STUDIES ON THE ISOLATED GAS-PERFUSED CAT HEART

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Lloyd P. Gabel

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ABSTRACT

Isolated cat hearts were perfused with warm, moist 95% oxygen-5% carbon dioxide by a modification of the Langendorff technique. Other hearts, perfused with substrate-free Krebs-Henseleit solution in a recirculating system, served as controls.

Gas-perfused hearts beat more forcibly, produced more work, and failed at a slower rate than did the controls under conditions of constant temperature, perfusion pressure and heart rate.

Both preparations were rapidly depleted of their carbohydrate stores and then utilized endogenous lipids, primarily phospholipids, as their source of energy during prolonged perfusion. The rate of phospholipid utilization was found to be a function both of the number of beats which occurred during the perfusion period and the isometric resting tension. This utilization rate was independent, however, of the heart rate, isometric developed tension, duration of perfusion, and the extent of failure.

Gas-perfused hearts, in contrast to their controls, showed a high incidence of spontaneous contractile alternation. This was sometimes accompanied by electrical alternation. A lower threshold to the arrhythmia-inducing effects of raised perfusion pressure was also observed during gas perfusion. Data obtained by the use of a procedure which presumably extracts electrolytes from the extracellular space during gas-perfusion indicated that the myocardial cells lost potassium and gained sodium at the expense of the extracellular space. These ionic shifts reached equilibrium within three hours of the initiation of gas perfusion. The extent of these shifts ~~were~~ was not significantly influenced by prior treatment of the hearts with "therapeutic" concentrations of ouabain.

Reactivity to injections of histamine, acetylcholine, adrenaline and isoproterenol was similar in liquid- and gas-perfused hearts. The actions of single injections of agents which were not susceptible to oxidation or enzymatic degradation (such as cocaine and pronethalol) remained constant in gas-perfused hearts unless the drugs were removed by short periods of liquid perfusion.

The greater contractile force and delayed onset of spontaneous failure observed in the gas-perfused heart could not be explained on the basis of any physiological or biochemical differences when comparisons were made to the relatively short-lived liquid-perfused preparation. A hypothesis was forwarded, based upon an early suggestion by A.J. Clark, that liquid-perfused hearts fail because their perfusate removes a factor which is necessary for the maintainance of optimum contractile force. It was reasoned that if this were so, the factor would accumulate in the extracellular space of gas-perfused hearts and should be obtainable in a concentrated form by perfusion with small volumes of liquid perfusate.

It was found that intermittent perfusion with small volumes of liquid (5.0 ml) at thirty-minute intervals caused rapid failure of gas-perfused hearts. These "washings" were recovered quantitatively and were found to exert a slight positive inotropic action on failed, isolated atria. Treatment of the washings with ammonium sulphate caused the precipitation of a material which was strongly inotropic to the test preparation. Pre-treatment of gas-perfused hearts with relatively low concentrations of ouabain (1×10^{-10} to 1×10^{-9} g/ml) decreased in a dose-related fashion both the quantity and specific activity of the recovered material. That this effect was related to the inotropic activity of the glycoside was demonstrated by the difference in potency

between digitoxin and dihydrodigitoxin. Relating these results to liquid-perfused hearts, it is suggested that spontaneous failure occurs in these preparations because of perfusate removal of an inotropic factor, and that the digitalis glycosides act to correct this by interfering with this removal.

This thesis is dedicated to my family, my wife Frances, Jonathan and David.

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"A systematic study of the changes in the energy transformation of the heart occurring in failure is feasible only in the isolated organ where the variables of circulatory dynamics cannot only be measured but where they can be controlled". A. Wollenberger, 1949

The literature concerning the physiology, pharmacology and biochemistry of the myocardium may be divided into two main classes: One in which the heart is studied in situ and the other in which it or some part is removed and maintained in vitro. In the former instance, the heart may be considered to be performing its usual function of perfusing itself and the body, while it is being influenced simultaneously by autonomic tone, arterial and venous pressure, and blood-borne factors such as hormones, metabolic substrates and end-products. Studies using intact preparations are presumed to yield information about the heart under conditions similar to those encountered in the normal life of the organism. By the same token, the very fact that the heart is operating within its usual environment means that the results of these experiments are influenced by a host of factors which may vary not only between laboratories but between individual animals and within the same animal. It is not uncommon to find reports in the literature with diametrically opposed results concerning a particular aspect of cardiac function. For example, Murrow (1) reported that the inotropic effect of ouabain is not affected by reserpine, whereas Tanz (2) concluded that reserpine prevented the ouabain - induced augmentation of contractility. These studies differed in choice of preparation, in that Murrow's data was obtained from in vivo experiments whereas Tanz studied the isolated papillary muscle.

Experiments with isolated tissues permit close control of those physical and chemical factors which influence the outcome of the experiment. By the selection of the proper saline medium or perfusion system,

one is able to maintain a more stable environment in regard to such factors as pH, pO_2, pCO_2, temperature, electrolyte concentration, osmolarity and drug or substrate concentrations. However, a review of the literature will reveal that even with these supposed advantages, reports of conflicting results still appear. For example, Kreisberg and Williamson (3), found that ouabain increased the uptake and utilization of glucose by perfused rat hearts, whereas Zacharia (4) found the uptake and utilization in the same preparation was unchanged.

The ability to control the supportive environment has fostered the proliferation of so many possible sets of experimental conditions that direct comparison between reports is again often impossible. One may cite the review by Lockwood (5) in which over 100 variations of Ringer's solution are described. The study in this report, unfortunately, offers no remedy to this situation but instead contributes further confusion to the field by suggesting a new technique for the maintainance of isolated hearts.

The Isolated Perfused Heart:

Langendorff in 1895 first described a preparation in which an isolated beating frog heart was maintained for prolonged periods by perfusing the tissue with either oxygenated serum or saline solution (6). Contractile force was measured either with a lever connected by a string to the unanchored portion of the heart or by determining the pressure developed within the ventricular chamber. Langendorff also showed that this preparation could be applied to mammalian hearts and determined the effects of temperature on the strength of contraction and the heart rate (7,8). A.J. Clark and co-workers, between 1913 and 1937, reported a series of studies in which the Langendorff preparation was used to examine the effects of ions, lipids, narcotics and metabolic poisons on the contractility and oxygen consumption of the frog heart (9-14). These studies culminated in the publication in 1938 of a monograph describing their work regarding the metabolism of the frog heart (15). Beside their monumental contributions to the understanding of cardiac metabolism, Clark and his co-workers pioneered the use of a perfusion system in which the perfusate was recycled after passage through the heart. Thus only a relatively small volume of perfusate was necessary and oxygen and substrate utilization could be determined by sampling the perfusate before and after passage through the heart.

The Langendorff preparation has since served as an extremely useful tool in studies concerned with the effect of various physical and chemical changes in the perfusate upon contractility, heart rate and metabolism. It has been applied to the study of avian, reptilian, amphibian and mammalian hearts. The isolated heart can be made to perform external work by pumping perfusate against a resistance (16). In this modification the heart perfuses

its vasculature. Work can also be determined by the lifting of a weight attached to the apex. The competence of the heart may be determined by imposing standard perfusate pressure loads. Cardiac efficiency can be measured by relating the power output to the minute oxygen consumption (efficiency = Power/O_2 consumption).

Perhaps the most outstanding problem to which the Langendorff preparation has been applied is the study of cardiac failure. Following cannulation and perfusion of the heart, the strength of contraction, although initially great, diminishes gradually until it reaches a level too low to be useful. The etiology and prevention of this gradual decline has been the subject of many reports. The term "failure" is generally applied to the gradual decline in isometric or isotonic contractility of hypodynamic preparations or to a decreased ability to perform external work under conditions of contant heart rate, imposed load and outflow resistance.

The removal or isolation of the heart from the animal appears to guarantee its eventual failure. The nature and composition of the perfusate is the prime determinant of the time for which an isolated heart remains essentially stable. The use of blood, which logically represents the ideal perfusate, simply delays the onset of spontaneous failure. The isolated heart perfused with blood from a donor animal in a recirculating system (17), and to a lesser extent the dog or rat heart-lung preparation (18,19), all demonstrate a long stable period of contractility before failure ensues.

When saline solutions are to be used for perfusion, the regulation of factors such as ionic and osmolar content, temperature, pH, oxygen and substrate content become matters of prime concern. The perfusate used must contain oxygen and organic substrates in quantities which provide a normal supply at the experimental coronary flow rates which are encountered.

The flow rate must also be sufficient to remove metabolic end-products whose accumulation is considered to be detrimental to myocardial performance.

Cardiac metabolism and contractile mechanism are a means of converting potential chemical energy into useful mechanical work. These functions may be subdivided into three general compartments (20): Uptake and/or storage of substrates such as oxygen, glucose and lipid for eventual use; conversion or conservation of substrate potential energy into the readily available potential chemical energy of high energy phosphate bonds in adenosine triphosphate (ATP) and creatine phosphate (CP); (21) and conversion of the energy of these phosphate bonds into mechanical power. These compartments, which may be delineated in terms of cellular structures such as membrane, mitochondrion, or myofibril, are interdependent. Alterations in each may be demonstrated during the onset and progression of heart failure. External manipulation to cause modification of the function of any one compartment can be shown to affect all of them. By the same token, any spontaneous alteration in the function of one compartment due to unknown factors which may be causative to heart failure should be reflected by measurable changes in the other compartments.

Oxygen and the Isolated Heart

The heart is incapable of contracting a significant oxygen debt (22) in spite of a small capacity for oxygen storage in the form of myoglobin (23). Thus the supply of oxygen can be a major determinant of the longevity and work production of an isolated preparation. Saline solutions equilibrated with oxygen at atmospheric pressure contain approximately 2 ml per 100 ml perfusate (24). The oxygen requirement for hypodynamic dog or cat hearts is 3-5 ml/100 g wet wt./min (18,25,26,27).

Since the heart is capable of extracting almost all the oxygen content of the perfusate (28) it is obvious that the minimum perfusate flow under these conditions must be 150-250 ml/100 g wet wt./min. This is generally equalled or surpassed at perfusion pressures of 30-60 mm Hg, (29,30). The isolated rat heart, which has an oxygen consumption approximately 3-4 times that of the dog or cat heart (27), has been shown to receive more than adequate supplies of oxygen when perfused with saline solutions as judged by metabolic criteria such as A-V_{O_2} differences, lactate production and glycogenolysis (27,31,32,33). Efforts to increase oxygen carriage of saline perfusate by the addition of haemoglobin (34) or erythrocytes (16, 35,36) have therefore been superfluous. These improvements contribute neither to the contractility nor to the longevity of the preparations. Furthermore, Douglas and co-workers (35,37) have shown that the inotropic effects of drugs which increase the oxygen consumption are similar when either plain or enriched media is used. They also noted that catecholamine-induced increases in oxygen consumption were similar in both instances but, as would be expected, drug induced increases in coronary flow were greater in hearts perfused with unenriched medium. Blinks and Koch-Weser (24) reviewed the literature pertaining to this question, and decided that although saline perfusates could support hypodynamic preparations, the oxygen supply would be inadequate were external work loads to be imposed. The oxygen requirement of working hearts is at least 2-3 times that of hypodynamic preparations (25,38,39) and it is in situations in which work is produced that the addition of haemoglobin or erythrocytes to the perfusate becomes useful. The type of work performed also bears directly upon the oxygen consumption, as work done against a large pressure or load increases the oxygen consumption more than does work in which large volumes of per-

fusate are pumped against a relatively small pressure (40).

