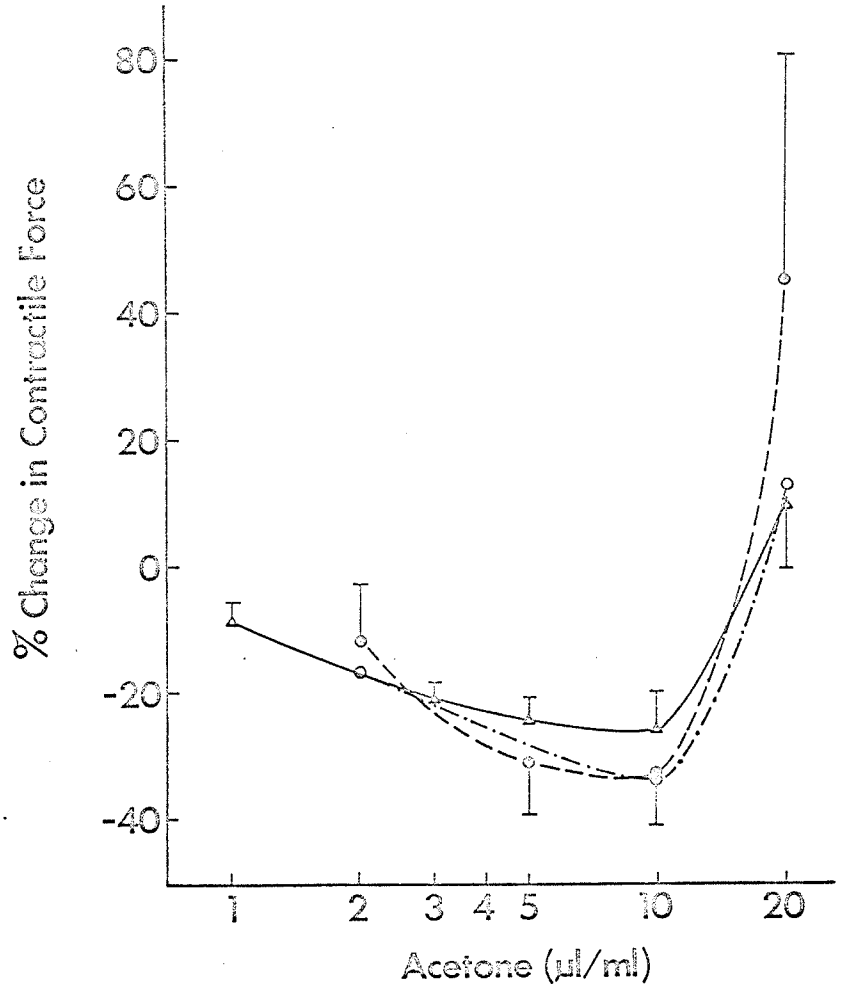

Figure 14. The effects of increasing doses of acetone on the contractile force of isolated left atria from guinea-pigs. The solid line represents the cumulative dose-response curve; the broken dashed line that to increasing single doses; the broken dot-dash line, the dose-response to single increasing doses added to atria taken from animals pretreated with reserpine. The bars represent the S.E.

Pronethalol treatment blocked the positive inotropic effects of acetone. In Figure 13, the 13 atria which before pronethalol (open bars) had responded with an increase in the strength of contraction all showed a decrease in contractile force after treatment (hatched bars). The 18 atria which prior to β-adrenergic blockade responded to acetone with a decrease in the strength of contraction now showed a significant further decrease in contractile force. Like kitten atria, the responses of guinea-pig atria to acetone is thus the summation of the direct depressant effect and the noradrenaline releasing effect. Unlike kitten atria, the negative inotropic effect is dominant in guinea-pig atria at the lower concentrations of acetone (< 10 µl/ml).

As with kitten atria, a significant increase in the strength of contraction of guinea-pig atria above control level was observed when 20 µl/ml doses of acetone were removed from the bath. This positive inotropic effect was decreased significantly by pronethalol treatment.

Repeated treatment of atria from guinea-pigs with high concentrations of acetone resulted in irreversible failure of the preparations. This failure by acetone was observed in atria from both normal and reserpine-pretreated animals. In 16 atria taken from normal animals the mean per cent of initial contractility was 51.1 ± 4.9; with the 10 atria taken from animals pretreated with reserpine the mean was 45.9 ± 6.8. This failure due to acetone was not observed in kitten atria.

In summary, guinea-pig atria respond to low concentrations of acetone with a negative inotropic effect. When the concentration of solvent is increased the response becomes varied until at high doses the positive inotropic effect becomes dominant. Doses which increased

the strength of contraction in right atria also increased the rate. Pronethalol treatment reversed or markedly decreased the positive inotropic and chronotropic responses in the atria. Depressant effect on rate and strength were most frequently observed in atria taken from reserpine pretreated guinea-pigs. The findings indicate that the inotropic and chronotropic effects are dose-dependent and are the summation of two opposing effects, a direct depressant one and an indirect stimulant one due to the release of noradrenaline from the nerve endings in the atria.

Guinea-pig atria appeared to be more suitable for studying the effects of the lipid because the amounts of solvent to be used had a consistent negative inotropic effect rather than the variable effect seen in kitten atria. In experiments of short duration this was the case. However, with experiments of longer duration the induction of failure in guinea-pig atria by repeated treatment with the solvent became a major consideration. Both preparations were therefore used in these studies.

The Inotropic Effect of the Lipid Material Extracted With Ether

Washings from 16 hearts were extracted with ether to determine whether the activity of WOF was due to lipids which are removed as lipoproteins. The pH of the washes were adjusted when necessary before solvent extraction was done. The general procedure for ether extraction is shown in Figure 15. Washings with GSH added were almost neutral (pH 6.9). The pH was adjusted with 3N HCl to pH 2 and with 3N NaOH to pH 9. The aqueous phase after ether extraction was neutralized before precipitation. Aqueous Phase I and Aqueous Phase II (see Figure 15)

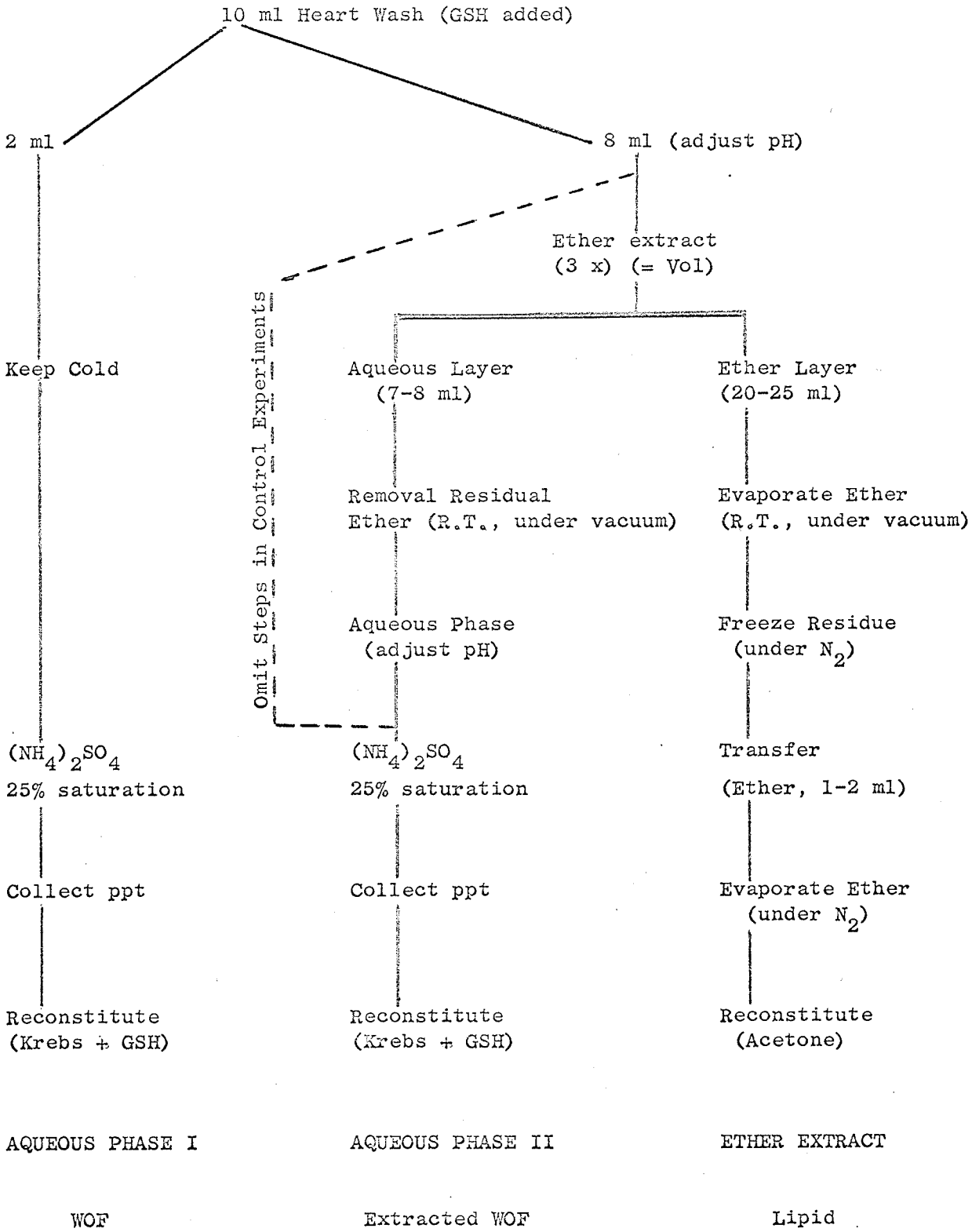

Figure 15. The diagrammatic presentation of the general procedure for ether extraction of the heart washings.

were precipitated with ammonium sulphate at the same time. After precipitation, Aqueous Phase I is WOF and Aqueous Phase II is "extracted WOF". The ether extract will be termed the lipid.

In one series of early experiments, done before the effects of acetone had been determined, the lipid was dissolved in ether and a known volume of Krebs solution was added and the ether evaporated off under N_2. Deposits appeared on the sides of the test-tube and the aqueous solution was not homogeneous. In these studies the inotropic activity extracted into the ether layer and that remaining in the aqueous layer after ether extraction at different pH are shown in Figure 16. The mean positive inotropic effect seen in 6 kitten atria failed by the standard method is expressed as per cent change in contractile force. The S.E. are indicated. The open bars (Aqueous Phase I) indicate the activity present in the washing without extraction (WOF). The hatched bars indicate the cardiotonic activity of the lipid material. Extraction of washings at pH 7 resulted in significantly less activity in Aqueous Phase II (see lined bars) than in Aqueous Phase I. The lipid had significant positive inotropic effects in each of 19 tests. However, as will be shown below, the mechanism of the cardiotonic effect of the lipid is not the same as that of WOF, so that no conclusions can be drawn concerning the distribution ot total activity. A considerable amount of a yellow material was found after evaporation of the ether layer following extraction at pH 7. Less of this material was seen in the flasks after ether extraction at pH 2 or pH 9. As shown in Figure 16, little or no cardiotonic activity was extracted into the ether phase at high or low pH. However, the aqueous phase after extraction (extracted

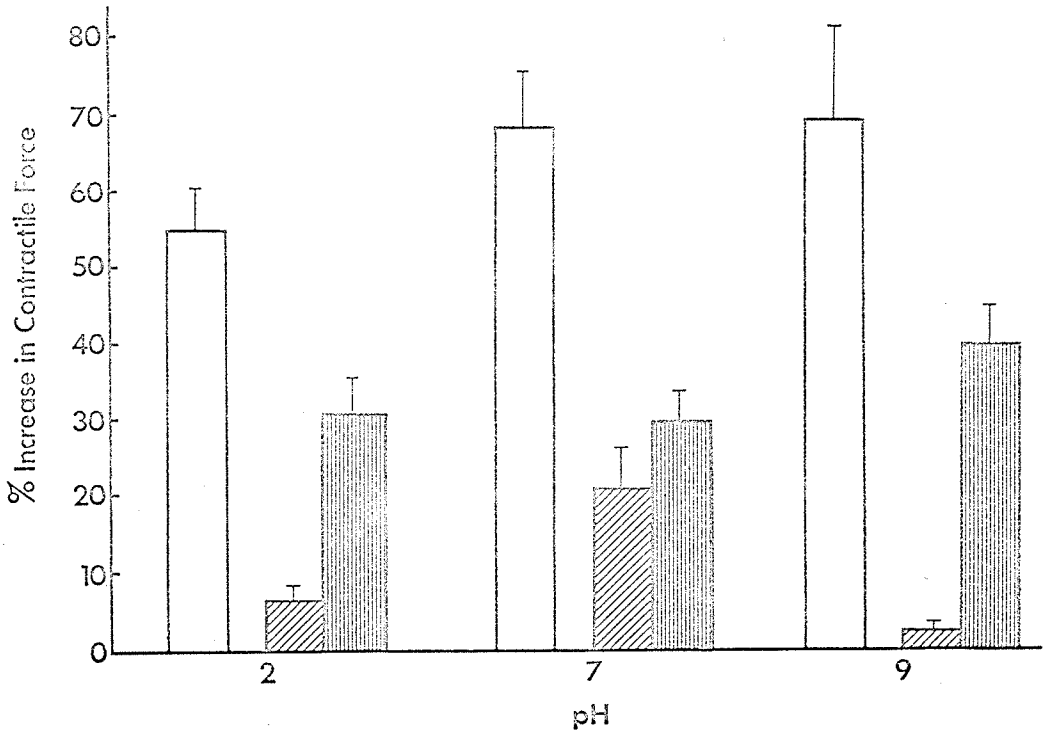

Figure 16. The effects of ether extraction of the heart washings at different pH on the cardiotonic activity. The mean per cent increases in contractile force ± S.E. are shown. The open bars indicate the activity present before extraction (WOF); the hatched bars the activity found in the ether layer (lipid); and the lined bars the activity remaining in the aqueous phase after extraction (extracted WOF). Extractions were done at pH 2, pH 7, and pH 9.

WOF) had less activity than before.

All further studies were carried out on lipid material extracted by solvents from washings at pH 7. The lipid was reconstituted in acetone.

The lipid extracted by ether from the washings of one gas-perfused heart was taken up in 0.3 - 0.6 ml of acetone. A constant volume of 100 μl (1/3 - 1/6 the total volume) was added to the 10 ml bath. Acetone, 10 μl/ml, was tested immediately before and immediately after the lipid. The average response to the solvent was compared with that to the sample. Good agreement between the 2 blanks in magnitude and direction of the inotropic effect was obtained.

The effects of 6 lipid extracts on the contractile force in 10 left atria from guinea-pigs are shown in Figure 17. The concentration of acetone in the blanks and the samples was 10 μl/ml. The mean inotropic effects are shown although some preparations responded with an increase, others with a decrease in contractility. The inotropic effect is expressed as per cent change from the control. In all 10 preparations the lipid (open bars) resulted in a greater positive inotropic effect or a less negative one than did the solvent blank (hatched bars). The differences were significant ($P < 0.05$). Additional ether extracts were tested. Out of 18 additions of lipid, 16 of the responses were either more positive or less negative than those to the solvent blanks. The effects of pronethalol (4×10^{-7} g/ml) on the positive inotropic effect of the lipid was investigated. The results are also shown in Figure 17. Pronethalol further decreased the negative inotropic effect of the solvent (6 of 10) and reversed the positive inotropic effect in the

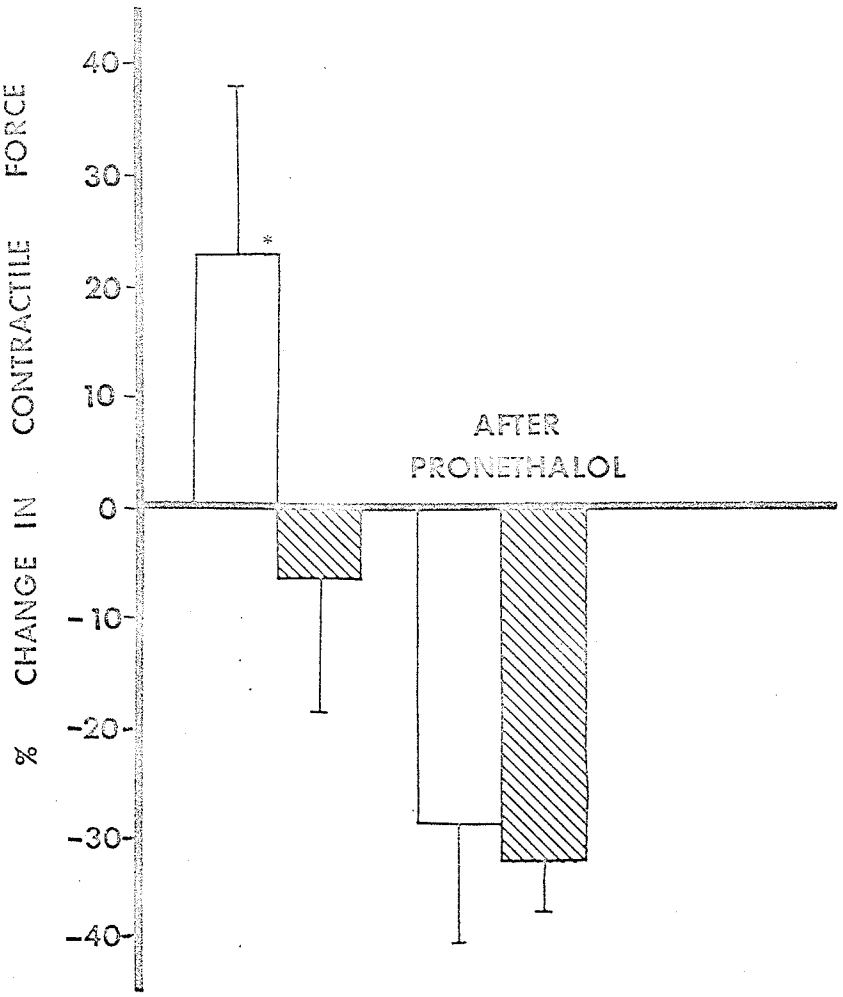

Figure 17. The effects of the lipid extracted from heart washings by ether on the contractile force of atria from guinea-pigs. Preparations failed by the standard method. Means of all the responses are shown. The S.E. are indicated. The open bars represent the mean response to lipid; the hatched bars those to solvent. Pronethalol concentration, 4×10^{-7} g/ml. * $P < 0.05$.

remaining 4 atria. The response to the lipid was markedly decreased in all muscles. The 6 preparations which had responded to the lipid with a positive inotropic effect before β-adrenergic blockade now responded with a negative effect. Those 4 atria which had previously responded with a decrease in contractile force showed a further decrease in contractility after pronethalol treatment. Following pronethalol blockade the degree of myocardial depression caused by the lipid and the blanks were not significantly different.

The positive inotropic effect of the lipid should be absent in atria taken from animals pretreated with reserpine provided that the lipid acts by releasing catecholamines. The effect of reserpine pretreatment on the responses was investigated using 6 samples. Three atria from normal and 3 from guinea-pigs pretreated with reserpine were failed by the standard method. Corrections were made for the acetone blanks. The response in atria from guinea-pigs pretreated with reserpine was markedly attenuated or absent in all cases. The mean response elicited by the lipid in the preparations from normal and animals pretreated with reserpine was 30.3 $\pm$ 6.4 and 6.8 $\pm$ 3.6 respectively. This difference is significant ($P < 0.01$). These findings indicate that the material extracted by ether from the heart washings caused an increase in the contractile force of atria from guinea-pigs by releasing noradrenaline from the nerve endings.

The effects of 14 lipid extracts were tested in 12 kitten atria failed by the standard method. In 24 of 27 tests, where 1/4 - 1/10 the total heart extract was added, the inotropic response to the lipid was either more positive or less negative than that to the corresponding blank.

The effects of increasing doses of acetone on the contractility

of isolated kitten atria are discussed on page 74. The effects of a constant volume of 8 of these 14 lipid extracts were studied in detail. Each extract was dissolved in 0.2 - 0.3 ml of acetone and a constant volume of 50 μl/ml (1/4 - 1/6 total volume) was added to the 10 ml bath. Acetone (5 μl/ml) was tested twice. Figure 18 shows the average response to the solvent blank (hatched bars) compared to that of the lipid (open bars) in each atrium. Two atria responded with an increase in contractile force when acetone was tested. The same preparations showed a greater increase in contractile force when the lipid was tested. A decrease in contractility resulted when the solvent was added to 5 of 7 atria. A smaller negative inotropic effect was observed in these preparations when the lipid was tested. Despite the solvent effects, the results demonstrated that the lipid had a positive inotropic effect. The responses to the lipid differed significantly ($P < 0.001$) from those of the solvent.

The effect of pronethalol (4×10^{-7} g/ml) on the increase in contractile force produced by the lipid was investigated. The results are shown in the lower portion of Figure 18. Pronethalol reversed the positive inotropic effect of the solvent and further decreased the negative inotropic effect in 5 preparations. A similar effect of pronethalol on the lipid responses was found. A significant difference ($P < 0.05$) in the lipid responses before and after β-adrenergic blockade was found. Following pronethalol treatment the myocardial depression resulting from the addition of the sample was not significantly different than that seen with the solvent ($P > 0.4$).

To study the effect of increasing doses of the lipid on the

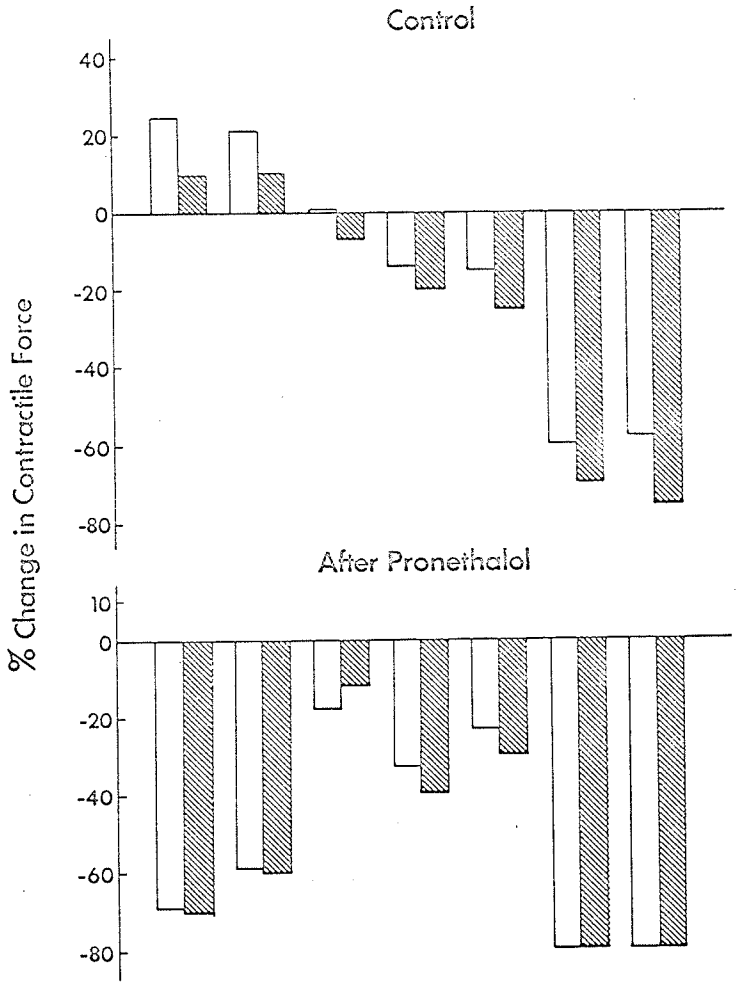

Figure 18. The effect of the lipid extracted from the heart washings by ether on the contractile force of kitten atria failed by the standard method. The open bars represent the individual responses to the lipid dissolved in acetone. The hatched bars represent the average effect of the acetone blank in the same atria. The responses are shown after pronethalol, 4×10^{-7} g/ml.

strength of contraction in kitten atria failed by the standard method, it was necessary to maintain a constant concentration of acetone (5 μl/ml). The lipid extracts from 3 hearts were combined and reconstituted in 0.3 ml of acetone. Separate additions of the lipid were made to the bathing medium of 2 atria. Correctionsfor the acetone blank were made. The positive inotropic effect of increasing amounts of lipid are shown in Figure 19. Additional acetone was added with each dose of lipid so that final concentration of acetone remained constant in each case. A dose-dependent increase in the strength of contraction was found. The results indicate that the dose-related, positive inotropic effect of the lipid is due to the release of noradrenaline from the nerve endings in kitten atria.

Attempts were made to extract WOF from Millipore filters with organic solvents. Both ether and acetone treatment resulted in disintegration of the filters. The resultant solutions had a marked negative inotropic effect in kitten atria. Possibly a myocardial depressant material was released from filters treated with these solvents.

The Inotropic Effect of the Material Extracted With Iso-amyl Alcohol

Ether extraction may remove a lipid material from a bound form. Ether is less effective in removing lipid from lipoprotein than several alcohols are. Burnstein (1967) found that iso-amyl alcohol removed 95 per cent of the lipids from serum proteins without causing denaturation of the proteins. The effect of extraction with iso-amyl alcohol on the inotropic activity of WOF was investigated. The washings from 7 gas-perfused hearts were used. The procedure followed is shown in Figure 20. Very little material was seen in the flask containing the

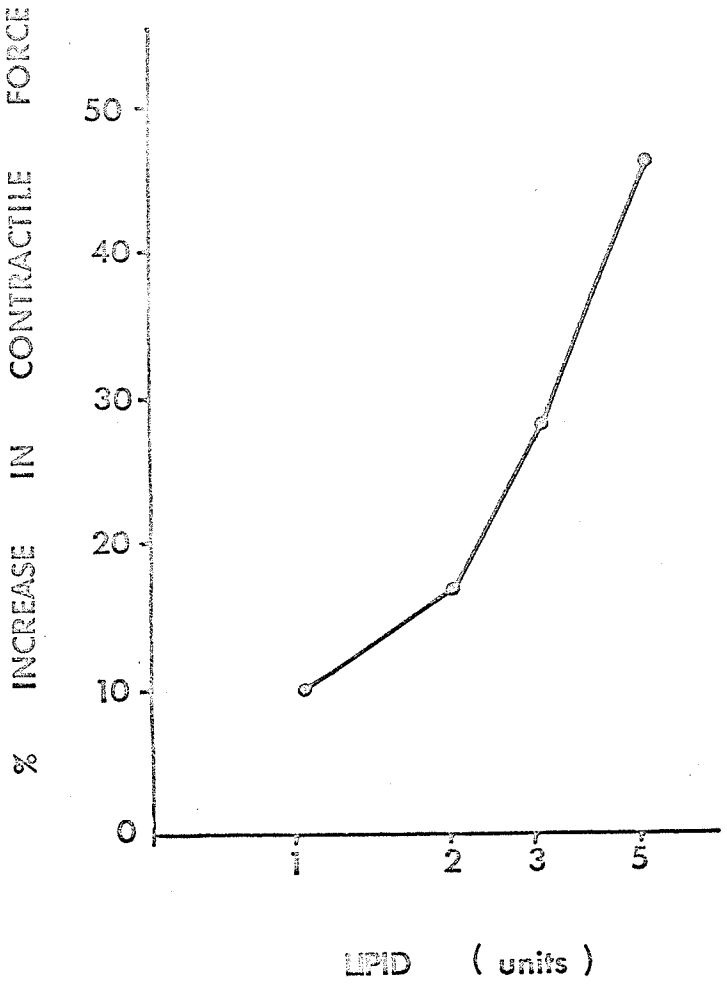

Figure 19. The effects of increasing doses of the lipid on the contractile force in 2 kitten atria failed by the standard method. The average response is shown. The volume of acetone in the lipid samples was maintained constant at 5 µl/ml. The results presented are the differences between the lipid response and the acetone blank response.