The rapid onset of spontaneous failure in isolated hearts perfused with saline solutions is accompanied by a decrease in oxygen consumption (10,11,41,42,43). Since contractile force and oxygen consumption decline in parallel, no change in efficiency occurs. Wollenberger (44) suggested that the oxygen supply might be the limiting factor in this instance. However, the recent studies of Zacharia (4) and Fisher and Williamson (27) show that increasing the coronary flow of isolated rat hearts by the addition of pentaerythritol tetranitrate prevents the fall in oxygen consumption, but not the decline in contractility. Spontaneous failure is delayed in hearts perfused with blood or artificial media which contain plasma or serum (45-51) and is then characterized by stable oxygen consumption during failure (10,11,52,53). Thus, failure can be accompanied by either decreased or unaltered efficiency, when judged solely by contractile strength and oxygen consumption.

During hypoxic or anoxic periods, contractile force diminishes, myocardial lactate (but not pyruvate) ion accumulates and the intra-cellular contents of ATP and CP fall precipitously (54-57). Myocardial glycogen stores are depleted rapidly during hypoxia and recovery of these stores is not always observed upon re-oxygenation (56,58). The restoration of contractile force following return to aerobic conditions depends to a large extent upon the species examined and the presence or absence of glucose. Winbury (59) has shown that although glucose does not alter the loss of contractility in anoxic cat papillary muscle, its presence during anoxia allows recovery of contractility following restitution of the aerobic state. It has been shown that cat ventricular muscle will contract during anoxia when stimulated by ouabain (60), epinephrine, or calcium (61) only

if glucose is present. Glucose can support the beating of the anoxic frog heart, but not that of the anoxic rabbit heart (62). Winbury's observations (59) would indicate that during anoxia, glucose permitted the maintenance of cellular integrity, if not contractile activity, of the cat papillary muscle. MacLeod and Daniel (63) confirmed Winbury's observation, and reported that large concentrations of glucose protected against the effects of anoxia on the membrane potential. Bing (64) and Bing and Michal (65) observed on increase in lactate production during anoxia, and suggested that anaerobic metabolism occurred. Certainly the beating of the anoxic frog heart would further support this idea, at least for amphibian hearts. Winbury (28) in reviewing the literature concerning anaerobic metabolism concluded that anaerobic glycolysis was an important source of energy during anoxia.

It is of interest to note that the Langendorff preparation is often used in studies concerning the pharmacological effects of drugs on the tone of the coronary vasculature. Commonly, fibrillation is induced in order to negate the effect of rhythmic contractility on coronary flow (66). The oxygen consumption during fibrillation is often 3-5 times that observed when normal work loads are imposed (38,67). The fibrillating Langendorff preparation may therefore be hypoxic. Valid conclusions cannot be made concerning the <u>in vivo</u> activity of a compound on the basis of this type of experimental data. Conclusions drawn from studies in which isolated hearts are arrested by increases in perfusate potassium content may also be questionable, because Whalen and Weddle (68) have shown that increased extra-cellular potassium content causes increased oxygen consumption in isolated rat ventricle.

Fawaz and co-workers (55,69) have reported that the ability

of the heart-lung preparation to oxygenate blood diminishes with time. Reports concerning spontaneous failure in this preparation (44) attended by decreased myocardial lactate utilization and increased glucose uptake may, in fact, be complicated by an unnoticed state of hypoxia.

Organic Substrates and the Isolated Heart

The heart derives its energy from the metabolism of exogenous organic materials. The hypodynamic and the working perfused hearts have been used in many studies of uptake and utilization of substrates. The isolated heart can utilize a host of organic substrates including carbohydrates, ketones, lactic and pyruvic acids, lipids and amino acids when these are present in the perfusate either singly or in combination (4,30, 32,58,70-78). The relative proportions to which each is utilized are dependent not only upon their concentration and molecular structure (76,79) but also on heart rate, cardiac work and temperature (5,58,73,75,78,80, for recent review, see Winbury (28). The previous dietary state of the donor animal can also influence the pattern of substrate consumption in the isolated preparation (28). The uptake of substrates by isolated hearts remains unchanged following the onset of spontaneous failure (44, 81). Reports that such failure may be secondary to decreased glucose uptake due to insulin loss 44) can be discounted, since addition of this hormone to the perfusate restores the normal rate of glucose uptake but does not influence the progression of failure (4).

The heart contains a relatively large store of glucose (400-500 mg %) in the form of glycogen, which serves as a readily oxidizable source of energy during periods of increased work, hypoxia, or in the absence of exogenous substrates (23).

The glucose utilization of hypodynamic hearts is approximately one-fifth of that observed when work loads are imposed. Neely, Liebermeister and Morgan (82) concluded that carbohydrate is used to a small extent in non-working heart, but its presence becomes of prime importance during periods of work. The literature concerning the eventual conversion of the potential energy of these substrates to ATP and CP via glycolysis and oxidative phosphorylation has been reviewed (83,84). Matsumoto and co-workers have recently demonstrated the presence of the pentose pathway in ventricular muscle, this source of energy being absent in atrial tissue (85,86). Olson (23,79) has determined that the isolated heart, although utilizing both glucose and free fatty acids (FFA), uses carbohydrate to a much greater extent. In the absence of glucose 75% of the extracted FFA was oxidized to CO_2, the remainder entering into myocardial lipid stores. The presence of carbohydrate reduced the magnitude of FFA uptake by 50%, with the fraction taken up almost entirely entering the lipid stores.

The relatively inefficient anaerobic breakdown of glucose to pyruvate via glycolysis yields but 2 moles of ATP per mole glucose. The subsequent conversion of pyruvate to CO_2 (via mitochondrial oxidative phosphorylation) yields the bulk of the total of 38 glucose-derived high energy bonds (83). It would seem logical that the highly efficient oxidation of either endogenous or exogenous lipids (1 mole palmitic acid yields 138 moles ATP) should be sufficient to furnish the necessary energy for contraction in the absence of glucose. Yet, in the absence of glycolytically derived ATP, work capacity diminishes.

The ATP contents of cardiac and other tissue may be considered to be in three interdependent compartments - the membrane fraction, the

cytoplasmic pool and the mitochondrial pool. ATP utilized at membrane and sarcoplasmic reticular sites is important in the operation of the sodium pumping mechanism (87) and the removal of calcium from actomyosin (relaxing factor activity) (88). The ATP utilized at this site originates in the cytoplasmic fraction. There is strong evidence that cytoplasmic and mitochondrial ATP production are separate processes which are linked by the transfer of phosphate from mitochondrial ATP to cytoplasmic ADP by an adenylate kinase present in the wall of the mitochondrion (89). Webb (90) showed that inhibition of glycolysis by fluoride ion greatly decreased contractility while causing only minimal decreases in cellular ATP content. Inhibition of mitochondrial respiration by cyanide or malate caused no greater decrease in contractility but very much greater decreases in ATP content. He suggested that ATP derived from glycolysis (cytoplasmic pool) is necessary for the efficient utilization of mitochondrial ATP by the contractile apparatus.

The presence of glucose in the perfusate has been shown to delay the onset of failure in cat heart muscle (91) and to maintain the cellular content of ATP and CP at normal levels throughout failure (91, 92,93,94). The failed heart cannot utilize lactate (44), an ion which is normally avidly removed from blood or perfusate and metabolized to pyruvate and CO_2 (23,65). Excessive lactate production causes increased intracellular levels of this ion which are considered to be injurious (44), possibly due to the release of intra-cellular cathepsins (95).

Spontaneous failure in the absence of substrates differs in many respects from the failure which occurs in their presence. During short periods of exposure to substrate-free fluid, glycogen is rapidly consumed (27, 96-99), but contractility remains essentially normal (90).

However, during prolonged periods (> 15 minutes) of activity in substrate-free medium, contractility and oxygen consumption decrease (4, 90, 91, 100). Changes in the electrocardiogram also take place (101, 102). Several workers (3,27,90,98,103) have suggested that some endogenous material was being utilized since the decrease in oxygen consumption following glycogen depletion is relatively small (approximately 25%) (27,32,91,103). Contractile force and oxygen consumption can be increased by adrenaline (32,98), and lactate is evolved if hypoxia is induced (96,97). It remained for Shipp and co-workers (99) to demonstrate a decrease in tissue lipids of rat hearts during substrate-free perfusion. They further reported that the phospholipid fraction contributed by far the major portion of this decrease.

The effects of substrates on hearts failed in the absence of substrates again depends upon such factors as the duration of perfusion with substrate-free medium, the substrate added, the species, and the bufferency system and temperature of the perfusate. Short periods (< 10 minutes) of perfusion with substrate-free Krebs-Henseleit solution which caused diminution of the contractile force of cat hearts do not appear to change the positive inotropic effects of subsequent perfusion with glucose-containing medium (see Section III). In cat papillary muscle maintained at 37°C for longer than 1 hour without substrate, the addition of glucose does not restore contractility (91,100) whereas pyruvate causes an increase in contractility, oxygen consumption, and also a return of ATP and CP levels to normal (91). Garb and Scriabine (104) have shown that when the temperature prior to and during failure is maintained at 27°C, glucose will restore the contractility.

Lowering the temperature of the perfusate not only affects

the ability of substrates to reverse failure but also has direct effects on contractility and metabolism. The direction of the effect depends on the species. The contractility of cat hearts increases with decreasing temperature as does the contractility of rat and rabbit hearts. The optimum temperature for rat hearts is 25°C and for rabbit hearts 28°C (24). The rate of failure in all hearts decreases with lowering of temperature. Metabolic studies have been carried out mainly in the rodent heart. Omachi and Ginski (105) have shown that rabbit hearts have elevated ATP and CP levels at 26°C. Shipp et al (58) demonstrated that rat hearts at low temperatures utilize exogenous and endogenous lipids in preference to exogenous glucose. These results would indicate that the dependence of contractility on ATP derived from glycolysis which was suggested by Webb (90) does not hold true at lower temperatures.

The type of buffer in the perfusate also bears directly on the rate of failure in the presence of glucose, and influences the response to substrates in glycogen-depleted tissues. Lee et al (60,106) reported that the ATP and CP contents of cat papillary muscle which had failed in the presence of glucose were similar to values obtained in fresh tissue. These results are in variance to the data of Greiner (54), who found that the content of these compounds fell during failure under apparently similar experimental conditions. Furchgott and de Gubareff (92), favoring Lee's results, attacked Greiner's data on the basis of his analytical methods. However, examination of their data indicates that Greiner's values for ATP content in fresh tissue are similar to those of Lee's and therefore Furchgott and de Gubareff's objections are themselves questionable. Further inspection shows that the only difference between Greiner's and Lee's methods resides in the choice of buffer in the per-

fusate. The low value of ATP and CP in failure reported by Greiner occurred in phosphate buffer, whereas Lee's group used bicarbonate-buffered solutions. It is also known that hearts maintained in phosphate-buffered solutions fail more rapidly than those in bicarbonate buffer (107-110). The positive inotropic effects of ouabain are unaccompanied by changes in cardiac ATP levels in bicarbonate buffer (to be discussed) whereas the glycoside causes a decreased ATP content under similar circumstances when phosphate buffer is used (60,91,106). Pyruvate is superior to other substrates for restoring contractility of rat hearts perfused with substrate free phosphate buffer, whereas glucose appears to be the most effective substrate for this purpose during perfusion with substrate free bicarbonate-buffered solutions (109). Metabolism differs in tissues maintained in phosphate and bicarbonate buffers. Phosphate-buffer appreciably increases the oxygen consumption of myocardial tissue (111). It also causes a high intracellular inorganic phosphate concentration (112), the level of which is thought to control the relative activity of ATP production by the glycolytic and respiratory pathways (89).