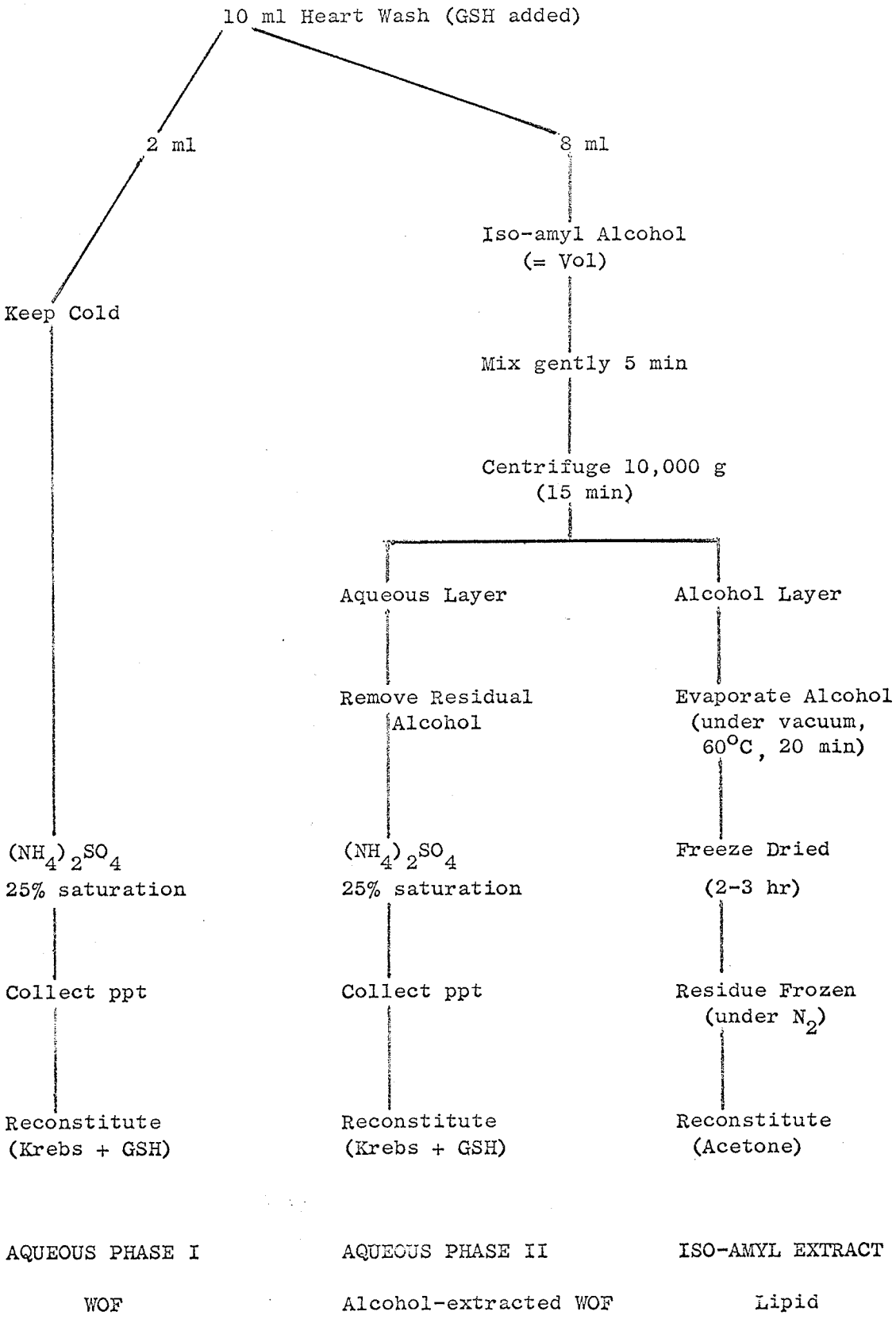

Figure 20. The diagrammatic presentation of the general procedure for iso-amyl alcohol extraction of the heart washings.

extract. The material extracted by the alcohol from the washings of one heart was taken up in 0.2 - 0.3 ml of acetone. A constant volume of 50 μl (1/4 - 1/6 of total volume) was tested in 6 kitten atria failed by the standard method. The average acetone blank response was compared with that of the alcohol-extracted material. No significant difference was found between the responses of the samples and those of the blanks ($P > 0.2$). Out of 14 comparisons, 10 responses to acetone were greater than those of the iso-amyl alcohol extracted lipid. The findings suggest that iso-amyl alcohol either did not extract any lipid or that the lipid extracted lacked cardiotonic activity.

The Inotropic Effect of the Aqueous Layer After Solvent Extraction

The aqueous phase after extraction with ether (Aqueous Phase II) contained a cardiotonic material which could be precipitated with ammonium sulphate (extracted WOF). Figure 15 (page 86) illustrates that the volume of heart washing of Aqueous Phase I was 2 ml and that of Aqueous Phase II was 7 ml. Both aliquots were eventually 25% saturated with ammonium sulphate, the precipitates collected and reconstituted in 2.5 ml of Krebs solution and equal volumes assayed. One would expect greater cardiotonic activity in the larger volume of washings than in the smaller one. To determine the magnitude of this difference, control experiments were carried out using the washings from 4 gas-perfused hearts. The washings from 2 hearts were combined and then divided into two unequal volumes in a ratio of approximately 1:3.5. These solutions were precipitated and filtered and reconstituted in the same way as those in the procedure for solvent extraction. In Figure 15, the broken line indicates the steps omitted in the control experiments. The WOF was

resuspended in 2.5 ml volumes of Krebs solution so as to mirror the comparison of Aqueous Phases I and II. The results are presented in Table IV. The increase in contractile force caused by the smaller aliquot of the washings was always less than that observed with the larger one. Either 1/5 or 1/10 of the total reconstituted material was tested. The ratio of the cardiotonic response of the smaller aliquot compared with that of the larger one was 1:1.35 for the higher dose and 1:1.31 for the lower dose respectively. These findings suggest a constant ratio for the positive inotropic effect when the volume ratio is 1:3.5. These findings also indicate that recovery of the cardiotonic material from the Millipore filters was poor since the activity of 3.5 times larger aliquots of heart washings, following precipitation and filtration is only 31% greater than the smaller aliquot treated in the same manner. However when twice the volume of reconstituted material is tested,the response is double that seen with the smaller volume.

The washings from 14 gas-perfused hearts were extracted with ether as depicted in Figure 15 on page 86. The cardiotonic activity of WOF (Aqueous Phase I) was compared with that of extracted WOF (Aqueous Phase II) in 6 kitten atria failed by the standard method. One fifth of the total reconstituted volume was added to the bath. From the control studies the expected ratio of cardiotonic activity was 1:1.35. The ratio however was changed to 1:0.68 indicating that ether extraction had decreased but not eliminated the activity in the aqueous layer. The mean per cent increase in contractile force was 49.7 $\pm$ 7.1 for WOF and 32.1 $\pm$ 5.4 for extracted WOF respectively. Pronethalol

Table IV

Control experiments showing the per cent increase in contractile force caused by the smaller and larger aliquots of heart washings (volume ratio 1: 3.5)

Fraction of Total Volume	No. of Tests	Smaller Aliquot	Larger Aliquot	Ratio of Activity Sm:Lg Vol.	P value
1/10	13	24.2 ± 2.3	31.8 ± 2.7	1:1.31	< 0.001
1/5	8	47.0 ± 3.4	63.6 ± 3.9	1:1.35	< 0.001

did not significantly decrease the effects of extracted WOF ($P = 0.5$). The mean per cent increase in contractile force was 32.8 ± 4.6 in the untreated muscles and 30.2 ± 4.2 in the pronethalol treated ones. These findings suggest that the protein, "extracted WOF", retained cardiotonic activity which was not due to the release of noradrenaline.

In summary, ether extraction of the washings resulted in the removal of a lipid material which had a positive inotropic effect in atria from kittens and guinea-pigs. This increase in the contractile force was blocked by pronethalol and was minimal or absent in atria taken from animals pretreated with reserpine. The lipid increased the strength of contraction by causing the release of noradrenaline from the nerve endings. The aqueous phase after ether extraction when 25% saturated with ammonium sulphate (extracted WOF) also had a positive inotropic effect in atria from kittens. Pronethalol had little or no effect on these responses.

Alcohol - extracted WOF

The aqueous phase after extraction of the heart washing with iso-amyl alcohol (Aqueous Phase II in Figure 20) also contained a cardiotonic material which could be precipitated with ammonium sulphate (alcohol-extracted WOF). The volume ratio of Aqueous Phase I: Aqueous Phase II was 1:3.5, the same as in the control experiments (see page 99). The ratio of cardiotonic activity with this volume ratio in the controls was 1:1.35. Comparison of the effects of WOF and alcohol-extracted WOF obtained with washings from 12 gas-perfused hearts were made in 6 standard preparations. The cardiotonic ratio was found to be 1:1.27, almost identical to that found with the controls. This finding suggested that

extraction with iso-amyl alcohol did not decrease the activity in the washings. When 1/4 - 1/6 the total reconstituted volume was added to the bath, the positive inotropic effect with the alcohol-extracted WOF was greater in 17 of 21 tests than that seen with WOF. A significant difference ($P < 0.02$) was found. The mean per cent increase in contractile force was 61.5 $\pm$ 10.2 for alcohol-extracted WOF and 48.7 $\pm$ 8.9 for WOF respectively. These results indicate that no cardioactivity was lost from the washings during extraction with iso-amyl alcohol. The lack of cardiotonic activity in the lipid extracts confirms this. It is concluded from these studies that the alcohol either did not dissociate the lipid from the protein or that the lipid was not soluble in the alcohol.

IV EFFECTS OF WOF IN PREPARATIONS OTHER THAN CARDIAC MUSCLE

STIMULATION OF 3-MG TRANSPORT BY WOF

The transport of glucose and several related sugars across the cell membrane of many tissues occurs by a specific mechanism. This mechanism is explained in terms of a reversibly operating carrier mechanism in the membrane (Levine, Goldstein and Huddlestun, 1949; Goldstein, 1959). In muscle, penetration of glucose by this process controls the rate of metabolism and is, in turn influenced by several factors. Insulin increases the transport of sugars independently of its effects on the subsequent metabolism of these materials (Levine, 1966). Anoxia has been found to accelerate transport in diaphragm (Randle and Smith, 1958), heart (Morgan *et al.*, 1959) and frog muscle (Ozand *et al.*, 1962). Randle and Smith (1960) proposed that anoxia may lower the level of ATP which normally restrains the activity of the transport system but this

has not been confirmed. Another explanation of this increase in sugar transport relates it to inhibition of active cation transport. Ouabain increases glucose uptake and metabolism in adipose tissue (Ho and Jeanrenaud, 1967) and in skeletal muscle (Bihler, in press). This effect occurrs at concentrations known to inhibit sodium transport and is also seen when sodium transport is inhibited by other means e.g. low extracellular potassium. These treatments also have other insulin-like effects, such as increased glycogen synthesis (Clausen, 1965, 1966).

Acceleration of transport in working muscle has been observed *in vivo* with severe exercise or by neural or electrical stimulation of muscle. It is a well known clinical fact that exercising diabetic patients require less insulin. Ingle *et al.* (1950) found that stimulation of the leg muscles of diabetic and normal rats caused a rapid fall in blood sugar. Studies done *in vivo* have shown that severe exercise also increased the transport of some non-metabolized sugars (Goldstein *et al.*, 1953; Helmreich and Cori, 1957). More recently, Holloszy and Narahara (1965) reported that the transport of 3-O-methyl-D-glucose (3-MG) was accelerated *in vitro* in frog muscle as the frequency of stimulation was raised and that the effect was independent of the amount of work done. In heart muscle, the opposite appears to be the case, transport increased proportionately to the work done when the rate was held constant (Neeley *et al.*, 1967).

The mechanism by which muscular exercise increases glucose uptake is unknown. Holloszy and Narahara (1967) have suggested that changes in sugar transport resulting from electrical stimulation may be related to changes in the intracellular concentration of calcium ion.

Goldstein et al. (1953) found that strenuous activity of only some of the dog's musculature led to a generalized cellular entry of non-utilizable sugars into the total body water. He considered the possibility of the elaboration of a circulating insulin-like material by strenuous exercise. In 1961, Goldstein provided evidence for the existence of such a humoral factor. The local insulin-like effects of muscle contraction have been generally confirmed by Helmreich and Cori (1957), Sacks and Smith (1958) and Dulin and Clark (1961). The latter two groups of workers suggested that the increased transport might be due to a localized hypoxia of the stimulated muscle. The presence of a circulating humoral factor was denied in these reports. These contradictory results could be reconciled by assuming a local release of an insulin-like material and the existence of a circulating inhibitor or destructive enzyme that usually limits the effects of such a factor to the region of its release. Recently Goldstein (1965) showed that cross-transfusion or transfer of lymphatic fluid from a working animal to a resting one stimulated transport in the recipient dog. He made many attempts to isolate and identify the "work factor" but was unable to develop a reproducible procedure for extracting a stable preparation because of the instability of the factor and the marked variation in activity from different animals. From the few successful preparations obtained, he was able to show that the material did exist and was distinct from known hormones. The "work factor" had the properties of a polypeptide in that it was acid soluble, non-dialysable and resisted protein precipitation techniques. Data from studies of exercise in man are also consistent with the existence of such a factor. Bergström

and Hultman (1966) found that glycogen depletion after strenuous exercise was followed by an enhanced resynthesis of glycogen over initial levels. The effect was restricted to the exercised muscle and persisted for several days. They suggested that the effect was on the cell membrane resulting from stimulation of sugar transport.

Although the results from *in vivo* experiments are suggestive of a humoral factor they are far from conclusive. However, for the *in vitro* results of R -Candela and R -Candela (1962) and Havivi and Wertheimer (1962 and 1964) no alternative explanation but a local humoral factor can be found at present. The studies of Havivi and Wertheimer give some information on its properties: the "muscular activity factor" (MAF) stimulated the entry into the cell of sugars and amino acids; it was released only from contracting muscle and not from resting muscle or other tissue; when the muscle was stimulated under conditions where it failed to contract (i.e. in an anaerobic environment or in sucrose solution) no MAF was released. They also found that fatigued muscle released less MAF and muscle in tetanus did not release any. Their results with uterine muscle agree with those obtained by R -Candela and coworkers (R -Candela and R -Candela, 1962; R -Candela *et al.*, 1962) who showed that hormone-stimulated uterine muscle released a product which enhanced glucose uptake by various tissues. Recently, Wertheimer (1965) reported the *in vivo* production of a factor, probably MAF, in rats adapted to swimming. When exercised, the trained rats released the material into the serum and excreted it into the urine. No such factor appeared in the serum and urine of untrained rats similarly exercised.