Current Concepts of the Relationship of Metabolism, Failure and Digitalis Action

Several closely similar hypotheses have been forwarded to explain failure in terms of energy metabolism. Wollenberger (44,113), Olson and associates (20,114,115), Furchgott and Lee (81), and Blain and co-workers (116) conclude that low output congestive failure is unattended by any disturbance in myocardial ATP production or content. They classified failure into two types on the basis of an impairment in either energy production or energy utilization. Failure of the former type includes

those due to thiamine deficiency, hyperthyroidism, anemia, hypoxia, and arterio-venous fistula. These are generally characterized by high cardiac output, and lower than normal cardiac ATP content. Wollenberger (44) has pointed out that these forms of failure are seldom corrected by administration of digitalis glycosides. Low output failure in vivo and spontaneous failure in the isolated preparation appears to involve changes in energy utilization and respond favorably to the digitalis glycosides. The most apparent effect of these glycosides are an increase in contractile force and an improvement of the low efficiency of conversion of oxidative metabolism to work. These effects are minimal in the absence of failure (44,114,117).

The widely divergent metabolic effects of digitalis have led to a proliferation of theories concerning its mechanism of action. It appears to be established clearly that exogenous substrates are required for the inotropic effect of digitalis. Several reports have shown the presence of glucose to be essential (4,118,119,120) and at least in the rat heart, pyruvate to be as effective as glucose (121). Surprisingly, there does not appear to be any information on the ability of lipids to permit an inotropic effect of digitalis.

There has been no evidence brought forward since Wollenberger's classical review (44) which would disagree with his conclusion that cardiac ATP levels remain essentially normal during (low output) failure and are not affected by digitalis in concentrations which reverse this failure. However, the levels of CP are reduced by digitalis (122), indicating an increase in the rate of ATP utilization. This also appears to hold true for the limited action of digitalis in high output failure. Schwartz

and Lee (123) and Feinstein (57) caused what must be considered to be high output failure in guinea pigs by progressive stenosis of the aorta. These hearts had decreases in ATP and CP levels during "failure". The administration of ouabain resulted in a further decrease in CP levels without affecting the level of ATP.

The decreases in high-energy phosphate levels in these experiments cannot be considered to be opposed to Wollenberger's thesis. It has long been known that cardiac 'pressure work' requires greater oxygen consumption than does 'volume work' (18,40). It has recently been demonstrated that the former but not the latter is accompanied by an immediate decrease in ATP levels (124,125). The changes in high-energy phosphate levels after chronic aortic stenosis are therefore predictable responses to high intraventricular pressure.

Oxygen consumption has often been reported to increase during reversal of failure with digitalis (3,44,60,126,127,128). This is probably not causal to the inotropic effect. Lee et al (91,129) have shown that the oxygen consumption of failed cat papillary muscles is not increased until toxic concentration of glycosides have been administered; and pentaerythritol tetranitrate increases oxygen consumption without affecting failure (4,27).

The positive inotropic effect of digitalis ~~are~~ is accompanied by an increase in glucose uptake (44,127,130,131,132). These are not usually considered to be related causally (44). Webb's hypothesis (90) linking glycolytically produced ATP to the efficient utilization of mitochondrial ATP for contraction may call for a reevaluation of this point. Kreisberg and Williamson (3) have shown that the increase in glucose uptake is not accompanied by increased glycogen levels, indicating the immediate use

of the extra carbohydrate absorbed into the cell. Kien and Sherrod (130) have shown that the inotropic effect of digoxin in failed dog hearts *in situ* is preceded by an increased uptake of glucose and a concomitant decrease in the uptake of lipids. Their results would tend to relate the action of digitalis in increasing the efficiency of ATP utilization to Webb's hypothesis of a glycolytic link in ATP utilization for contractility. This is further supported by the observation that the inotropic effect of digitalis is prevented by either fluoride (118) or iodoacetate (133) inhibition of glycolysis.

The action of digitalis on a number of enzyme systems in cardiac muscle has been examined. In general, these findings have not been thought to be of importance to its mechanism of action. Digitalis in therapeutic concentrations has no effect on anaerobic glycolysis, succinic dehydrogenase, cytochrome oxidase or lactic acid dehydrogenase (see (134) for review). Govier *et al* (135) reported inhibition of coenzyme nucleotidase, an action which would tend to protect NAD (nicotinamide adenine dinucleotide) from destruction and thereby correct a hypothetical failure-inducing mechanism. Digitalis also inhibits ATP-deaminase and adenosine deaminase (136,137). It is difficult to see how this might be causal to its inotropic effect in view of the well substantiated lack of effect on ATP levels of the failing heart. The action of digitalis on the various ATP-ases will be considered in some detail below.

The effect of digitalis on the relationship between frequency and contractile force has also been investigated (for reviews see 138, 139). Kruta (140) suggested in 1937 that the height of contraction of atrial tissue, which varied with the frequency of stimulation, was

influenced by two "factors" with opposing inotropic actions. Blinks and Koch-Weser (141,142) described the strength-interval relationship of atrial and ventricular tissues of various species in terms of the accumulation of a negative inotropic effect of activation (NIEA) and a positive inotropic effect of activation (PIEA). They speculated that activation of the muscle produced two temporary changes with opposing effects on contractility, and that the amplitude of contraction was a function of the ~~algebraic~~ algebraic sum of these two forces. Their bathing solution contained 4 mM concentrations of fumarate, glutamate and pyruvate, in addition to glucose. These tricarboxylic acid cycle intermediates were previously shown by Webb (90) to cause decreased contractility in isolated atria, and it is likely, therefore, that the atria employed by Blinks and Koch-Weser were maintained under somewhat less than optimal conditions. They showed clearly that digitalis changed the strength-interval relationship in a dose-related manner, causing its virtual abolition in concentrations within the 'therapeutic' range. This was not a specific effect, for it was shared by noradrenaline (143).

Farah and coworkers also studied the frequency-force relationship, with additional attention to the 'post-stimulation' potentiation and post-extrasystolic potentiation. They suggested that these phenomena were associated with changes in the level of a potentiating substance within the myocardial cell, which substance was carried into the cell with each contraction and was removed from it or inactivated by a relatively slow process. The strength-interval relationship established by these workers spans only the high-frequency portion of the total curve studied by Blinks and Koch-Weser. Not only do the two curves differ in

configuration, but there is disagreement concerning the effect of digitalis on them. Blinks and Koch-Weser show no effect of digitalis on the shape of the high-frequency portion of their curve. Tuttle and Farah (144) show that the shape is altered so as to abolish the frequency-force relationship. They also showed that the same concentrations of digitalis which abolish the "normal" strength-interval relationship will reestablish this relationship after it has been altered by procedures which reduce the contractility of isolated myocardium. These included the prolonged incubation of the tissue in the bathing solution, the addition of pentobarbital to the solution and, possibly of greatest significance, the lowering of the calcium concentration of the solution. Both the strength-interval relationship and the action of digitalis are very sensitive to the concentration of calcium in the medium (145). The two groups worked at quite different concentrations of this ion, Blinks and Koch-Weser using a concentration of 2.4 mM as compared to the 1.8 mM calcium concentration used in the work in Farah's laboratory. It is most unfortunate that despite our knowledge of the many factors affecting the heart, even modern groups seem to make an effort to confuse the concepts by utilizing different bathing solutions.

The need for calcium ion for cardiac contraction (140,146-148) has been recognized since the work of Ringer (149). Much evidence has accumulated in recent years indicating that calcium represents one of the major steps in excitation-contraction coupling. Calcium entry into the cell occurs from the transverse tubules and/or the sarcoplasmic reticulum (150,151,152). The inotropic action of digitalis is also

dependent on the calcium concentration. This relationship has been summarized most recently by Farah and Witt (153). The metabolic effects of digitalis also require the presence of calcium (3,111,128). The inotropic effect of digitalis has been shown to be a function of the total number of heart beats during exposure to the drug and may thus be correlated with increases in calcium exchange coincident with cardiac contractions (154,155). The inotropic action of the glycosides appears to be related to an increase in the rate of calcium exchange and not to a change in the total tissue calcium content. This has been shown to be true at both 'therapeutic' concentrations of digitalis and at toxic concentrations (156,157). Although Farah and Witt (153) have suggested that digitalis acts at some step involved in the transfer of membrane calcium to the contractile elements of muscle, it would appear possible that the same net increase in calcium at this level could be caused by interference with the mechanism for its removal, i.e. relaxation. This mechanism is thought to be localized in the sarcoplasmic reticulum and has been given the name "relaxing factor". It is thought that depolarization of the cell membrane is conducted deep within the cell by the transverse tubules (158). Depolarization of the transverse tubules causes calcium entry from this site but may also cause the release of bound calcium from the sarcoplasmic reticulum. The ion is necessary for the interaction of myosin, actin and ATP which causes shortening of the contractile units. During repolarization, muscle relaxation is caused by the re-binding of calcium by the sarcoplasmic reticulum, a process requiring ATPase activity (for reviews see 152,159). Isolation of sarcoplasmic reticulum granules has allowed the detailed study of re-

laxing factor activity. The binding of calcium and the presence of calcium-dependent ATP-ase were demonstrated. Further studies showed the interdependence of the two processes.

Relaxing factor activity in relation to the contractile elements has been studied by observing the interaction of ATP and calcium in partially glycerinated muscle fibers or the action of isolated sarcoplasmic reticulum granules on the syneresis of isolated myofibrils. Lee and coworkers showed that the effect of ATP on glycerinated fibers was potentiated by digitalis only in the presence of relaxing factor (160) but that glycosides had no action on the inhibition of syneresis by relaxing factor (161). They also showed, however, that relaxing factor activity was inhibited by the passage of electrical current through the mixture of granules and myofibrils, and that low concentrations of digitalis potentiated this inhibition. They suggested that this action is sufficient to explain the inotropic effect of digitalis.

Several studies which attempt to localize the site of action of digitalis by localizing the sites of its binding within the heart are of interest in this connection. Again, there is considerable disagreement between various groups of workers. Infused radioactive digoxin has been localized either in particular fractions of cardiac cells (162) or in the soluble fraction (163,164,165). Combination of autoradiographic and electronmicroscopic localization led Tubbs et al (166) to localize digitoxin at the contractile elements (A-band) whereas Fozzard in a later study considered the sarcoplasmic reticulum to be a more important site of localization (167) and Luchi and Conn showed that isolated contractile proteins do not bind the drug (168). Whether such studies can

even be fruitful is in doubt, since the area of greatest concentration of drug need not necessarily be the site of its primary action.