Havivi and Wertheimer (1964) concluded that MAF was a protein or protein-bound substance since it is non-dialysable, precipitated by ammonium sulphate and perchloric acid, and is inactivated by proteolytic enzymes. They found a positive correlation between the protein concentration of the material released from working muscle and the sugar uptake.

Unlike insulin, MAF was thermolabile, was elaborated by chronic diabetic animals, had no effect on glycogen synthesis and its effects on sugar transport were not decreased by phloridzin. Like insulin, MAF increased the cellular uptake of sugars and the non-metabolized amino acid, α-aminoisobutyric acid (AIB). Both agents increased the incorporation of alanine, leucine and glycine into muscle protein.

Havivi and Wertheimer did not investigate the effect of MAF on the contractile force of isolated heart muscle. Recently Frederickson, Bihler and Dresel (submitted) tested the bathing media of rat hemidiaphragms incubated under the conditions used by Havivi and Wertheimer in their study of MAF release. They found that a factor, apparently MAF, which increased sugar uptake was released from contracting muscle only, whereas a factor which increased the contractile force of atria failed by pentobarbital was release both by contracting muscle and by muscle at rest. They concluded that the material released from working muscle was a mixture of at least two biologically active substances.

WOF, the cardioactive material removed from gas-perfused hearts resembles MAF in many respects. WOF is released from contracting muscle and is precipitated by ammonium sulphate and perchloric acid. The effect of WOF on the transport of non-metabolized sugars was

investigated by Bihler and Dresel (1966) who found that the uptake of 3-MG and D-xylose was stimulated by this material. The effect of WOF on transport was specific since it was blocked with phloridzin (2.0 mM), a specific inhibitor of sugar transport mechanisms. WOF did not affect the entry of ^{3}H-mannitol, the extracellular marker. Preliminary experiments had shown that WOF also increased the transport of AIB into rat skeletal muscle. Both MAF and WOF were shown to be immunologically distinct from insulin. Unlike MAF, WOF increased the glycogen synthesis in muscle (Bihler, unpublished).

WOF possesses two biological activities, the increase in the strength of contraction of the standard preparation and the insulin-like effect on sugar transport. It was, therefore, of interest to determine whether any correlation between these two activities existed. Concurrently further studies on the insulin-like effects of WOF were carried out.

To investigate whether any correlation existed between the two biological activities of WOF, 10 samples of the material were tested both for cardiotonic activity in 10 standard preparations and for the stimulation of transport in intact hemidiaphragms. In these experiments only, the heart material collected on the 8.0 μ and 0.8 μ filters was reconstituted separately into 2.5 ml of Krebs solution. The effect of WOF on transport was expressed as per cent increase from transport in the control hemidiaphragm. The effect of WOF on the contractile force was expressed as per cent increase from the control level. Two different volumes, 0.2 and 0.4 ml, of both the 8.0 μ and the 0.8 μ filtered material were tested in duplicate preparations.

To determine whether the insulin-like activity of WOF was evenly distributed between the 8.0 μ and the 0.8 μ filters the responses resulting from the addition of each material were compared. The mean response resulting from the addition of the smaller and the larger volume was 35.2 $\pm$ 18.0 and 11.2 $\pm$ 9.5 for the 8.0 μ filtered material and 39.2 $\pm$ 24.1 and 10.0 $\pm$ 10.9 for the 0.8 μ filtered material, respectively. In 6 of 10 preparations the 8.0 μ filtered material resulted in greater stimulation of transport than that of the 0.8 μ filtered material. However, the difference was not statistically significant and therefore the results obtained with the materials from the two types of filters were pooled.

With the smaller dose (0.2 ml) of the materials from both filters, the sugar uptake of hemidiaphragms was compared in duplicate muscles. The variation between groups was not statistically significant ($P > 0.4$) but there was considerable difference in the magnitude and the direction of the responses to different samples of WOF. Out of 9 paired trials, both duplicates showed stimulation of transport in 5 pairs and no change in 2 pairs. In the remaining 2 pairs the effects were in opposite directions. The results clearly indicate that the transport of sugar is increased *in vitro* in muscle by WOF. When the same amount of each sample was added to duplicate atria preparations no significant difference ($P > 0.8$) between the responses was found but considerable variation in the magnitude of the effects on contractility were observed. Out of 9 paired trials, both duplicates showed increases in contractile force in 8 pairs and no effect in one pair. This marked variability of the cardiotonic activity of WOF obtained from different hearts has been

discussed in detail (see page 56). The results presented here indicate a similar variability of the effects of different samples of WOF on 3-MG transport. The mean per cent increase in 3-MG transport was 33.0 ± 10.1 and that in contractility was 12.7 ± 2.4. Since the same amount of WOF was added to 2 ml in testing the effect on sugar transport and to 10 ml in testing the effect on contractile force, it appears that WOF is about 3 times more potent in causing a positive inotropic effect than in stimulating glucose uptake.

A dose-response relationship was demonstrated for the cardiotonic effects of WOF (see page 58). The mean per cent increase in glucose uptake resulting from the addition of 0.2 and 0.4 ml (pooled data from 8.0 μ and 0.8 μ filtered material) of WOF to the incubation media was 36.9 ± 13.7 and 11.7 ± 6.1 respectively. In 6 of 9 tests the higher dose resulted in less sugar uptake than the lower dose. Thus, an inhibitory effect appears at high doses of WOF. These results suggest that an inhibitory factor may be present in WOF and that this may obscure any dose-response relationship. It is possible that a test of smaller doses might have shown such a dose-action relationship.

To ascertain whether the two biological activities of WOF were associated, they were measured simultaneously in the same samples. A total of 31 comparisons were made. The results are shown in Figure 21. The mean per cent stimulation of sugar transport was 27.7 ± 7.4. Seven of 31 additions resulted in a decrease in 3-MG uptake when compared to control and 2 of 31 had no effect. The mean per cent increase in contractile force was 19.6 ± 3.3. No correlation between the 2 activities was found ($r = 0.038$). These results indicate that the effects on sugar

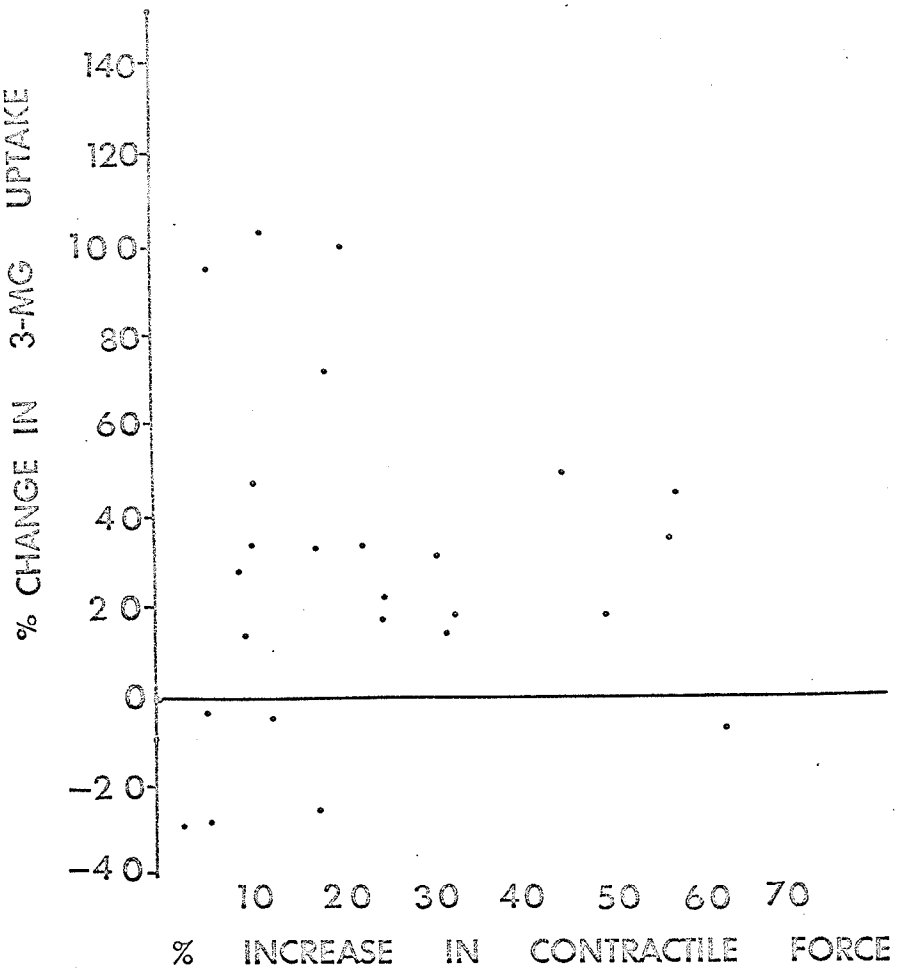

Figure 21. The relationship between the cardiotonic effect (abscissa) and the insulin-like effect on transport (ordinate) of WOF. Activities expressed as per cent increase over control. Total number of comparisons, 31. r value = 0.038. $P > 0.05$.

transport and the cardiotonic activity of WOF are independent of each other.

Havivi and Wertheimer (1964) found a positive correlation between the protein concentration and the increase in sugar transport of MAF. With the material from resting muscle at concentrations of protein greater than 0.4 mg/ml they found a decrease in glucose uptake. Figure 22 shows the mean per cent increase in sugar transport related to the protein concentration of samples from both 0.8 and the 8.0 μ filters. The protein concentration in the media was divided into 5 ranges and the number of tests in each range is given in brackets. The stimulation of transport appeared to increase progressively as the protein concentration was increased to 20 μg/ml but further increases resulted in a lesser stimulation of uptake in 18 of 39 tests. These findings would be consistent with the presence of an inhibitory factor in WOF. In this regard, the effect of WOF would appear to resemble more the effect of the resting muscle media of Havivi and Wertheimer (1964) than that of MAF.

To investigate whether the stimulation of transport by WOF is associated with a protein or with a protein-bound lipid, the heart washings were extracted with ether. Both the lipid and extracted WOF (see Figure 15, page 86) had been shown to increase the contractile force of kitten atria failed by the standard method (see pages 92 and 98).

Samples of lipid extracted from the washings of 9 hearts were used. The lipid was dissolved in acetone and varying volumes from 1/7 - 1/28 of the total material from one heart were added to the hemidiaphragms. Sugar transport was stimulated in 20 of 28 tests. Considerable variation in the magnitude of the response occurred, in part due to the different

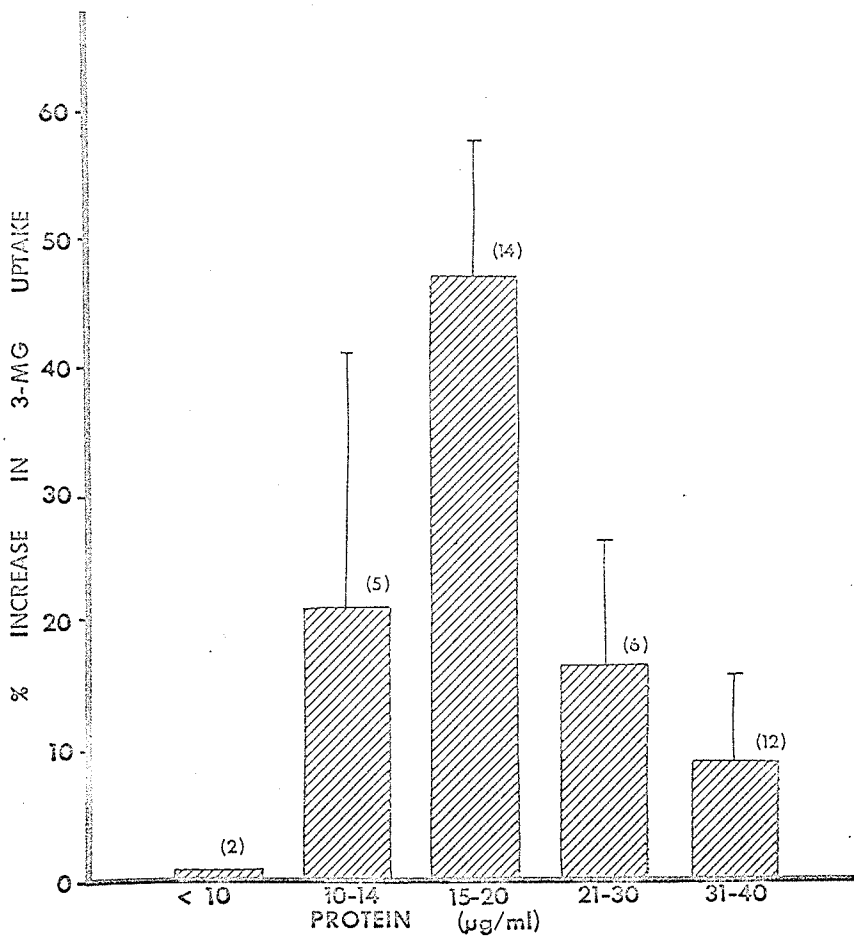

Figure 22. The relationship between the protein concentration (abscissa) and the insulin-like effect of WOF on 3-MG transport (ordinate). The concentration of protein (µg/ml) is divided into 5 ranges. The mean response of all the tests are given. The S.E. are indicated. The number in brackets indicates the number of tests with protein concentration within each range.

amounts of total heart lipid added but also due to the marked variation in activity of samples from different hearts. Better agreement between duplicate preparations was found with the lipid than with WOF. All 9 additions in 18 tests increased the 3-MG uptake. As larger volumes of the lipid appeared to elicit a larger increase in transport than smaller ones it appeared that a dose-response relationship might exist. To investigate further the effect of graded doses of the lipid on sugar transport an additional experiment was carried out. The lipid material from 2 hearts was pooled and dissolved in 0.7 ml of acetone and was tested on twelve rat hemidiaphragms. The same volume of material was tested for cardiotonic activity in 2 standard preparations. The average responses of each volume of lipid tested in duplicate are presented in Table V. The amount of lipid added is expressed as the fraction of the total material from one heart. The results show clearly that increasing doses of the lipid resulted in progressively greater responses in both types of preparations. As with WOF, the final concentration of lipid required in the incubation medium to stimulate sugar transport is several times that required to produce a positive inotropic effect. High doses of lipid did not result in decreased stimulation of transport as had been observed with high doses of WOF. This would suggest that any inhibitory factor is not soluble in ether.

Although only 6 comparisons of the two biological activities in the lipid were made, it appears that there may be some correlation between them ($r = 0.877$). The dependence of the one activity on the other is greater than would be expected to occur by chance ($P < 0.005$).

Only one sample of extracted WOF was tested for its effect on

Table V

The effect of increasing doses of the lipid material on 3-MG uptake in skeletal muscle and on contractile force in heart muscle.

Fraction of One Heart Extract Added	Per Cent Increase	
	3-MG Transport	Contractile Force
1/17.5	No change	10
1/7	44	28
1/3.5	75	58

sugar transport. The 0.8 μ and the 8.0 μ filtered material increased the sugar transport in 8 of 11 tests. The results indicate that the sugar transport stimulating activity was present in both the lipid and the extracted WOF. Thus these studies did not separate the two biological activities of WOF and did not resolve the question of the chemical nature of the factor.

The data can be summarized as follows:

1) WOF, extracted WOF and the lipid material all increased 3-MG transport in the rat hemidiaphragm. No dose-response relationship was found with WOF but some indication of dose-dependence was seen with the lipid.

2) No correlation between the protein concentration and the effect of WOF on sugar transport was found. In fact, the results at protein concentrations greater than 20 μg/ml, suggest the presence of an inhibitory factor.

3) No correlation was found for the two biological activities of WOF. However, with the lipid, there appears to be a positive correlation of the two biological activities.

4) Following ether extraction of the heart washings the transport enhancing activity was present in both the lipid and extracted WOF. These studies failed to separate the two biological activities of WOF.

THE EFFECTS OF WOF IN ISOLATED SMOOTH MUSCLE PREPARATIONS

Bohr and Johansson (1966) studied the contraction of vascular smooth muscle in response to plasma. They found that concentrations of plasma too small to produce contraction potentiated the tension developed

in response to stimulation by adrenaline, angiotensin and KCl. The responses to plasma were compared to those of several known vasoactive agents of plasma including adrenaline, noradrenaline, serotonin, angiotensin, vasopressin, bradykinin and histamine. They reported important differences. Dorevitch, Nayler and Lowe (1967) recently studied the actions of Kinekard, a cardioactive fraction isolated from human plasma, on isolated smooth muscle. In rabbit thoracic aorta, rabbit ileum and guinea-pig uterus the action of Kinekard resembled that of adrenaline. However, they concluded Kinekard and adrenaline were different, and that Kinekard did not act on smooth muscle by liberating endogenous stores of catecholamines. The effects of WOF on three types of smooth muscle preparations; gut, vascular and uterine muscle was investigated.

The effects of 6 samples of WOF were studied in 16 segments of ileum from 4 guinea-pigs. Ten sections were non-terminal and 6 were terminal. Of the latter preparations, 4 of 6 segments contracted when stimulated with adrenaline (1×10^{-6} g/ml). The mean response was 9 mm. All 16 segments of ileum responded to acetylcholine (ACh), 3×10^{-8} g/ml, histamine (H), 1×10^{-7} g/ml, and 5-hydroxytryptamine (5-HT), $2 - 3 \times 10^{-7}$ g/ml. The mean response in mm obtained with each agonist was 72, 78 and 39 for ACh, H and 5-HT respectively. No response to WOF was seen in any of the 6 terminal segments of ileum. With the non-terminal preparations, a small contraction (mean 5 mm) occurred 50% of the time. Three samples of WOF had no effect. The effect of WOF on the spontaneous activity of the preparations was unpredictable. When spontaneous activity was present, WOF tended to eliminate it. If it was absent, WOF tended to induce it.

Treatment of the ileum segments with phenoxybenzamine (POB), 1×10^{-6} g/ml, completely abolished the responses to the agonists and to WOF. KCl ($2.5 - 3.5 \times 10^{-3}$ g/ml) was used to show that the lack of response after POB treatment was not due to the loss of contractility of the tissue. Lower concentrations of POB (2×10^{-7} g/ml) also markedly decreased the responses.

Untreated washings from 2 gas-perfused hearts were tested in 4 non-terminal segments of ileum. No response was seen. A similar lack of activity was found when 2 samples of the cardioactive lipid were tested. The preparations were responsive to the usual doses of ACh, H and 5-HT.

The effect of WOF on isotonic and isometric contractions of 12 helical strips of rabbit thoracic aorta was studied. WOF neither caused contraction nor relaxation of the vascular smooth muscle. No potentiation or antagonism of the noradrenaline response was observed. The effect of WOF on the relaxation of the noradrenaline contraction resembled that of isoproterenol ($1 \times 10^{-6} - 1 \times 10^{-7}$ g/ml). This effect of WOF on relaxation was found to be due to the presence of GSH and ammonium sulphate. Untreated washings from 3 gas-perfused hearts were therefore tested. In no case was any contraction or relaxation, nor potentiation or antagonism, or effects on relaxation observed with these washings in the aortic strips.

The effect of WOF in four uterine strips from rats was studied. WOF was inactive in these preparations. The heart material did not induce contraction in quiescent strips nor did it inhibit contractions induced in the muscle by ACh (1×10^{-7} g/ml). In these preparations,

adrenaline (1×10^{-6} g/ml) always inhibited both spontaneous and ACh-induced contractions.

The results indicate that the effects of WOF on smooth muscle preparations are insignificant or absent at concentrations of the material which have a marked positive inotropic effect on depressed heart muscle. Of the 4 smooth muscle preparations investigated, only non-terminal ileum responded 50% of the time to WOF with a minimal contraction. POB blocked this response. No relaxation or contraction was seen in terminal segments, aortic strips or uterine smooth muscle when WOF was studied. Nor did WOF either potentiate or antagonize the responses of these preparations to the usual agonists.

V MECHANISM OF ACTION OF WOF ON HEART MUSCLE

THE EFFECTS OF WOF ON THE PARAMETERS OF ISOMETRIC CONTRACTION

Changes in contractility caused by WOF have thus far been described simply by increases in the developed tension of the preparation. The tension generated depends on two factors: the rate of tension development and the length of time during which tension is generated. The rate of tension development (maximum, $\frac{dT}{dt}$ or mean, $\frac{\Delta T}{\Delta t}$) reflects the intensity of the active state i.e. the position of the force-velocity curve of the contractile element; and the time required to reach peak tension (TTP) is directly proportional to the duration of the active state (Sonnenblick, 1967).

The papillary muscles used in these studies were not failed with pentobarbital because this agent markedly changes the contour of the isometric contraction curve by reductions in both $\frac{dT}{dt}$ and TTP (Buccino *et al.*, 1967). Koch-Weser and Blinks (1962) have shown that ouabain increased

the strength of contraction of normal papillary muscle when the preparations were stimulated at low frequencies. Preliminary studies with WOF were carried out to investigate its positive inotropic effect on unfailed papillary muscles stimulated at a frequency of 0.5 sec^{-1}. When 3 samples of WOF were tested in 3 unfailed papillary muscles from kittens the mean absolute increase in peak tension (PT) was 0.43 g/mm^2. In relative terms, the mean per cent increase was 54% over the control baseline. These findings indicated that WOF increased the strength of contraction in unfailed papillary muscle when the preparations were stimulated at a low frequency. The time-tension studies were therefore carried out on normal preparations. As discussed earlier (see page 54) papillary muscles were less responsive to WOF than were atria. Twice the volume of WOF had to be added to the papillary muscle to elicit equivalent responses. To eliminate excessively large additions of the reconstituted material to the bath, the samples were reconstituted in 2.5 ml instead of 5 ml of 0.9% NaCl solution. Usually 0.25 ml was added to the 10 ml bath. It was shown previously that the positive inotropic effect of WOF was temperature dependent (see page 61). The bath was therefore maintained at 37 $\pm$ 0.5°C despite the fact that most time-tension index studies reported in the literature were done at 30°C. The frequency of stimulation and the temperature of the bath are two physical factors known to influence the parameters of isometric contraction (Buccino *et al.*, 1967). The short duration of action of WOF necessitated the use of a higher frequency of stimulation than that commonly employed in studies of this type (Buccino *et al.*, 1967). The resting tension was maintained at 1 g.

To characterize further the inotropic effect of WOF, the changes induced by this material in the three interrelated variables of isometric contraction, PT, $\frac{dT}{dt}$ and TTP were investigated. Any muscle with cross-sectional area greater than 1.3 mm^2 was excluded from the investigation. The mean cross-sectional area of all the muscles $\pm$ S.E. was 1.01 $\pm$ 0.28 mm^2. Peak isometric tension was corrected for cross-sectional area and was expressed in grams per square millimeter (g/mm^2); time to peak tension was measured from the onset of tension development and expressed in milliseconds (msec). The maximum rate of tension development (maximum slope of the tension curve; $\frac{dT}{dt}$) was expressed in grams per square millimeter per second ($g/mm^2/sec$). The method of measurement is illustrated in the insert in Figure 23. The maximum rate of tension development was measured twice and if good agreement was obtained, the average measurement was taken. When poor agreement existed a third measurement was made and the 2 closer values used to calculate the $\frac{dT}{dt}$. The total developed tension (TDT) was estimated by determining the area under the curve (cm^2) with a Polar Planimeter (Keuffel and Esser Company). The total duration of contraction (TD) was expressed in msec. The mean responses $\pm$ S.E. are expressed in absolute values. In measuring responses to WOF and pharmacological agents each muscle was used as its own control. In describing the effects of WOF on papillary muscle taken from kittens pretreated with reserpine, data from different muscles were grouped and compared with those from a group of normal muscles studied under the same conditions.

The effect of 8 samples of WOF on the parameters of isometric contraction in 12 cat papillary muscles was investigated. The effect of

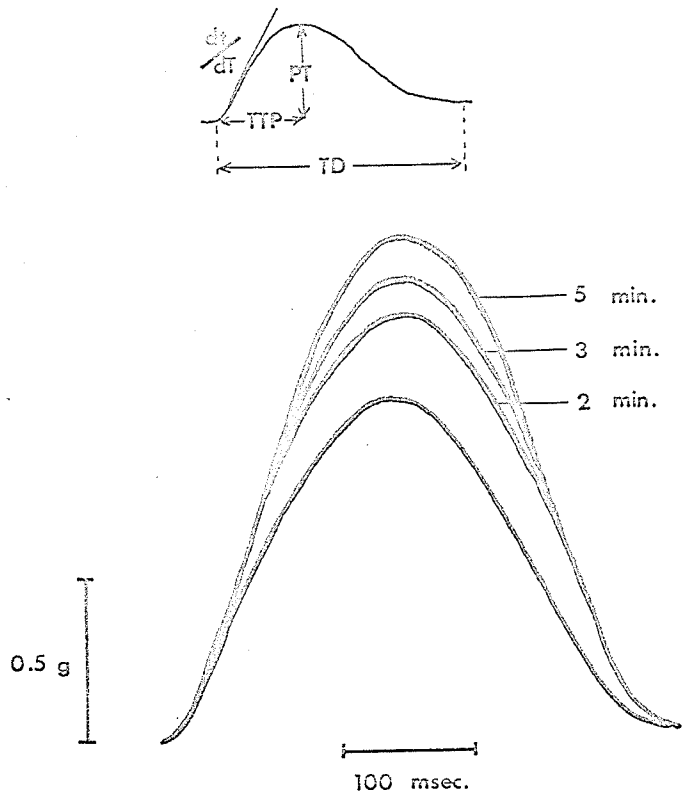

Figure 23. The effects of WOF on tension development by cat papillary muscle. Responses are shown 2, 3 and 5 min after addition of 0.25 ml of WOF. Temperature, 37°C. Frequency, 0.5 sec^{-1}. Cross-sectional area, 1.25 mm^2. Superimposed tracings made from enlarged 35 mm film records. The horizontal bracket indicates time; the vertical bracket, force.

Insert at the top indicates the method of measurement of the different parameters of isometric contraction under investigation.

	Control	2 min	3 min	5 min
TTP (msec)	176	192	192	196
PT (g/mm^2)	1.34	1.66	1.80	1.96
dT/dt ($g/mm^2/sec$)	11.2	12.6	14.6	16.0

time on the response to WOF is shown in Figure 23. This tracing is typical of several obtained under similar conditions. The changes in the 3 parameters of isometric contraction were measured at 3 time intervals after the addition of WOF. The PT and $\frac{dT}{dt}$ progressively increased from control at each time interval studied. The TTP was prolonged to maximum at 2 min and either remained the same or decreased slightly after longer exposure to the material. The total duration (TD) of the isometric contraction was unchanged by WOF. In all further experiments, the measurements were made 5 min after the addition of WOF.

The effect of increasing concentrations of WOF on the 3 parameters of isometric contraction is shown in the typical records traced in Figure 24. Two serial 0.25 ml additions of WOF were made 5 min apart. An increase in the concentration of WOF in the bath resulted in further increases in all parameters tested i.e. PT, TTP and $\frac{dT}{dt}$. Neither dose changed the TD of isometric contraction.