Changes in the extracellular potassium and sodium concentrations have long been known to affect myocardial contractility (for reviews see 134,145). Intracellular potassium concentrations decreases while sodium concentrations increase when isolated tissue are maintained in "physiological" salt solutions (169,170,171). Hajdu and Leonard (172) reviewed the literature on myocardial potassium and sodium ion movements during failure and subsequent restitution of contractile force by digitalis. They concluded that the loss of intracellular potassium caused by digitalis was causal to the inotropic effect.

A great deal of work has been done since their review on the inhibition of the sodium-activated membrane ATP-ase by digitalis. This inhibition is so specific that digitalis is now being used to determine the relative sodium-dependent activity of mixed preparations of ATP-ase. There is still a great deal of dispute concerning the causal relationship of this action to the inotropic effect. Indeed, there is doubt whether inhibition of ion transport mechanisms takes place at all in the presence of "therapeutic" concentrations of digitalis (87,173). Much evidence has been presented that the inotropic actions of "therapeutic" concentrations are accompanied either by no change in intracellular potassium content (60,174-180) or by an increase in potassium (110, 134,174). One of the most recent experiments concerning the causal relationship is that of Muller (181) who observed a close quantitative and temporal relationship between potassium loss from sheep trabeculae and increased strength of contraction due to small concentrations of ouabain.

One crucial question has been ignored throughout this controversy: if digitalis causes its inotropic effect by causing the loss of potassium, does it differ in this respect from other inotropic drugs? The only studies available on the specificity of this effect are those of Melville and Korol who showed that loss of potassium from the heart is a non-specific effect of positive inotropic agents (xanthines, catecholamines) and that potassium shifts into cells are a non-specific effect of agents with negative inotropic activity (182,183).

Tanz has suggested recently that release of catecholamines by ouabain may be causal to its inotropic effect in vitro (2,184). Spann et al (185) have recently demonstrated that isolated papillary muscles depleted of their catecholamines by reserpine are indeed insensitive to the inotropic effect of digitalis glycosides, thus corroborating Tanz. However, they also showed that muscles depleted to an equal extent by chronic denervation reacted to the glycosides to the same extent as did muscles containing the normal amounts of catecholamines. They concluded that the presence of catecholamines was not essential for the positive inotropic effect of digitalis in vitro. Other reports indicate that endogenous catecholamines do not play a role in the contractile response to digitalis in vivo (1). However, catecholamine release has been implicated in the changes in cardiac conduction caused by digitalis glycosides in vivo (1,186).

Removal of inotropic materials from the heart by perfusing solutions

Artificial perfusates, although they contain concentrations of ions identical to those found in the extracellular fluid, do not con-

tain any of the multiple other constituents of serum or extracellular fluid which represent part of the cellular environment in vivo. It is not surprising therefore that equilibration with such artificial solutions results in the removal of a large number of substances from the perfused tissue. The possibility that this removal is causative to the certain failure of isolated preparations is an aspect of the problem which I believe has not received nearly the attention commensurate with its importance.

The relatively prolonged state of stable contractility in preparations perfused with blood supplied by a donor animal (17,22) indicates that blood may contain a factor or group of factors which are necessary for optimum cardiac performance.

As early as 1872, Bowditch (45) demonstrated that the beat of hypodynamic frog hearts was strengthened when serum was added to the perfusate. This was later confirmed by Ringer (46) and others (15,187, 188). Besides failure, prolonged perfusion of cardiac tissues in the absence of serum or plasma results in the loss of the frequency-force relationship (144,189,190) and post-stimulation potentiation (PSP) (144). Addition of serum to the perfusate restores both the contractile strength (47,187,188,191) and the PSP (187). 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 (188). The addition of serum to the perfusates of hypodynamic hearts also causes an increase in glucose and oxygen utilization (see reference 15, chapter 5). Although the signs of

failure eventually return, these hearts do not show the decreases in oxygen consumption usually observed during failure in the absence of serum, as mentioned previously (see page 7).

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 (4,118). In the rat heart perfused with substrate-free bicarbonate buffer, pyruvate becomes superior to glucose in restoring contractility when serum is present (4,100), whereas glucose is the superior substrate in the absence of serum (109). The action of serum is not species-specific, since bovine serum improves the contractility of cat papillary muscles (192) and human serum affects the frog heart (193). 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. They also antagonize the negative inotropic effects due to increased perfusate potassium content, as does serum (194). Zacharia (4) 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 cardio-active lipid substance in serum to the reversal of failure.

Hadju and coworkers (187) were also interested in the positive inotropic effect of serum. They isolated β-palmitoyl lysolecithin from

plasma (195) and suggested that this was the material responsible for the inotropic actions of plasma. They reported that its effects on the hypodynamic frog heart were similar to those of the digitalis glycosides (193,196). Kahn and Schindler (197) examined the premise of a digitalis-like action of β-lysolecithin in two test systems. They showed that this lipid did not inhibit the ATP-ase of red blood cells, an action typical of digitalis. They also showed that the lipid did not increase, but rather decreased the strength of contraction of isolated guinea pig hearts. Their negative results cannot be accepted fully, however, because, for example, they gave no information concerning the extent of failure of their preparations or their method of solubilization of the phospholipid. The results of Green, who showed a positive inotropic effect of a crude lipid extract of liver rich in lysolecithins (198) would tend to support Hajdu's earlier results.

Serum proteins have also been shown to have positive inotropic activity. Green et al (192) found the major component of activity in Fractions IV and V of human and bovine plasma. Activity in the globulin fraction has also been demonstrated by Hajdu and Leonard who have described three components termed Cardioglobulins "A", "B" and "C" (193, 196,199,200,201). They demonstrated that one of these, Cardioglobulin "C" occurred in increased amounts in patients with essential hypertension and aortic stenosis (200). Conversely, decreased amounts were found in patients with congestive heart failure. By labeling Cardioglobulin "C" with radioactive calcium, they demonstrated that this fraction was bound to the myocardium only after prior treatment with Cardioglobulin "B", and Cardioglobulin "A" then presumably released "C"

into the cell where it exerted an inotropic action (201).

Nayler and her coworkers (188,202-206) have recently described the isolation of a peptide called "Kinekard" from human plasma. This evokes a positive inotropic response in isolated dog, rabbit, monkey and rat ventricular tissue. They report 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~~ after the cardiac action potential. After injection into intact anesthetized rabbits, increases in the systolic and diastolic blood pressures as well as increases in cardiac output and left and right ventricular diastolic pressures were noted (204). A direct pressor action was also noted (203). The substance also increased the heart rate in animals pretreated with pronethalol. The inconsistency and diversity of the actions exerted by Kinekard and the lack of correlation between blood levels of this substance and clinical syndromes (203) engender doubt concerning both the isolation methods used and the suggested physiological role.

Very little has been done to identify the sources of the cardioactive materials identified in plasma. It has long been known that the useful life of heart-lung preparations is increased by the inclusion of the liver in the circuit (207-210). Kako et al (211) have recently reinvestigated the several factors contributing to the failure of heart-lung preparations and have shown that inclusion of the liver in the preparation delayed the onset of decreases in substrate and oxygen utilization and of spontaneous failure. Possibly of greater importance were their observations that addition of the liver to the perfusion circuit of a failed heart-lung preparation, while not reversing the

changes in substrate and oxygen utilization, caused an increase in the contractile force and therefore an increase in the efficiency of cardiac contraction. They suggested that the liver provided the preparation with a substance of "digitalis-like" action.

Attempts to isolate cardioactive materials from liver homogenates are guaranteed to be successful because this organ contains huge numbers of materials. For example, Green (198) and Green and Nahum (212) investigated a number of liver fractions and identified tyramine, methionine, menadione and dicumerol as cardioactive materials contained in the liver. Hajdu et al (195) studied the tissue distribution of β-lysolecithin and found the liver to be one of the organs containing relatively large amounts of this material. They also demonstrated concentration of this substance in heart, adrenal medulla and plasma. Nothing has been done to identify the site of synthesis of the Cardioglobulins, but it would appear reasonable that the liver is the site of origin of these substances.

Clark observed that recirculation of the perfusion fluid delayed the onset of spontaneous failure (15). This is the origin of the hypothesis that perfused hearts lose a substance which is necessary for prolonged viability. Clark thought that he might be dealing with a lipid. Since his time, a large number of substances have been shown to be removed from the heart by perfusates. These include many small water-soluble molecules such as noradrenaline (213), glycerine (214) and creatine (215) as well as various enzymes (216,217,218). The significance of these losses has been clouded by two main difficulties: the

multitude of cellular constituents which have been shown or thought to be lost, and the great dilution of these materials by the perfusate which causes great difficulty in their identification.

A simplifying assumption is that water-soluble materials will be removed by the perfusate. On this basis, Robb (219) felt that loss of B-vitamins might explain spontaneous failure and showed that the addition of small concentrations of an undefined mixture of these vitamins delayed the onset of failure. Similarly, Calder (220) showed that nicotinic acid and nicotinamide increase the contractility of failed hearts. It is obvious that this type of experiment does nothing to establish causality.

The occurrence of adenosine and adenylic acid in the perfusate from isolated hearts has often been reported (93,94,218,221,222). The use of substrate-free perfusates hastens the loss of these compounds (221,223). Inosine and hypoxanthine have also been reported to occur (93,94,218). The enzyme adenosine deaminase has also been found in perfusates (218), an observation which may account for the presence of these compounds. ADP and ATP have not been reported to be removed from the heart. However, the presence of nucleoside phosphorylase (218) indicates the possibility that they were decomposed and determined as adenylic acid. The adenylates are potent relaxants of coronary artery smooth muscle (224) and the fact that they are lost into the perfusate in increased amounts during periods of hypoxia has led Berne (225) to suggest that they are the prime determinants to the coronary resistance. However, the significance of the losses incurred by the heart to the genesis of spontaneous failure is probably minimal. These compounds are

apparently synthesized by the heart at rates equal to their loss, for Lee et al (60,91) reported that the myocardial content of total adenylates was unchanged during failure.

It was also shown by Lee et al that myocardial creatine phosphate level was unchanged during failure. Since the heart cannot synthesize creatine (226), the observation that creatine is lost into the perfusate of isolated hearts implies that the level of total creatinine must be decreasing with time. Creatine phosphate represents approximately one-half of the total myocardial creatine pool. Davies et al (227) have suggested that cardiac contractility may depend as much on free creatine content as on the content of creatine phosphate. If they are correct, loss of creatine into the perfusate could well cause failure in the presence of normal ATP and CP levels. Further studies on the importance of this compound in spontaneous failure may prove rewarding.

One of the consistent and puzzling features of spontaneous failure is the loss of the ability of the heart to utilize exogenous lactate. This impuissance may be explicable in perfused hearts. Several important constituents of the lactate-pyruvate system have been found in the perfusates of isolated hearts, including lactic acid dehydrogenase (see Sheinin and Cohen, 216). Several workers have shown that the cofactor for this enzyme, NAD, is also lost by the perfused heart (228,229). De Barbieri et al (230) have shown that the addition of lactic acid dehydrogenase to the perfusate of failed hearts causes an increase in contractile force. The addition of this enzyme to the perfusates of normal hearts has no effect. All these factors would

indicate that this aspect of the failure of perfused hearts might be explained by losses of essential enzymes and cofactors into the perfusate. However, De Barbieri's work is open to considerable criticism on the following basis: he used a recirculating perfusion system into which one may expect significant amounts of lactate to be lost from the failing heart (15). It is known that pyruvate can be utilized by failed hearts which are losing lactate (113), and that addition of pyruvate improves contractility (228). It would appear possible that addition of lactic acid dehydrogenase to his system might well have caused the formation of pyruvate in the perfusion fluid with a resultant increase in contractile force, rather than increasing contractility by a direct action to improve cellular function.

Spontaneous failure is accompanied by a decrease in myocardial permeability to glucose (4). Wertheimer and coworkers (231,232,233) have recently described the isolation of a protein which causes an increase in glucose and oxygen uptake by skeletal muscle. Their material was obtained from the bath or perfusion fluids of isolated skeletal muscle, heart and smooth muscle. They noted that the material could only be obtained after the preparations had been contracting rhythmically and therefore named the material "MAF"- Muscle Activity Factor. They also showed that MAF causes an increase in amino acid uptake by skeletal muscle (232). The relationship of this material to myocardial failure remains uninvestigated.

A.J. Clark suggested in 1913 that some necessary material was lost from the frog heart perfused with Tyrode solution and that this loss was causative to failure (146). He thought that he might be

dealing with a lipid substance which originated at the cell membrane, and showed that alcoholic extracts of serum would restore the contractile force of failed preparations. He also demonstrated that impure, but not pure lecithin had the same effect (15).

Gas Perfusion

The suggestion of use of gaseous perfusates usually engenders thoughts of embolism, and immediate cardiac standstill. However, injections of oxygen gas into peripheral arteries may produce beneficial effects in cases of peripheral diseases such as Reynaud's disease atherosclerosis (234), intermittent claudication (235) and in the treatment of amputation stump ulcers (236).

Magnus (237) reported in 1902 that the beat of an isolated cat heart could be maintained for one hour if it was perfused with gaseous oxygen. Cattell (238) suspended frog skeletal muscle in an oxygen environment and showed that contractile responses to electrical stimulation could be obtained for several days. Reflex activity in the frog spinal cord can also be maintained by perfusion with gas (239). Talbert and coworkers (240) perfused the kidneys of dogs with oxygen for four hours and reported that the organs functioned normally *in situ* when perfusion with blood was re-instituted. Burns *et al* (241) confirmed the results of the heart perfusion experiments by Magnus, maintained the organ for four hours, and described the relationship between pressure and gaseous flow through coronary arteries. They also extended the use of gas perfusion to the nerve-muscle preparation in the cat hind limb. Sabiston and coworkers (242,243) showed that isolated dog hearts con-

tinued to beat for several hours when perfused with oxygen via either the coronary arteries or coronary sinus and that cardiac electrical activity persisted for periods up to eight hours. These authors, however, perfused the heart with sodium chloride solutions equilibrated with 95% O_2 - 5% O_2 before instituting gas perfusion. They therefore, utilized a preparation which previously had been compromised by a surprisingly inept liquid perfusion technique. Carter and Sabiston (244) measured glycogen and lactate contents of gas perfused dog hearts, and reported that the levels of these fell rapidly during the first hour of perfusion. In none of these reports, however, were any physiological or pharmacological comparisons made between gas-perfused hearts and the more usual liquid-perfused preparations.

SECTION II

METHODS

A. Perfusion Technique

Kittens, of either sex, weighing between 0.4 - 1.2 Kg were obtained commercially and fed a diet of meat, fish and eggs ad libitum for at least 3 days before use. This weight range and diet regimen were chosen because the hearts of young animals contain proportionally greater stores of energy-yielding lipids then do older animals (245), and because fasting reduces the myocardial content of these substrates (15). The animals were killed by a blow on the head. The heart was removed and placed in a beaker of cold (4°C) oxygenated Krebs-Henseleit solution. Anticoagulant or anesthetics were not employed because of the possible effect of tissue metabolism (246,247). The hearts were cleaned of extraneous tissue, and the aorta cannulated.

The technique of gas perfusion requires the heating (to 37.5°C) and humidification of a gas mixture consisting of 95% O_2 - 5% CO_2 and its delivery at constant pressure to the coronary vasculature of an isolated heart. Preliminary results obtained using the apparati described by Burns et al (241) and Sabiston et al (243) were found to be highly variable. In their techniques the gaseous perfusate was heated before it entered the aortic cannula by either bubbling it through warm water or by passage through a double-jacketed condenser. We found that the temperature of the perfusate treated this way was uneven and fell at times by 10°C under conditions of varying flow. An apparatus was therefore constructed which permitted close temperature control of the perfusate at various flow rates, as well as permitting rapid switching from liquid to gas perfusion. This is shown in Fig. 1.

The heart is perfused by the Langendorff technique (6). The cannula inserted into the aorta had four side-arms close to its tip. Two thermistor probes (YSI models 402 and 403) extended directly into the perfusate flow through side-arms A. One probe was used to monitor the perfusate temperature, the other functioned in conjunction with a temperature regulator (YSI model 63) which controlled the flow of electrical current through a heating coil placed around the glass tube leading to the cannula. Fluctuations in the current flow through this coil served only as a final temperature adjustment (see below). It has been possible to maintain the temperature of both liquid and gas perfusates at 37.5 ± 0.4°C with this arrangement. During the gas perfusion, sidearm B was connected by way of stopcock S-3 and a trap to a gas pressure regulator (PR) constructed by immersing a glass tube into a column of water to a depth corresponding to 60 mm Hg. The trap served to catch liquid perfusate contained in the system at the time of change from liquid to gas perfusion. Sidearm C maybe connected to a Statham P-23AC pressure transducer (PT). A thin polyethylene tube used for injection of drugs passed through sidearm D and terminated in the cannula close to its point of insertion into the aorta.

Two liquid perfusates of different composition could be stored in reservoirs placed level with the heart, and were pressurized to 60 mm Hg with the equilibrating gas. The liquid passed through a water-jacketed spiral condenser for warming and then through stopcocks S-1 and S-2 before reaching the cannula. Stopcock S-1 selected either reservoir. Stopcock S-2 selected either gas or liquid perfusate.

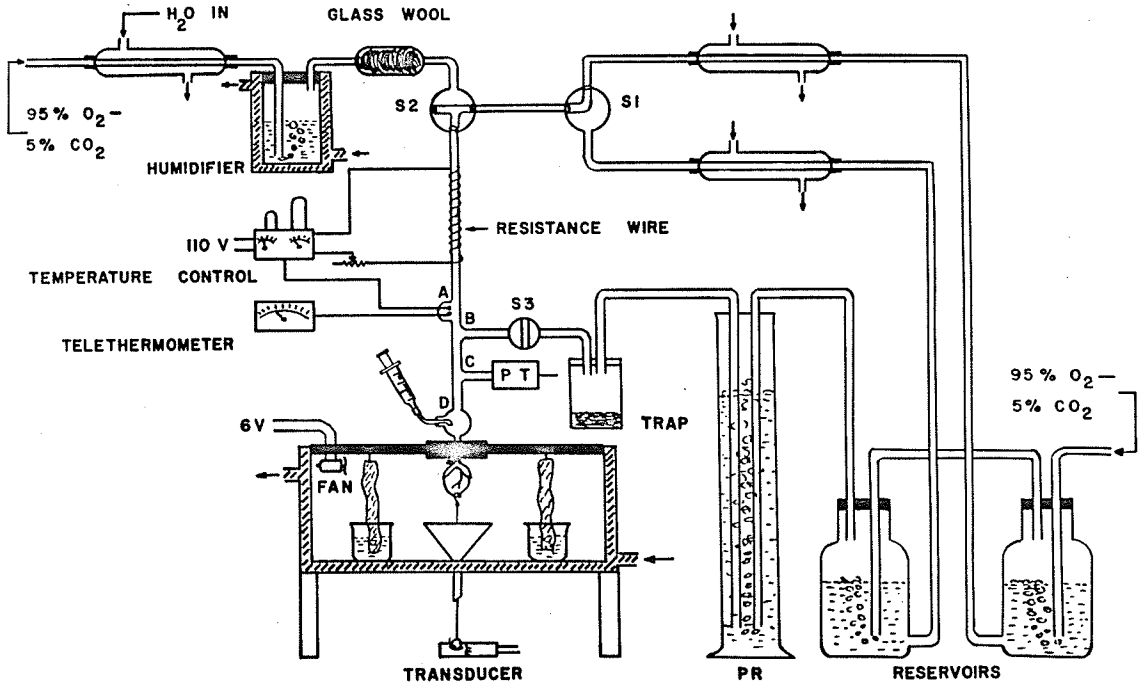

Figure 1. Apparatus for gas and liquid perfusion. For explanation, see text.

The liquid perfusate used in most of these studies was substrate-free Krebs-Henseleit of the following composition:

	g/l	mM
NaCl	6.90	118.0
KCl	0.35	4.7
KH_2PO_4	0.16	1.2
$CaCl_2$	0.37	3.3
$MgSO_4$	0.29	2.4
$NaHCO_3$	2.20	26.0

The perfusate was saturated with 95% O_2 - 5% CO_2 before use. The pH was 7.4. In a few experiments in which work was determined, glucose, 2.0 g/l was added as substrate.

Compressed 95% O_2 - 5% CO_2 was supplied from a tank through a pressure-reducing valve. It was preheated in a water-jacketed condenser and humidified in a water-jacketed gas scrubbing bottle containing warm distilled water. It then passed through glass wool in order to remove mist and was led to stopcock S-2.

The heart was enclosed in a water-jacketed lucite box. High humidity in the box was maintained by several vertical strips of moist filter paper, the ends of which dipped into small beakers filled with distilled water. A funnel under the heart passed through the bottom of the box for recovery of liquid perfusate. The water circulating through all jacketed components was supplied from a constant temperature bath at 40.0 ± 0.5°C.

Hearts were cannulated and perfused with liquid for five

minutes. During this time each ventricle was incised and electrodes and recording apparatus placed. Gas perfusion was initiated by the simultaneous turning of stopcocks S-2 and S-3. The liquid perfusate which remained between stopcock S-2 and sidearm B is caught in the trap. The liquid perfusate in the cannula between sidearm B and the aorta passed into the heart due to the pressure of the gas above it. Unless used for subsequent chemical analysis, the hearts were weighed to ± 0.05 g at the end of each experiment.

In later experiments the cannula was modified in order to supply two hearts simultaneously. During experiments in which liquid perfusate was used exclusively, the apparatus was modified so that the perfusate efflux was returned to a reservoir by a pump after passage through a glass-wool filter. The total volume of liquid in the system ranged from 35 to 50 ml/g (wet weight) of heart. At the termination of such experiments, the vasculature was cleared of fluid by perfusion with gas for ten seconds before the heart was weighed or prepared for chemical analysis.

B. Measurement of physiological parameters

Isometric contractile force was measured with Grass FT-03 force-displacement transducers. A stainless steel hook pierced the apex of the perfused hearts and string attached to the hook was led through the stem of the funnel to the transducer (Fig. 1). Adjustment of resting tension was simplified by mounting the transducer on a rack and pinion. Resting tension was defined as the diastolic tension exerted on the apex in this arrangement. Heart rate was counted from the recordings of contractile force. Electrograms were recorded through clip electrodes

placed on the right atrial appendage and the ventricular apex. All recordings were made with a Grass polygraph.