Ouabain (5×10^{-7} g/ml) and adrenaline (5×10^{-7} g/ml) were also studied in these experiments. The results are summarized in Table VI. The mean values are shown for each agent with the S.E. given in the line below. The means of the differences are shown. All 3 agents had a significant effect of PT, $\frac{dT}{dt}$ and TTP. The effects of WOF, ouabain and adrenaline were similar on PT and $\frac{dT}{dt}$. Adrenaline increased PT by markedly increasing $\frac{dT}{dt}$ since TTP was shortened with this agent. With WOF and ouabain the increase in PT was caused by increases in the two interrelated variables $\frac{dT}{dt}$ and TTP. When the same data were used to determine the mean rate of rise ($\frac{\Delta T}{\Delta t} = \frac{TTP}{PT}$) the magnitude of the mean differences was slightly less than that of $\frac{dT}{dt}$ (maximum) for all 3 agents. It was therefore decided

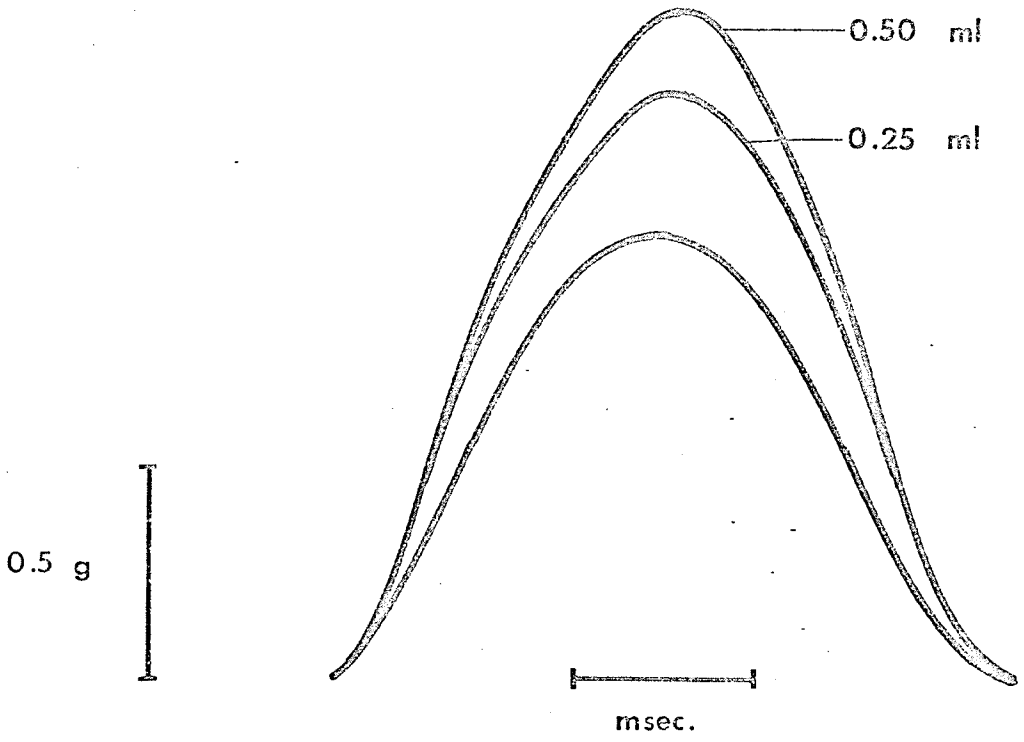

Figure 24. The effects of two doses of WOF on tension development by cat papillary muscle. Doses of WOF, 0.25 and 0.5 ml. Temperature, 37°C. Frequency, 0.5 sec^{-1}. Cross-sectional area, 1.25 mm^2. Superimposed tracings made from enlarged 35 mm film records. The horizontal bracket indicates time; the vertical bracket, force.

	Control	0.25 ml/10 ml.	0.5 ml/10 ml.
TTP (msec)	180	196	204
PT (g/mm^2)	1.34	1.77	2.04
dT/dt ($g/mm^2/sec$)	10.6	12.6	16.4

Table VI

The effects of WOF, ouabain and adrenaline on the parameters of isometric contraction in cat papillary muscle.

Agent	N		Peak Tension g/mm^2			Maximum Rate of Tension Development $g/mm^2/sec$			Time to Peak Tension msec		
			Control	Exp.	Diff.	Control	Exp.	Diff.	Control	Exp.	Diff.
WOF (0.25 ml/10 ml)	20	Mean	1.08	1.88	0.82*	6.8	10.9	4.0*	172	184	12 **
		S.E.	0.22	0.25	0.15	0.7	1.0	0.5	11.3	8.5	4.7
Ouabain (5×10^{-7} g/ml)	6	Mean	0.87	1.48	0.61*	6.1	10.6	4.5*	170	181	11 **
		S.E.	0.22	0.19	0.09	1.0	1.4	0.8	14.9	16.0	3.6
Adrenaline (5×10^{-7} g/ml)	6	Mean	0.81	1.33	0.52*	5.5	10.6	5.1*	162	144	-18 **
		S.E.	0.22	0.23	0.09	1.2	2.0	1.0	9.4	5.5	7.9

N indicates the number of tests

Diff. = Mean of the differences between control and experimental

* $P < 0.01$ by paired data analysis

** $P < 0.05$ by paired data analysis

to calculate $\frac{dT}{dt}$ rather than $\frac{\Delta T}{\Delta t}$. The total developed tension (TDT) was very similar in each case. The mean increase in TDT $\pm$ S.E. was 5.2 $\pm$ 1.0, 5.1 $\pm$ 1.3 and 5.2 $\pm$ 1.8 for WOF, ouabain and adrenaline respectively. Changes in TD did not reach statistical significance after any of the agents. Adrenaline however, shortened TD slightly in each test, ouabain slightly prolonged TD and WOF caused variable effects. The effects of ouabain on TTP differed from those reported by Buccino et al. (1967) who found that ouabain significantly decreased TTP. However their assay conditions varied considerably from those employed here. They used a temperature of 30°C and a contraction frequency of 0.2 sec^{-1}.

Untreated washings from 3 gas-perfused hearts were tested and the effects on these parameters of isometric contraction analyzed. The washings were warmed briefly to 37°C before adding 1.0 ml to the 10 ml bath. This volume would be expected to yield an amount of WOF equivalent to 0.125 ml. The results are presented in Table VII. The mean of 7 responses $\pm$ S.E. are shown. The changes were similar to those of WOF in all experiments.

Preliminary experiments had indicated that the effects of WOF on contractility in cat papillary muscle may be due in part to the release of noradrenaline from the nerve endings (see page 65). Therefore, a number of samples of WOF were tested in 2 groups of papillary muscles: one group of 8 muscles from normal animals in which the sample was tested both before and after pronethalol (5×10^{-7} g/ml) and a group of 4 muscles obtained from animals pretreated with reserpine. Figure 25 shows the differences in the parameters of isometric contraction caused by WOF in these preparations. The increase in peak tension (PT) was clearly the

Table VII

The effects of untreated heart washings on the parameters of isometric contraction in cat papillary muscle.

Parameter	Control	Experimental	Difference
Peak Tension (g/mm^2)	1.00 ± 0.05	1.22 ± 0.06	0.22 ± 0.05**
Maximum Rate of Tension Development ($g/mm^2/sec$)	9.0 ± 0.3	12.3 ± 0.6	2.8 ± 0.27**
Time to Peak (msec)	160 ± 5.7	172 ± 5.1	11 ± 5.1*

Difference = Mean of the differences between control and experimental

* $P < 0.01$ by paired data analysis

** $P < 0.05$ by paired data analysis

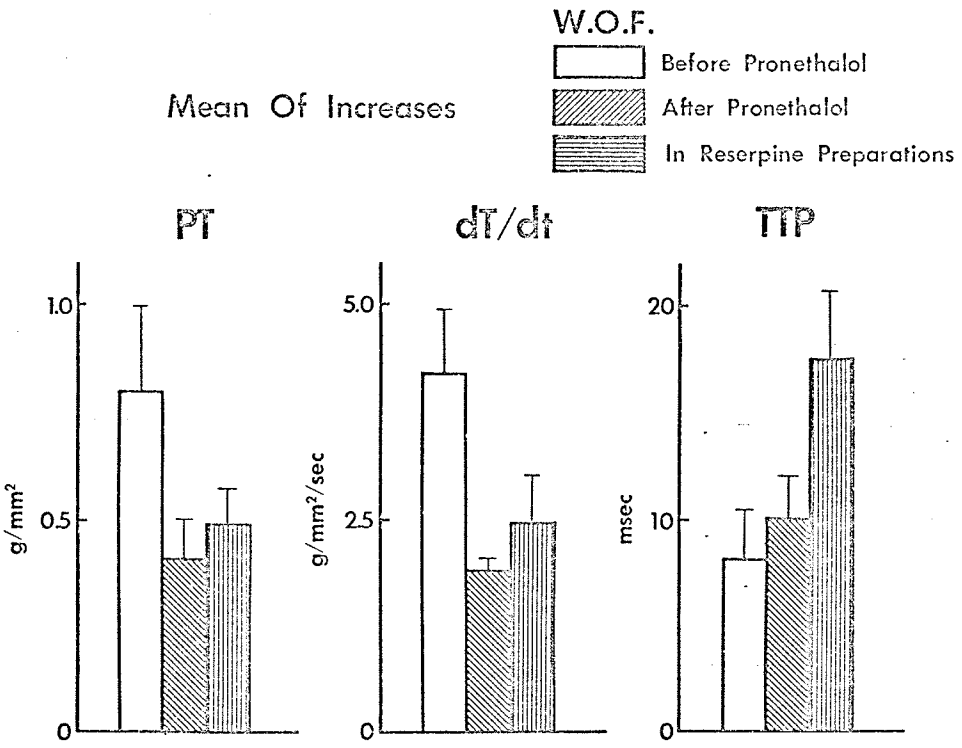

Figure 25. The effects of WOF on the parameters of isometric contraction in cat papillary muscle taken from normal animals and those pretreated with reserpine. Mean ± S.E. of the differences are shown.

greatest in the normal, untreated preparations (open bars), being reduced by 30 - 50% by either β-adrenergic blockade or pretreatment with reserpine (lined bars). The residual effect of WOF after elimination of adrenergic influences was, however, significant ($P < 0.01$). Similar decreases in the effect of WOF on the rate of tension development, dT/dt, were also seen when the adrenergic influences were eliminated. Again, a significant residual effect remained. The time to peak tension, TTP, of normal preparations was prolonged by WOF in these as in the previous experiments but in this small group the increase did not reach statistical significance. Neither pronethalol blockade nor previous treatment with reserpine caused a decrease in the response to WOF in this parameter. The muscles from cats pretreated with reserpine, although showing some increase in TTP when compared to controls in the absence of WOF, were significantly more sensitive to this aspect of its action.

Thus WOF appears to have two effects on contractility in cat papillary muscle; the first is due to release of stored noradrenaline from the nerve endings, the other is a direct effect. The two effects act in the same direction when PT and dT/dt are measured but oppose one another when TTP is considered. Accordingly, removal of the adrenergic influences decreases the changes in the first two variables but increases the change on TTP due to WOF.

The effect of extracting the heart washings with ether were discussed on pages 92 and 98. The lipid increased the strength of contraction in atria by causing the release of noradrenaline from the nerve endings. The extracted WOF still contained a cardiotonic factor which could be precipitated with ammonium sulphate. The inotropic activity of

this material in atria was not affected by pronethalol treatment and was present in preparations taken from kittens pretreated with reserpine. Preliminary studies with papillary muscles had indicated that the cardiotonic response to extracted WOF remained unchanged ($P > 0.8$) after β-adrenergic blockade. In 5 of 11 tests pronethalol slightly increased the positive inotropic effect. It was therefore decided to investigate in considerable detail the effects of 6 samples of extracted WOF on the parameters of isometric contraction of papillary muscle taken from normal cats and those pretreated with reserpine. If all the noradrenaline releasing material is extracted into the ether phase one would expect extracted WOF to have similar effects in both groups of muscles. The results are presented in Figure 26. Only the means of the differences are considered. The S.E. are indicated. The effect of extracted WOF on PT was not changed significantly by pronethalol treatment or by previous treatment with reserpine. However, the change in $\frac{dT}{dt}$ was significantly greater in the normal papillary muscles (see open bar) than in the other two groups. It should be noted that the mean increase in rate of tension development was only approximately 50% of that found with non-extracted WOF (see previous Figure). The TTP was prolonged in all 3 types of preparations. If noradrenaline release was involved in the effect of extracted WOF on $\frac{dT}{dt}$ in normal muscle it did not decrease TTP as would be expected. No significant difference in the mean changes in TTP between the normal group and either treated group was found.

These findings suggest that a large part of the noradrenaline releasing activity is lost from the aqueous phase during ether extraction. The larger increase in $\frac{dT}{dt}$ caused by extracted WOF in normal papillary

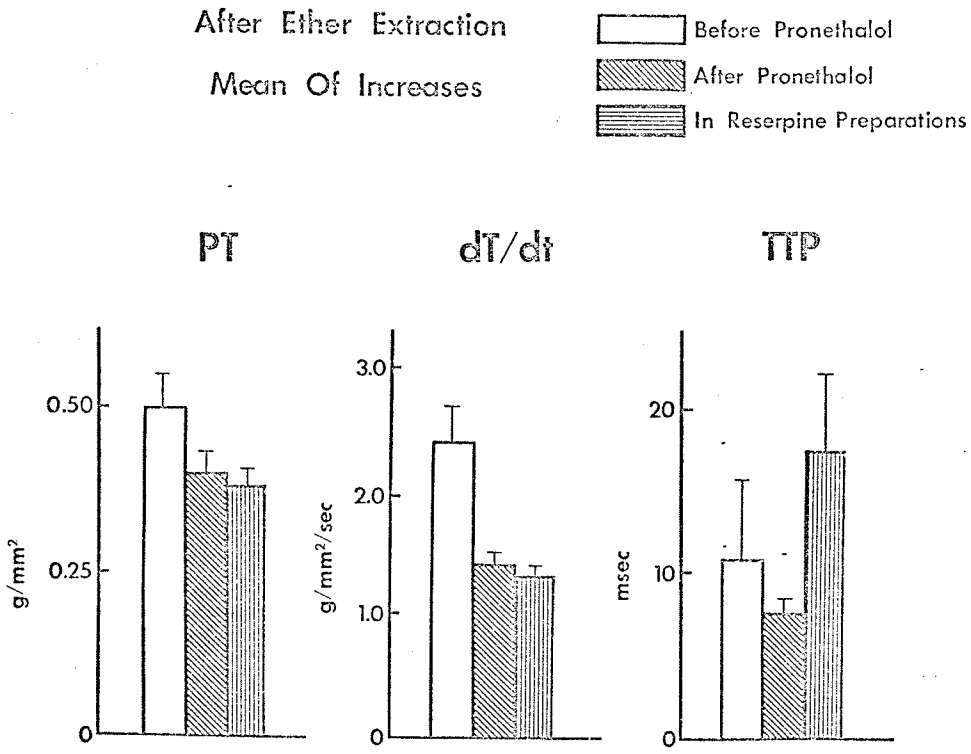

Figure 26. The effects of extracted WOF on the parameters of isometric contraction in cat papillary muscle taken from normal animals and those pretreated with reserpine. Means $\pm$ S.E. of the differences are shown.

muscle suggests, however, that not all of the noradrenaline releasing activity is removed.

No attempt was made to evaluate the effect of the lipid material on these parameters of isometric contraction.

The results from these time-tension index studies clearly indicate that the increased strength of contraction caused by WOF is due to increases in both $\frac{dT}{dt}$ and TTP. If these parameters reflect changes in the intensity and duration of the active state as has been proposed (Buccino et al., 1967; Sonnenblick, 1967; Koch-Weser and Blinks, 1962) then one may conclude that WOF increases PT by increasing both the intensity and the duration of the active state in ventricular muscle. The increase in $\frac{dT}{dt}$ observed with WOF was also seen when the muscles were exposed to either ouabain or adrenaline and probably reflects an increased intensity of the active state. The increase due to WOF persisted after β-adrenergic blockade, was present in muscles taken from animals pretreated with reserpine and occurred with samples of extracted WOF. Under these conditions, the changes in $\frac{dT}{dt}$ were less pronounced but the findings show conclusively that the increase in the rate of tension development is not due only to a noradrenaline releasing activity. Prolongation of TTP by WOF was observed in muscles from normal animals, before and after β-adrenergic blockade and from kittens pretreated with reserpine. The noradrenaline released by WOF did not shorten TTP as is typical of this agent. WOF effects on TTP were more variable than those caused by extracted WOF when tested in the normal muscles. The less predictable effects of WOF were probably due to the presence of different amounts of the noradrenaline releasing activity in samples of WOF from various hearts.

Under the conditions of assay, WOF resembled ouabain in its effects on all the parameters of isometric contraction measured.

THE EFFECTS OF WOF ON THE INTERVAL-STRENGTH RELATIONSHIP

The importance of the interval between contractions of heart muscle in determining the strength of contraction is well documented (Koch-Weser and Blinks, 1963). The term "interval-strength relationship" (ISR) encompasses all direct effects of cardiac rate and rhythm on myocardial contractile performance. ISR includes among other manifestations the influence of frequency on the strength of contraction, the time course of contractions, as well as the transient changes in myocardial contractility ("staircase") which follow sudden changes in frequency. The staircase may be ascending or descending dependent on the interval change and on the species. Several cardioactive substances have been reported to "abolish the staircase" either in the sense that a change in frequency of contraction which had altered the steady-state force of contraction before the substance was applied did not do so afterwards, or that the rate of change to the new steady state was altered. The slope of the decay or rise in contractility following an abrupt decrease or increase in frequency is a sensitive measure of drug activity and has been utilized frequently to study the inotropic activity of cardiac drugs (Hajdu and Szent-Györgyi, 1952; Moran, 1963, 1967).

Nine samples of WOF were used and each was reconstituted in 2.5 ml of Krebs solution (GSH added). The short duration of action of WOF limited the number of frequencies which could be tested. The following protocol was used: A constant volume (0.25 ml) of sample was added to the bath while kitten papillary muscles were stimulated at a frequency

of 1.0 sec^{-1}. At the peak of the response to WOF, the frequency was abruptly decreased to 0.5 sec^{-1}. When contractile force had reached a steady state at this new frequency, the rate was increased to 2.0 sec^{-1}. The frequency was abruptly decreased to 1.0 sec^{-1} after equilibration at 2.0 sec^{-1}.

The upper record in Figure 27 shows that typical responses. The ISR in 15 of 16 control tests was 2.0 sec^{-1} > 1.0 sec^{-1} > 0.5 sec^{-1}. The lower record (Figure 27) shows the effect of WOF on ISR in the same muscle. Here the staircase is reversed at the frequency change from 1.0 sec^{-1} to 0.5 sec^{-1} and is markedly reduced at the other changes in rate.

The rate of decay of contractility following an abrupt reduction in frequency (frequency-reduction test, FRT) is an indication of the action of a drug on contractility (Moran, 1963, 1967). Two FRT's were performed in each muscle, that from 1.0 sec^{-1} to 0.5 sec^{-1} and that from 2.0 sec^{-1} to 1.0 sec^{-1} (see above). Table VIII shows the effect of WOF on FRT. The contractile force of the sixth contraction is shown as a percentage of the first contraction at the new frequency. The effects of WOF on FRT were compared with those of ouabain (3×10^{-7} g/ml) and adrenaline (1×10^{-7} g/ml). All 3 agents significantly reduced the slope of FRT tested between 1.0 sec^{-1} and 0.5 sec^{-1}. WOF and ouabain reversed the slope in 50% of the experiments; no reversal was seen with adrenaline. When the frequency is reduced from 2.0 sec^{-1} to 1.0 sec^{-1} only ouabain affected FRT significantly. However both WOF and adrenaline decreased the changes from those of the control groups. The greater variability in the latter FRT as indicated by the larger standard errors suggest that a

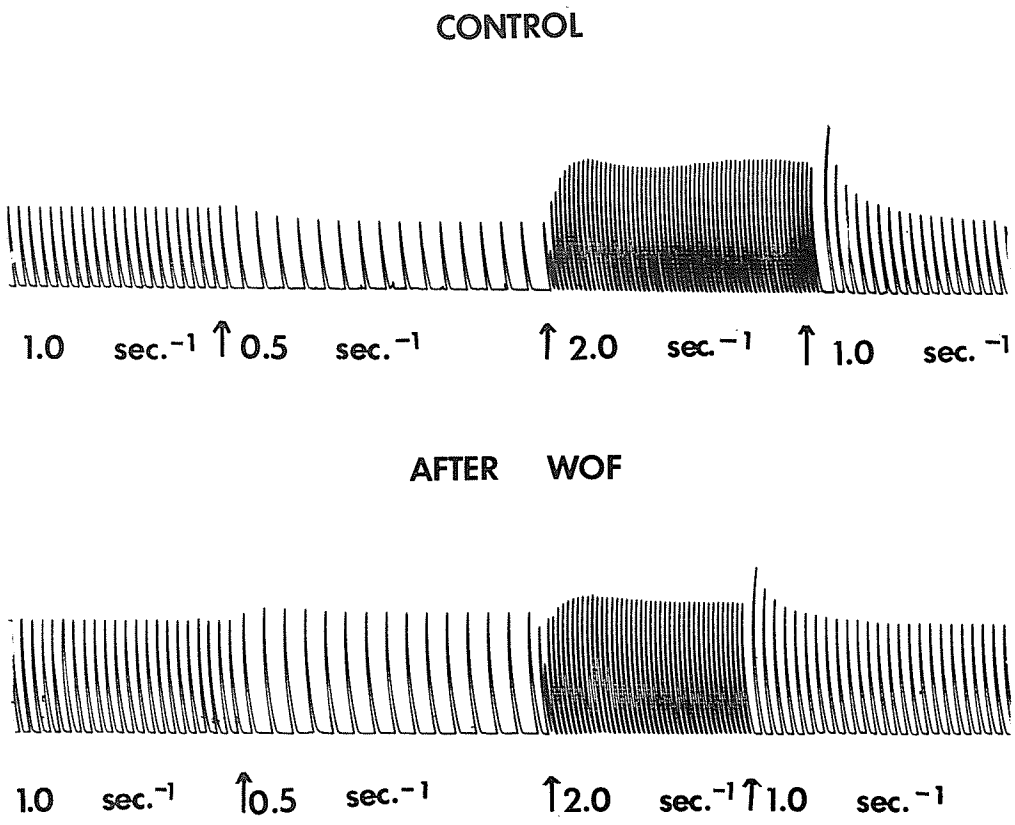

Figure 27. Record of the typical interval-strength relationship (ISR) in kitten papillary muscle with 3 changes in frequency. The sequence of changes in rate was 1.0 sec^{-1} to 0.5 sec^{-1}, 0.5 sec^{-1} to 2.0 sec^{-1} and 2.0 sec^{-1} to 1.0 sec^{-1}. Arrows indicate change in frequency.

The effects of WOF on the ISR in the same muscle is shown in the bottom record. Dose of WOF, 0.25 ml/10 ml.

Table VIII

Effects of WOF, ouabain and adrenaline on the frequency-reduction test (FRT) in cat papillary muscle. Contractile force at beat 6 as per cent of initial beat at new frequency.

Agent	N	1.0 sec^{-1} to 0.5 sec^{-1}		2.0 sec^{-1} to 1.0 sec^{-1}	
		Control	Treated	Control	Treated
WOF (0.25 ml/10 ml)	12	87.7 ± 1.4	95.9 ± 2.8*	78.4 ± 2.5	82.9 ± 5.9
Ouabain (3×10^{-7} g/ml)	20	85.2 ± 1.8	104.2 ± 3.4*	74.9 ± 8.7	92.7 ± 3.3*
Adrenaline (1×10^{-7} g/ml)	4	85.5 ± 1.8	95.0 ± 2.4**	76.8 ± 6.0	86.3 ± 8.1
Papillary Muscle from Kittens Pretreated with Reserpine					
WOF (0.50 ml/10 ml)	7	76.1 ± 4.6	87.1 ± 3.5*	69.0 ± 3.4	80.8 ± 2.9*

* $P < 0.01$

** $P < 0.05$

FRT with changes in frequency from 1.0 sec^{-1} to 0.5 sec^{-1} may be a more reliable measure of drug activity on contractile force. The effect of WOF on FRT is not due only to noradrenaline release because the same effect was seen in papillary muscle taken from kittens pretreated with reserpine. No additional information concerning the mechanism of action of WOF was obtained from these studies since FRT did not distinguish between the effects of ouabain, adrenaline and WOF.

Another way of presenting these results is to show the effects of the agents on steady state contractile force at different frequencies. The changes observed with WOF at the 3 rates of contraction are clearly seen in the record of a typical experiment shown in Figure 27 (bottom record). In this experiment, the per cent increases in contractile force at different frequencies were 37, 75 and 3% at ratesof stimulation of 1.0 sec^{-1}, 0.5 sec^{-1} and 2.0 sec^{-1} respectively. Steady state contractile force was considered, therefore, only at the 2 lower frequencies since WOF had minimal effects at the highest rate.

The changes in the steady state contractile force caused by WOF were compared with those of ouabain (3×10^{-7} g/ml), adrenaline (1×10^{-7} g/ml) and tyramine (1×10^{-6} g/ml) at the 2 lower rates of stimulation. The results are presented in Figure 28. The mean responses are expressed as per cent increase in the contractile force from the control. The open bars indicate the responses obtained at the frequency of 0.5 sec^{-1} and the hatched bars those at 1.0 sec^{-1}. The S.E. are indicated. In 11 of 12 tests, ouabain showed the greater effect at the lower frequency. This agrees with the literature. WOF resembled ouabain. With all 14 additions of WOF, the response was greater at 0.5 sec^{-1} than that observed at 1.0 sec^{-1}.

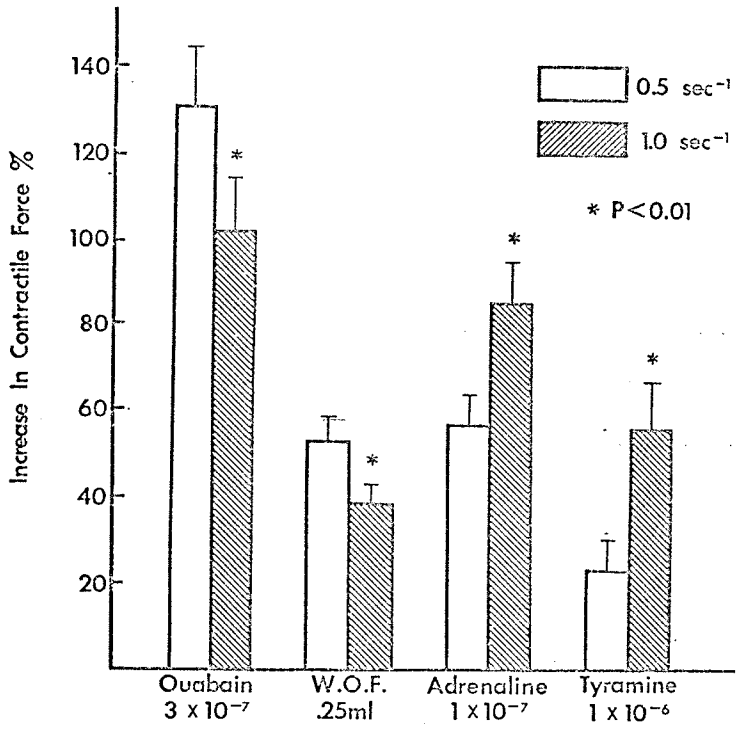

Figure 28. The effects of four cardioactive agents on the steady state contractile force of kitten papillary muscles stimulated at two frequencies. Mean ± S.E. are indicated. Increases in contractile force expressed as per cent.

Adrenaline and tyramine showed the opposite in that the responses were greater at the higher of the two frequencies. With adrenaline, 9 of 10 responses were greater at 1.0 sec^{-1} than those at 0.5 sec^{-1}. In the case of tyramine, all 8 responses were larger at the higher frequency of stimulation. The responses obtained at the 2 frequencies were significantly different ($P < 0.01$) with the 4 cardiotonic agents.

When a larger volume (0.5 ml) of the same samples of WOF was tested in the same preparations, all 6 tests resulted in greater responses at the higher frequency. The mean positive inotropic response was 100.8 ± 8.3 and $79.2 \pm 6.2\%$ at rates of 1.0 sec^{-1} and 0.5 sec^{-1} respectively. The difference was significant ($P < 0.01$).

The observed change in the frequency at which the larger inotropic response occurred with high concentrations of WOF might be due to an increased release of noradrenaline from the nerve endings. To test this hypothesis, an equal volume (0.5 ml) of the same samples of WOF was tested in muscles taken from animals pretreated with reserpine. These preparations showed the maximum response at the lower frequency. Here, the mean positive inotropic effect was 29.9 ± 2.1 and $44.6 \pm 6.8\%$ at frequencies of 1.0 sec^{-1} and 0.5 sec^{-1} respectively. The difference was significant ($P < 0.005$). These findings indicate that more noradrenaline is released by the higher dose of WOF and that the frequency for the greater response is now the same as that of adrenaline and tyramine (see Figure 28). When the preparations are depleted of their catecholamine stores, the larger dose of WOF resulted in a greater response at the lower frequency.

These results clearly indicate that the maximum effect of WOF

on contractility occurred at the lower frequency of stimulation whether low doses were tested in papillary muscles from normal kittens or high doses were tested in preparations taken from animals pretreated with reserpine. The responses under both these conditions resemble those of ouabain. Larger doses of WOF when tested in normal muscles showed the maximum response at the higher frequency, typical of that seen with adrenaline and tyramine. This shift in the greater effect to the higher frequency was due to an increased release of noradrenaline.