The contractile force and heart rates observed at the end of the 5-minute liquid preperfusion were taken as the control or initial values and subsequent changes were expressed as a percentage of these. In experiments in which liquid perfusion was employed exclusively, the readings obtained after the first 5 minutes of perfusion were considered as the control values.

A simple apparatus was constructed to determine isotonic contractility and the work done by perfused hearts. A thin rigid steel rod having an eyelet in its center was suspended from the apex of a perfused heart (Fig. 2). Isotonic displacement was measured by means of a second rod which passed through the eyelet and transmitted motion to the movable leg of a Walton-Brodie (248) strain gauge. A permanent magnet was attached to the lower end of the rod which hung from the heart. The total weight of the rod and magnet was 10 g. Additional weights (10-175 g) were hung on hooks soldered above the magnet. The load imposed by the isotonic recording rod was approximately 100 mg, and was considered negligible in relation to the weight load. The magnet moved freely within an air-core solenoid with a motion imparted to it by the movement of the apex during each cardiac cycle. The output, E_o of the coil is given by:

$$E_o = - N \frac{d\phi}{dt} \text{ volts}$$

where ϕ = the flux of magnetic induction in Webers, and N is the number of turns linked by the flux ϕ. The area under the voltage curve is proportional to the product of the velocity of the magnet and the time

for which such velocity is maintained. The total area under a series of coil outputs for the polarity which corresponds to an upward displacement of the magnet is thus directly proportional to the sum of the heights through which the magnet is lifted and is independent of the rate at which the force develops.

The coil was a secondary winding from a discarded Harvard Inductorium, potted in paraffin wax for waterproofing. The coil output was amplified by a Grass P5 amplifier and the output of this fed into the integrating circuit shown in Fig. 2. The gain of the amplifier was usually set at 50 X, but was changed as required by the contractile properties of individual preparations. High frequency roll-off was set to be half gain at 2KC, thus precluding any high-frequency interference or feedback. Sixty-cycle (A.C.) interference was easily eliminated by shielding the input loads to the coil and by taking reasonable precautions to keep A.C. sources some distance from the coil. The coil was connected to the amplifier so that an upward movement of the magnet resulted in a positive signal to the integrator. The diode D_1 transmitted only positive signals which in turn charged the integrating capacitor C_1. Thus the charge on the capacitor accumulated in stopwise fashion with each upward movement and at any instant the accumulated charge on the capacitor was a measure of the total magnitude of upward movements. The charge on the capacitor was erased at regular intervals by a timer. The output from the integrating circuit was a function of the charge on the capacitor and was recorded on a Grass polygraph. Unless the diode D_1 is biased to offset the small non-conducting portion of its forward characteristics, a portion of each rectified waveform will be lost before it is deposited on C_1. If the bias voltage is too

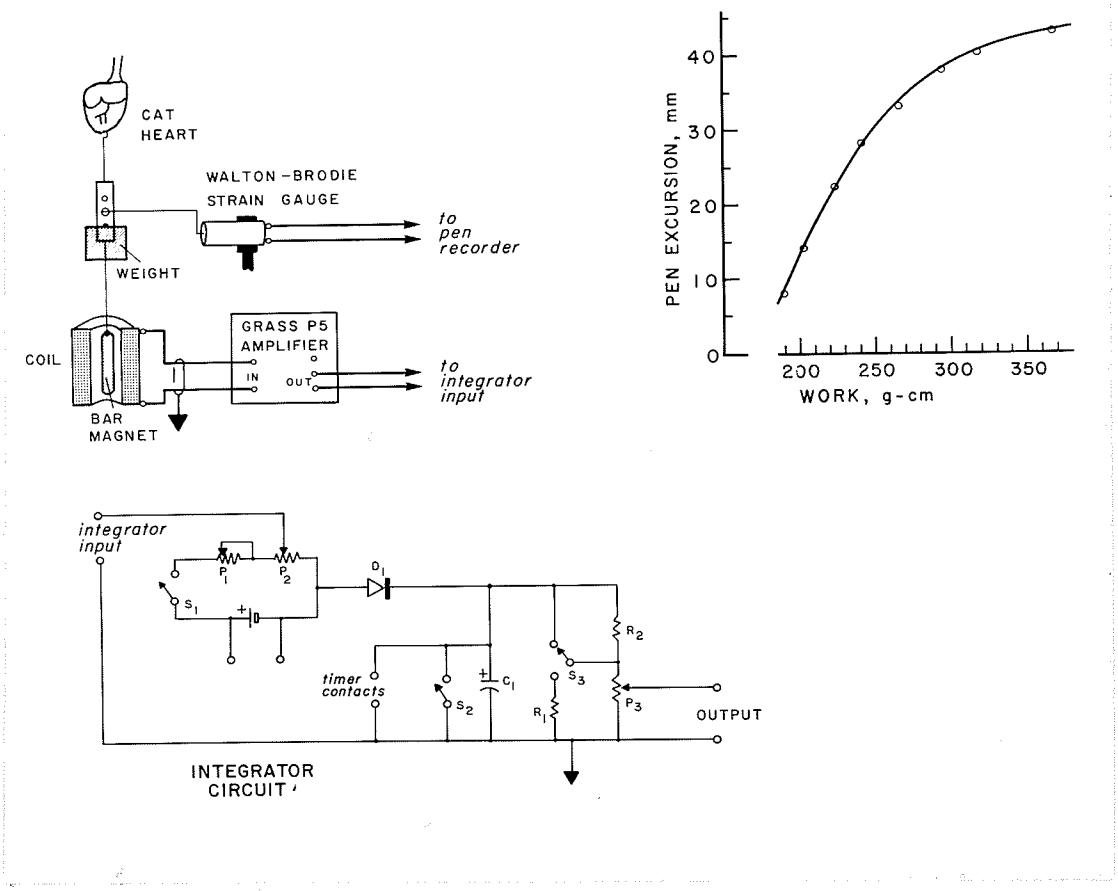

Figure 2. Diagram of work apparatus and integrator circuit. P_1 = "BIAS adjustment, FINE" potentiometer, 100 ohms, 1-watt. P_2 = "BIAS adjustment, COARSE" potentiometer, 500 ohms, 1-watt. P_3 = "OUTPUT LEVEL" potentiometer, 50K ohms, 1-watt. All potentiometers linear wire-wound or type AB. R_1 = 5600 ohms, R_2 = 47K ohms, both $\frac{1}{2}$-watt. D_1 = Philips silicon diode, PH1021. C_1 = Electrolytic capacitor, 1000 mf, 25WVDC. S_1 = "BIAS ON-OFF" switch, shown in "OFF" position. S_2 = "MANUAL RESET" switch, normally open pushbutton. S_3 = "OUTPUT LEVEL X1 - X10" switch, shown in X10 position. S_4 = timer contacts. Bias cell = 1.4 V, mercury.

Upper right: plot of electronically integrated work output vs. work calculated from isotonic excursions at constant load (25 g). Timer contacts shorted every five seconds.

high, however, a charge will accumulate on C_1 in the absence any waveform. The bias voltage was adjusted with controls P_1 and P_2 so that no polygraph pen movement occurred, even at higher amplification, in the absence of a signal at the amplifier input.

Isotonic displacement was calibrated by manual movement of the displacement transmitting rod along a millimeter scale. The integrator was calibrated using an isolated heart driven electrically at several rates. The timer was set to discharge the capacitor every five seconds and the maximum height reached by the recorded pen during this interval was related to the work determined from the isotonic record (load) X (isotonic displacement/beat) X (number of beat/5 seconds) . A typical calibration curve is shown in the inset to Fig. 2. This calibration curve was found to be constant in subsequent experiments during the same day; the calibration was repeated daily.

The action of 0.1 μg of adrenaline on a perfused heart which was driven electrically at a constant rate is shown in Fig. 3A. The record of work was found to vary not more than 5% from measurements taken from the isotonic contraction record. The determination of work during the periods of rapidly changing heart rate or arrhythmias is difficult when recordings of isotonic motion alone are available. In Fig. 3B, the ability of the integrating circuit to follow the contractions of a failed heart during periods of spontaneous contractile alternation and tachycardia is demonstrated. In this experiment, the amplifications of both channels were increased, and thus only the early linear portion of the calibration was used.

Meyler et al (249) have described the use of a magnet and coil transducer similar to the present one to measure what they referred

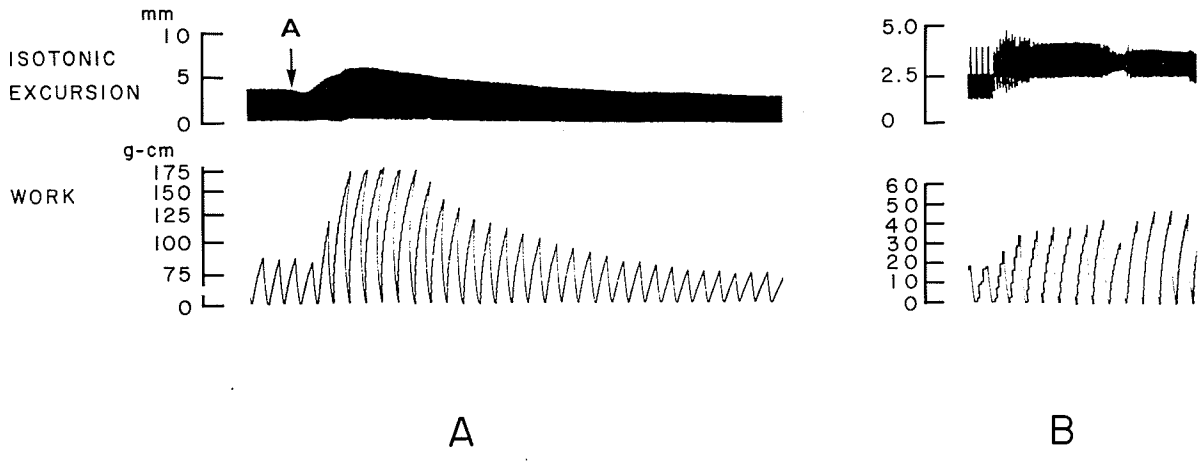

Figure 3. Correlation of work record and isotonic excursion record.
A: Response to adrenaline. At "A" - 0.1 µg adrenaline added to perfusate inflow. Heart rate held constant (168/min). Load = 25 g.
B: Record obtained during spontaneous arrhythmias. Load = 25 g.

to as the "contraction height of the heart". They took the peak of the coil output to be proportional to the height of contraction. This is erroneous because the coil output is a function only of velocity which can vary independently of height.

It should be noted that the circuit does not provide an ideal integration because there is a leak of charge through the output resistance network and because of the leakage inherent in electrolytic capacitors.

C. Other procedures

When desirable, the heart rate was controlled by electrical stimulation through electrodes placed on the right atrial appendage and the ventricular apex. Just suprathreshold pulses (4-8 volts) of 5 millisec. duration were supplied either from a Grass SD-5 stimulator or from a Tektronix stimulation unit whose output was led through an isolation transformer.