DISCUSSION

DISCUSSION

The inotropic effect of WOF was characterized by time-tension index studies on unfailed cat papillary muscles. When the effects of WOF on peak isometric tension (PT), the maximum rate of tension development $\left(\frac{dT}{dt}\right)$ and on the time to peak tension (TTP) were compared with those of ouabain and adrenaline, the agents were found to change the parameters of contraction under investigation. The concentration of the latter two drugs (5×10^{-7} g/ml) was chosen to give effects on PT similar to those of WOF. All three increased $\frac{dT}{dt}$ to an equivalent degree.

The significance of these findings are best appreciated when they are related to the basic mechanics of muscular contraction. The peak tension that can be generated in an isometric contraction depends upon two factors: the rate with which tension is developed $\left(\frac{dT}{dt}\right)$ and the time provided for this tension to be generated (TTP). Muscle contraction reflects the interaction of an active contractile element and a passive elastic component while rate of tension development depends on the properties of both of these components. The contractile element is characterized by its force-velocity relation, and its speed and force of contraction are the mechanical reflections of the intensity of the active state. Since the series' elastic component of heart muscle does not vary with inctropic interventions, increments in $\frac{dT}{dt}$ will reflect an increase in the intensity of the active state (Sonnenblick, Morrow and Williams, 1967).

Adrenaline and ouabain have previously been reported to increase $\frac{dT}{dt}$ (Buccino *et al.*, 1967; Koch-Weser and Blinks, 1962). The results presented here show that WOF also caused increments in $\frac{dT}{dt}$. All three

agents increased the intensity of the active state.

However, the PT also depends on TTP which reflects the duration of the active state (Sonnenblick, 1965, 1967). Adrenaline in agreement with the literature, shortened TTP. Ouabain under the conditions of this assay prolonged TTP. The effects of ouabain on TTP differed from those reported by Buccino et al. (1967) who found that ouabain significantly decreased TTP. However their conditions of assay varied considerably from those employed here. They used a temperature of 30°C and a frequency of stimulation of 0.2 sec^{-1}. WOF, like ouabain, significantly prolonged TTP. If these parameters reflect changes in the intensity and duration of the active state as has been proposed then one may conclude that WOF increases PT by increasing both the intensity and duration of the active state in ventricular muscle. It is possible that both WOF and ouabain would not affect TTP if the frequency of stimulation and the temperature of the bath were lower. Frequency and temperature are physical factors known to affect the parameters of isometric contraction (Buccino et al., 1967).

The effect of WOF on PT and on $\frac{dT}{dt}$ were found to be decreased by approximately 50% when the adrenergic influences were eliminated. The effect of WOF on TTP was unaffected by pronethalol treatment and was clearly potentiated in muscles from animals pretreated with reserpine.

These results indicate that WOF has as part of its mechanism of action, an effect of releasing noradrenaline in tissues which is more marked in cat papillary muscle than had previously been observed in left atria. However, this indirect action does not account for all the cardiotonic activity. As will be discussed presently, most but not all of this

noradrenaline releasing activity is removed by extraction with ether.

The results from the comparison of the effects of WOF, adrenaline, ouabain and tyramine on steady state contractile force at two frequencies of stimulation (0.5 sec^{-1} and 1.0 sec^{-1}) showed that WOF, like ouabain, had a relatively greater positive inotropic effect at the slower frequency (Koch-Weser and Blinks, 1963). The effect of all four substances on the slope of the frequency reduction test (Moran, 1967) was the same, indicating that change in "staircase" is not an adequate description of the effects of inotropic drugs.

The ether extraction experiments (pH 7) have shown that the ether layer contained a yellowish material which was insoluble in aqueous solution but partially soluble in acetone. The investigation of the action of acetone on heart muscle showed that the solvent has a dual action. With kitten left atria a stimulant effect was found at low concentrations (1 - 5 μl/ml) and a depressant effect at higher ones. The stimulant effect was blocked completely or reversed by pronethalol and was absent in atria taken from animals pretreated with reserpine. The depressant effect was potentiated by these treatments. From these studies, it is concluded that acetone has a direct depressant effect on contractility and an opposing stimulant action due to the release of noradrenaline. The effects of acetone on left atria from guinea-pigs could be interpreted similarly. However, low concentrations caused depression of contractility which was reversed as the highest concentration tested (20 μl/ml) was reached.

The effect of the lipid was compared to the effect of its appropriate acetone blank. The results clearly showed that the positive

inotropic effect of the lipid was due to the release of noradrenaline since its effect was blocked completely by pronethalol and was absent in atria taken from animals pretreated with reserpine. The action of the lipid would account for the partial blockade of the effects of WOF by pronethalol as discussed previously.

The aqueous phase after extraction with ether still contained a cardioactive substance which could be precipitated in the same manner as WOF. Extracted WOF had about 50% of the cardioactive potency of WOF. The effect of extracted WOF on PT was unaffected by pronethalol or by pretreatment with reserpine. The changes in dT/dt caused by extracted WOF were significantly blocked by pronethalol suggesting that not all the noradrenaline releasing activity was extracted with ether. Extracted WOF increased TTP, an action which was increased by pronethalol or reserpine pretreatment.

These results indicate that following ether extraction two cardioactive substances are present and that the mechanism of action of each is different. The lipid acts by releasing noradrenaline from the nerve endings and the "protein" apparently has a direct effect on contractility. The results have indicated an adrenergic component of the action of WOF but have shown clearly that this is not the only mechanism of its positive inotropic effect.

The data from the study of the insulin-like effects of WOF in rat hemidiaphragm indicate clearly that this material increases the *in vitro* transport of 3-MG, a non-metabolized sugar. Although the biological activity of WOF was shown to be distinct from insulin, it is of interest to consider the similarities between the two agents. The

present data show that, like insulin, WOF enhances the transport of 3-MG; and that phloridzin blocks the increased uptake. Bihler and Dresel (1966) have shown other insulin-like effects on transport including increased uptake of D-xylose and alpha-aminoisobutyric acid. WOF also increased the incorporation of ^{14}C-glucose into glycogen; this could be the consequence of either the increased uptake or an insulin-like effect favoring the glycogen-storage pathway. To distinguish between these possibilities an independent measure of total glucose uptake is required. The 'intact' rat hemidiaphragm is unsuitable for such studies since the extraneous tissue attached to the ribs also consumes glucose. Additional experiments with different tissue preparations are needed to clear up this point. A different approach to the same problem would be to investigate whether WOF stimulated the activity of glycogen synthetase. If an increase in the activity of this enzyme was found without a concurrent increase in phosphorylase activity one might then attribute an intracellular influence to WOF and not merely an effect on transport. Insulin administration promotes protein synthesis by augmenting incorporation of amino acids into proteins, independent of the transport effects, perhaps WOF would exert a similar protein anabolic influence.

The release of a factor from contracting muscle which stimulated sugar transport has been described in vivo (Goldstein, 1953, 1965) and in vitro for skeletal, heart and smooth muscle (Havivi and Wertheimer, 1962, 1964). Such a humoral factor could have a physiological role in increasing transport of glucose in response to increased metabolic demands during work. Goldstein proposed that the labile, circulating hypoglycemic factor was readily destroyed or neutralized by some pre-existing plasma

inhibitor. Accordingly, he has recently (1966) demonstrated that severe exercise in the near absence of normal plasma proteins resulted in plasma which exhibited marked sugar transport activity. It is probable that the ability to demonstrate a stable factor in vitro in the present studies was due to the absence of normal plasma constituents.

WOF resembles MAF, the "muscular activity factor" of Havivi and Wertheimer (1962, 1964) in many respects. Both factors are released from contracting muscle, increase the membrane transport of sugars and amino acids yet are distinct from insulin. The two factors are precipitated by ammonium sulphate and perchloric acid, and the biological activity is thermolabile. However certain differences between WOF and MAF exist. MAF was reported not to increase glycogen content of the hemidiaphragms, the effects on transport of sugars were not blocked with phloridzin (Wertheimer and Beck, 1963) and the transport stimulation was dependent on the protein concentration. WOF, on the other hand, increased the incorporation of ^{14}C-glucose into glycogen, the effects on transport of 3-MG were blocked by phloridzin and the stimulation of transport of sugars was not correlated with the protein concentration. In fact high concentrations of protein in these studies resulted in a lessening of the insulin-like effect of WOF and these results resemble more the resting media results of Havivi and Wertheimer than those of MAF. As yet the effect of WOF on O_2 consumption and incorporation of ^{14}C-labelled amino acids into muscle protein has not been studied. MAF has been reported to increase both (Wertheimer and Beck, 1963).

The data shows that there was no correlation between the inotropic and the sugar transport stimulating effects of WOF. This

conclusion is supported by the results of Frederickson, Bihler and Dresel (submitted) who showed that MAF is released in vitro only from contracting diaphragm whereas an inotropic material could be demonstrated in the media of resting muscle. Both activities were also present in extracted WOF and the lipid, suggesting the existence of 2 factors. The presence of activity in both layers suggests that an intact lipoprotein complex is not required for activity. However, in these few experiments the possibility is not excluded that the effect of the ether extract is due to a non-specific stimulation by the lipids since the total activity of WOF was not decreased during the process of solvent extraction. It is also possible that acetone which had no effect on transport alone may have changed the sensitivity of the muscle to the active component in the lipid. Acetone in similar concentrations to those used here has been reported to potentiate the effects of acetylcholine in amphibian skeletal muscle (Chang and Lin, 1949; Zeleny and Kozak, 1958) and at higher doses to change the responses of several mammalian tissues to biogenic amines (Ehrenpreis, Hazra and Bigo-Gullino, 1968). These questions will have to remain unanswered until a more satisfactory method of reconstituting the lipid is found.

The limited data with the lipid suggests that a positive correlation exists between the two biological activities. Confirmation of this dependence would require additional experiments. Ether extraction no doubt removes several lipids, so whether or not the same lipid possesses both activities or two lipids are involved has not been determined. Until the lipids are separated by thin layer chromatography and the pharmacologically active lipids identified this question cannot be answered.

WOF was virtually inactive in all the smooth muscle preparations

in which the material was tested. This lack of activity distinguishes WOF from the component in plasma reported by Bohr and Johannson (1966) and from the cardioactive polypeptide, Kinekard, isolated from plasma by Nayler and her co-workers. Although the activity of Kinekard in gut, vascular and uterine muscle resembled that of adrenaline, the material was shown not to act on smooth muscle by liberating endogenous stores of noradrenaline (Dorevitch, Nayler and Lowe, 1967).

The data available to date have failed to provide clear-cut information on the chemical nature of the active material. It cannot be taken for granted that because WOF is precipitated with ammonium sulphate or perchloric acid that the active material is a protein. Such findings are equally compatible with the substance being a lipoprotein or even a coprecipitated lipid. It is highly unlikely that the material precipitated as WOF is a single substance. This becomes obvious when the concentration of protein in the bath is compared to the biological activities. A positive correlation between the protein concentration and the cardiotonic activity was found. However, no correlation was found with sugar transport activity. Increased transport tended to occur until concentrations of 20 μg/ml were reached and then transport dropped toward control levels with further increases in protein concentration. These findings suggest that an inhibitor of the insulin-like effect might be present at high doses of WOF.

Very little evidence was found in the present studies to support the suggestion of Gabel (1965) that WOF might be a lipoprotein. The centrifugation studies suggest that a cardioactive material might be released from the particulate into the soluble fraction. The results from the ultrasonic treatment of the washings are inconclusive because of the conflicting reports in the literature regarding the effects of disintegration

with ultrasound on lipoprotein complexes. Dependent on the purity of the preparations, lipoproteins may be dissociated or lipids complexed to proteins by this treatment (Napier and Olson, 1965; Searcy and Bergquist, 1965). Following ether extraction of the washings, the solvent layer contained a yellow-brown oily material which was insoluble in water. This finding and the fact that most lipids are not sufficiently polar to circulate freely in aqueous solution suggests that the lipid was solubilized in the washings from the gas-perfused heart by an interaction with protein. At the present time it is unknown whether WOF is a lipoprotein or a lipid associated with a protein.

Adjustment of the pH of the washings to pH 2 or pH 9 markedly decreased the extraction of the lipid. The decreased activity in the aqueous phase following partition at extremes of pH was probably due to structural changes in the proteins. Extraction of washings with iso-amyl alcohol, an agent reported by Burnstein (1967) to strip 95% of the lipids from serum lipoproteins without denaturing the protein moiety, proved ineffective. In these experiments, no decrease in the cardiotonic activity in the aqueous phase was found. No activity was present in the alcohol extract. Some variation in the amino acid composition of soluble and membranous lipoproteins have been reported (Hatch and Bruce, 1968) but it is unlikely that this could account for the observed differences. A more plausible explanation would be that the dissociation of the lipid-protein complex was incomplete since the experiments reported here were carried out in the cold and solvent extraction is in general slower at lower temperatures.

At present nothing is known concerning the chemical nature of

the lipid. In future studies with this material priority should be given to the chemical identification of the lipid. The material extracted by ether from the heart washings is probably a mixture of several lipids. The usual analytical methods of lipid chemistry could be used, notably thin layer chromatography. It should be possible to separate and identify, at least pharmacologically, the active lipid from the inactive constituents. If the active material was found to resemble known materials in chromatographic behavior these materials could be tested for biological activity. Recently Govier and Boadle (1967), have shown that lysolecithin increased the strength of contraction of isolated heart muscle by releasing noradrenaline.

It would be of interest to recombine the lipid with a known protein by the method of Stein and Stein (1965) and to determine whether this changes the mechanism of the inotropic response from noradrenaline release back to a mechanism not involving this mediator. It might be feasible to increase the quantity of the active lipid removed from the gas-perfused hearts by adding low concentrations of albumin to the Krebs solution used for intermittent perfusion. Stein and Stein (1965) increased the removal of lecithin from rat hearts in this manner. Although the identification of the lipid may be investigated with very small quantities of material, study of the protein component will require accumulation of larger amounts of WOF. Perfusion of skeletal muscle has not proved to be a source of this material (McNeill and Dresel, unpublished). Preliminary experiments with gas-perfused dog hearts have indicated that a substance like WOF is released so perhaps this preparation will provide a source of sufficient quantities of WOF to allow

investigation of the protein component. Should the two components be isolated and identified it might be possible to recombine the lipid with the protein remaining in the aqueous phase after extraction and thus finally determine whether WOF is in fact a lipoprotein. These studies might also serve to separate the sugar transport from the cardiotonic activity.

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