Electrical release of catecholamines from myocardial stores (250) was accomplished by increasing the stimulation voltage to driven hearts by a factor of 10 for a period of 30 seconds. A Walton-Brodie strain gauge arch sewed to the left ventricle was used to monitor contractile force in these experiments.

Drugs were administered to gas-perfused hearts by rapid injection of 1.0 ml of liquid into the cannula. It was important that the gas pressure drive this liquid through the coronary circulation as a single bolus. The formation of bubbles caused irreversible changes in the perfusion because of the high interfacial tension.

Stock solutions of the following drugs containing 10 mg/ml of the base were made in 0.9% NaCl acidified to 0.1 N with HCl:

Acetylcholine chloride	Calbiochem
Adrenaline bitartrate	Sterling-Winthrop or Regis
Atropine sulfate	British Drug Houses
Cocaine hydrochloride	Nutritional Biochemical
Digitoxin	" "
Histamine diphosphate	" "
Isoproterenol hydrochloride	Sterling-Winthrop or Regis
Noradrenaline bitartrate	Calbiochem
Ouabain	Nutritional Biochemical
Pronethalol hydrochloride	Ayerst, McKenna and Harrison
Dihydrodigitoxin	synthesized by Drs. Chimere Ikoku

and P.E. Dresel by catalytic hydrogenation of digitoxin (251). Infrared spectrum analysis indicated that the product, after extensive recrystallization from methanol-water, was 85-95% pure.

Dilutions were made with the same vehicle on the day of the experiment. 0.05-0.1 ml of the appropriate dilution were brought to 1.0 ml with warm Krebs-Henseleit solution immediately before injection.

The extracellular electrolyte contents of gas perfused hearts ~~was~~ were determined as follows: Hearts were perfused for 5 - 180 minutes with gas after 5 minutes of liquid perfusion. Resting tension was maintained at 10 g. Heart rate was not controlled. One group was pre-perfused for 5 minutes with liquid containing ouabain, 1.0×10^{-9} g/ml and then perfused with gas for 180 min. After the desired period of gas perfusion, each heart was perfused with 35 ml of iso-osmotic sucrose solution (10.2%). The sucrose effluent was collected in fractions of 1 - 4 ml. The hearts were weighed and then discarded. The sodium and potassium content of the individual sucrose fractions were analysed with a Patwin internal standard flame photometer.

D. Analytical Methods

Perfusion was terminated by cutting off the ventricles below the fat pad surrounding the circumflex artery and dropping them into a beaker of liquid nitrogen. The frozen tissue was then placed into a steel test tube cooled in liquid nitrogen and pulverized by striking it several times with a cooled steel pestle. The frozen powder was mixed by stirring with a cooled spatula and stored at -20°C.

Weighed aliquots of frozen tissue powder were analyzed as follows:

1. Water content: 100-200 mg of powder were dried in vacuo to constant weight at 80°C. in a Precision Heater Vacuum Desicator. The value for water content was used to calculate the dry weight of aliquots used for other analyses.

2. Carbohydrates: (a) Total carbohydrate was determined by the method of Kemp and Kits von Heijningen (252). Samples of 100-200 mg were homogenized using a ground glass pestle with the addition of 5% (w/v) trichloroacetic acid containing 0.1% (w/v) $AgSO_4$ (253). Following centrifugation for 10 minutes at 5000 xg, 3.0 ml of 96% (w/v) sulfuric acid was added to a 1.0 ml aliquot of deproteinized supernatant and the mixture heated in a boiling water bath for 6.5 minutes and then cooled under tap water. The absorbance was measured at 520 mμ and compared to glucose standards. All readings were made with a Hitachi Perkin-Elmer model 139 Spectrophotometer. Results were expressed as μ moles glucose/g dry wt.

3. Lactate and Pyruvate: 50-100 mg of tissue were homogenized in 7.0 ml of 6% (w/v) Perchloric acid. The homogenate was centrifuged and the

supernatant treated as follows:

(a) Lactate was measured by the coupled enzymatic reduction of βNAD (254). 3.0 ml of the supernatant were incubated for one hour at 37°C with Lactic dehydrogenase, βNAD, and semicarbazide. The reaction:

$$\text{Lactic acid} + \text{NAD} \rightleftarrows \text{Pyruvic acid} + \text{NADH}$$

proceeds to the right very slowly unless the pyruvic acid formed is converted to pyruvic acid semicarbazone. The NADH was measured spectrophotometrically at 340 mμ. Standard solutions of sodium lactate were analyzed concurrently.

(b) Pyruvate was determined by following the disappearance of βNADH in the same reaction (255) which proceeds rapidly to the left in the presence of concentrations of lactate which are usually encountered. 1.0 ml of 1.1 M K_2HPO_4 was added to 3.0 ml of the perchloric acid supernatant and the precipitated potassium perchlorate removed by centrifugation. The supernatant was then added to a cuvette containing βNADH in phosphate buffer. After determining the optical density at 340 mμ, lactic dehydrogenase was added and the optical density again recorded after 5 minutes incubation. Solutions of sodium pyruvate served as standards.

The tissue lactate and pyruvate contents were expressed as μ moles/g dry wt. of tissue. The materials for these two procedures were obtained in kit form from Sigma Chemical Co., St. Louis, Mo.

4. Lipid: A chloroform - methanol extract of 1-2 g tissue was prepared by the method of Folch et al (256). The final volume of the extract was adjusted to 25.0 ml. Aliquots of the extract were used as follows:

(a) Total lipid: 5.0 ml of the extract were pipetted into tared test tubes and the solvent removed by evaporation under nitrogen. The

residue was weighed and the results expressed as mg/g dry wt. of tissue.

(b) Phospholipids: The residue from the total lipid determination was digested with 50% (w/v) sulfuric acid and the phosphorus determined spectrophotometrically at 700 mμ by the method of Zilversmidt and David (257) as modified by Connerty et al (258). Stock solutions of KH_2PO_4 served as standards, and results were expressed as μ moles phosphorus/g dry wt. of tissue.

(c) Esterified, non-esterified and total cholesterol: The solvent in two 2.0 ml aliquots of the extract was evaporated to dryness under nitrogen. In one sample, total cholesterol was determined by the method of Zlatkis, Zak and Boyle (259). In this method, 4.0 ml of a Sulfuric acid - Ferric ammonium sulfate reagent was added to the sample and heated in a water bath at 56°C. The resulting color was measured spectrophotometrically at 560 mμ against cholesterol standards. The second evaporated sample was dissolved in 3.0 ml of isopropanol containing 1% (w/v) digitonin. The precipitated non-esterified cholesterol (258) was isolated by centrifugation and washed with acetone. Determination of cholesterol content was as described above.

Total and non-esterified cholesterol were calculated as μ moles/g dry wt. tissue, and esterified cholesterol calculated as the difference between the two and expressed as percent of total cholesterol content.

(d) Free fatty acids: Duplicate 2.0 ml portions of the extract were evaporated under nitrogen. Free fatty acids in the residue were extracted with a two-phase heptane-isopropyl alcohol-water system (260). This procedure removes some interfering substances such as acetic and lactic acid but not phospholipids. For that reason, the heptane layer

containing the fatty acids was further extracted with 0.02N sulfuric acid to remove phospholipids (261). The resulting solution of fatty acids was titrated under nitrogen with 0.02N sodium hydroxide solution using thymol blue as an indicator (260). Appropriate dilutions of oleic acid were titrated as standards and results expressed as μ moles/g dry wt. of tissue.

(e) Triglycerides: The presence of phospholipids in the extract causes falsely high values when triglycerides are determined by a method measuring ester bonds (262). They were removed by the method of Van Handel and Zilversmit (263). Eight ml of extract were evaporated under nitrogen to dryness in a glass stoppered test tube. 2g of heat activated zeolite (Duocil®, W.A. Taylor Co.) and 10 ml of chloroform were added and the resultant slurry shaken by hand for 15 minutes and centrifuged. Phospholipids are bound to the zeolite whereas triglycerides remain in the chloroform supernatant. A 5.0 ml aliquot of the chloroform extract was then evaporated to dryness and redissolved in 3.0 ml of a 3:1 ethanol-diethyl ether mixture. Triglycerides were then determined spectrophotometrically by the hydroxylamine method of Stern and Shapiro (264). Solutions containing known concentrations of triolein were run as standards. Results were expressed as μ moles/g dry wt. of tissue.

The zeolite-chloroform supernatant was found to be free of measurable quantities of phosphorus using previously mentioned techniques. In other experiments, 97-103% of triolein subjected to the zeolite procedure were recovered.

E. Additional methods applicable to Section IV

1. "Wash-out" Procedure

Hearts were gas-perfused by the method previously described.

In all experiments the duration of perfusion with liquid was 5 minutes. At the end of the first hour of gas perfusion, 5.0 ml of oxygenated Krebs-Henseleit solution (37.5°C) was injected into the cannula. This procedure was repeated at 30-minute intervals thereafter, for a total of four "washings". Fresh portions of liquid were used for each "washing". Gas perfusion was maintained for a minimum of 30 minutes following the last "washing". The hearts were then removed from the cannula and weighed.

2. Precipitation of the cardioactive material

The liquid used for "washing" the heart was collected in calibrated test tubes and the volume adjusted, when necessary, to 5.0 ml with fresh Krebs-Henseleit solution. This generally required the addition of less than 0.5 ml of solution. Powdered ammonium sulphate was then added in quantities sufficient to produce saturations of 25 percent. The amounts necessary were computed from a nomogram (265). After stirring, the tube was emmersed in an ice-water bath (0.5°C). The buoyant fine precipitate which formed was filtered in the cold room by suction through an 8.0 μ pore diameter Millipore® filter. The precipitate was washed with fresh ammonium sulfate solution of the same saturation used for its precipitation. After drying by suction the filter paper containing the precipitate was placed in a covered beaker and stored at -20°C. The filtrates in some of the earlier experiments were treated with ammonium sulfate to saturations of 50,75 and 100%. These treatments did not result in collection of active precipitates and saturation to 25% only was adopted as standard in all the later experiments.

3. Chemical analyses

Protein analysis were performed by the method of Lowry et al (266). Samples were treated with cupric tartrate solution, following

which Phenol reagent (Fisher Scientific Co.) was added. The intensity of the resultant blue color was measured spectrophotometrically at 500 mμ. Samples of bovine plasma albumin (Calbiochem, Lot 52064) were analysed concurrently and the results expressed as μg protein.

Adenine nucleotide analysis was performed on samples in which protein was precipitated by 5% (w/v) perchloric acid. The precipitate was removed by centrifugation. The optical density of the supernatant was measured at 250 mμ. Standard solutions of 5-adenylic acid (Calbiochem) were also analysed and the results expressed as μg adenylate.

Lipids, i.e. triglycerides plus phospholipids were determined by the hydroxylamine method as described in Section D above. No attempt was made to fractionate the lipids by the use of the zeolite absorbtion procedure.

All chemicals and solvents used were reagent grade. The water used had been distilled before passage through a mixed-bed deinonizer column.

4. Assay for inotropic activity

Atria of kittens were suspended in Krebs-Henseleit solution containing 2 g/l of glucose. The bath volume was 10 ml, the temperature 37.5°C. The solution was oxygenated continuously with 95% O_2-5% CO_2. Contractions were obtained by electrical stimulation through platinum electrodes placed close to, but not touching the atria. Suprathreshold rectangular impulses (5-12 volts, 5 millisec. duration) were supplied by a Tektronix stimulator. Isometric contractile force was measured with Grass FT-03 force-displacement transducers and recorded on a Grass polygraph. Resting tensions were maintained at 2 g.

The standard rate of stimulation was 60/min. The initial

value of contractile force was taken at the time this stabilized at the standard rate (20-30 min). The atria were then driven at the rate of 300/min for several 1/2-hour periods between which 5-minute periods of stimulation at the standard frequency were interposed. The bathing solution was changed every 15 minutes throughout. The contractile force reached 30-35% of initial after 2-3 hours of this treatment and high frequency driving was terminated. The failed atria then responded to adrenaline, to increased calcium concentration and to the unknown cardioactive material from the "washings" with increases of contractile force which were expressed as percent of the difference between the initial and the failed contractile force.

The unknown inotropic material deposited on Millipore® filters was dissolved in 0.9% NaCl. In all but the earliest experiments the solution contained 1×10^{-3} g/ml glutathione. The solution was kept in an ice-water bath throughout the assay. The volume was usually 2 ml. Samples for protein and lipid analysis were taken from the same solution and were frozen until analyzed.

SECTION III

RESULTS

THE GAS-PERFUSED CAT HEART

A. Gas Perfusion

The perfusion apparatus provides warmed, humidified gas at a constant pressure of 60 mm Hg. The total flow is divided between the perfused heart and the overflow in the pressure regulator. The flow through the heating coil must be greater than 300 ml/min for proper gas temperature regulation. When flow falls below this figure the mean temperature remains at 37.5°C, but cyclic variations of ± 1 or more degrees are observed. Measurements made during preliminary experiments indicated that the gas flow through hearts weighing 6 - 15 g was 200 to 300 ml/min. These values are similar to those found by Burns et al (241). The period between removal of the heart from the animal and the initiation of perfusion was found to be critical. When this period was held below 5 minutes (the heart being kept in liquid at 4°C for most of that time) the heart would begin to beat immediately upon perfusion with warm liquid. Prolongation of this period inevitably resulted in preparations which beat weakly and which usually fibrillated soon after gas perfusion was initiated. For this reason the general procedure of slitting the ventricles and attaching recording electrodes and transducers was delayed until after cannulation of the aorta and initiation of liquid-perfusion.

The change from liquid to gas perfusion must be rapid so that a sharp interface forms between the two phases in the cannula. The change in perfusate is then essentially instantaneous in the coronary vasculature. Formation of bubbles causes a rapid increase in resistance to flow due to the high surface tension between such multiple interfaces and the coronary vessels. In practice, a sharp interphase may be observed moving through the translucent walls of coronary arteries lying on the myocardial surface.

The injection of small volumes of drug-bearing liquid during gas perfusion must also be done rapidly so that the liquid passes through the vasculature as a single unit.

In preliminary experiments, the hearts of several rabbits, rats and guinea pigs were perfused with gas. Their contractile force declined rapidly after the initiation of gas perfusion. Since the kitten heart responded well to gas-perfusion, all subsequent experiments were performed using this species.

The 60 mm Hg perfusion pressure was chosen because preliminary experiments had indicated the occurrence of ventricular extra-systoles at higher pressure (to be discussed). 95% O_2 - 5% CO_2 was chosen as the gaseous perfusate because Sabiston's and Burns' reports had indicated that this mixture was superior to pure oxygen for prolonged administration. Their findings are purely empirical, and no explanation is immediately forthcoming, except perhaps that the 5% CO_2 may have aided in maintenance of extracellular pH. It is obvious that gas-perfusion does not provide the heart with exogenous substrates. Comparison with liquid-perfused hearts has therefore been made using substrate-free Krebs-Henseleit solution as the control perfusate.

B. Effects of Perfusion with Liquid and Gas on Contractility and Heart Rate

Isometric contractile force at a constant resting tension of 10 g remained stable for 10 minutes in a group of 6 hearts perfused with liquid in a recirculating system. Thereafter, contractility decreased and had fallen to 50% of control at the end of 80 minutes of perfusion. The contractile force fell thereafter at a faster rate (1%/min vs. 0.6%/min) and reached 10% of initial after 120 minutes of perfusion (Fig. 4).

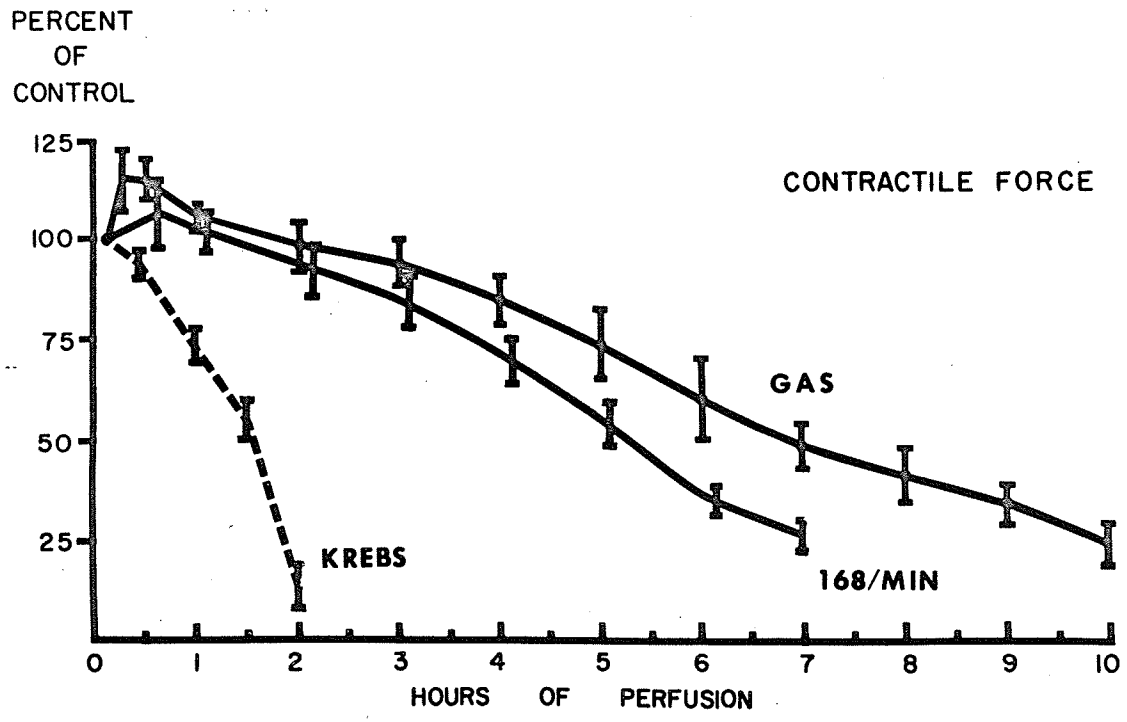

Figure 4. Time course of isometric contractile force. The group labelled 168/min was electrically driven during gas perfusion. Bars show S.E.

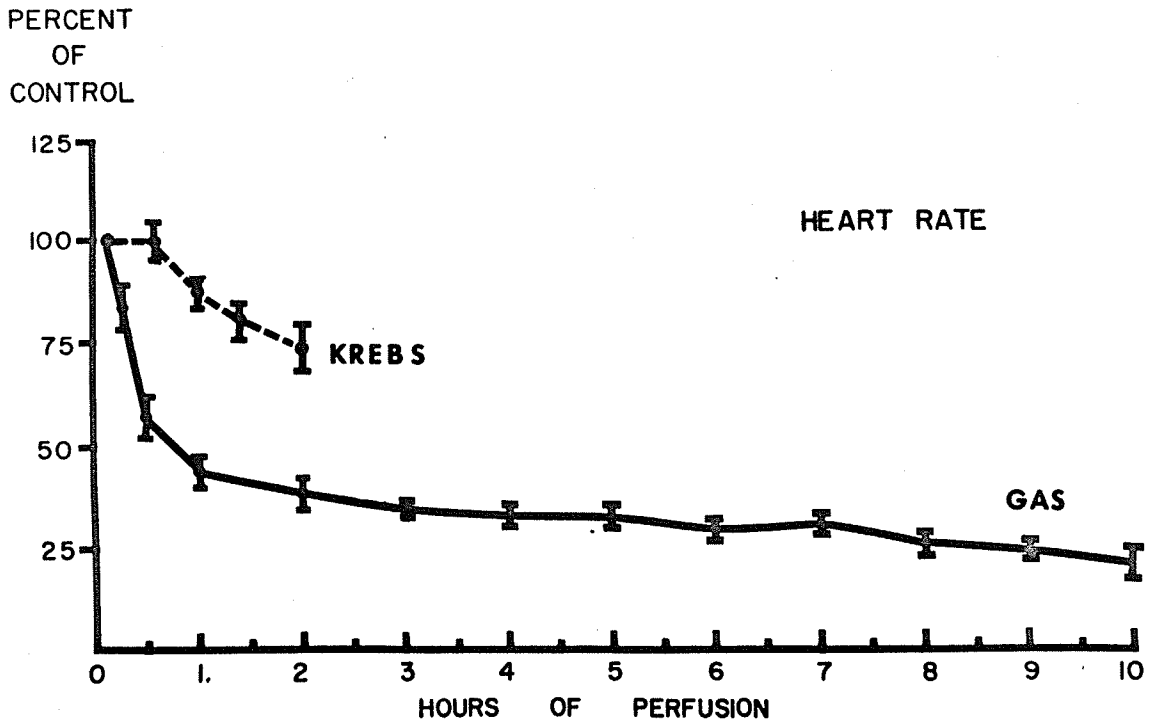

Figure 5. Time course of heart rate during perfusion. Same hearts as in Fig. 4. Bars show S.E.

The heart rates in this group (163 ± 8 beats/min) declined gradually during this period to 118 ± 9 beats/min, this representing a decrease to 73 ± 6% of the initial value (Fig. 5).

The contractility of 4 gas-perfused hearts increased during the first 30 minutes of perfusion and remained elevated (15 ± 5%) for a further 30 minutes. The mean did not fall below the initial value for three hours. Contractility then fell gradually (-0.2% min), reaching 50% of initial after 7 hours and 25% of initial at the end of 10 hours perfusion (Figure 4). Heart rates in this group fell precipitously during the first hour of perfusion to 43 ± 3% of initial, but remained essentially stable for the remaining 9 hours.

The difference in the rate of decrease in contractile force might have been due to the great difference in heart rates. Therefore, four hearts were perfused with gas while being electrically driven at 168 beats/min. The increase in contractility during the first 30 minutes was only slightly less than that of the other group but was not statistically significant. The subsequent gradual decrease in contractility was similar to that of the undriven gas-perfused group. The strength fell to 50% of initial after 5 hours and to 25% of initial after 7 hours (Fig. 4).

In agreement with the observations of Cattell and Gold (107) and Luisada and Weiss (267), myocardial tissue maintained with liquid showed an apparent shortening or "contracture" with time, necessitating periodic adjustment of the force displacement transducer in order to maintain a constant resting tension. This was not the case during gas-perfusion. Two hearts which had been perfused with liquid for two hours were then perfused with gas. The diastolic tension decreased in

both cases. The maximum decrease was reached within 3 minutes. When resting tension was returned to 10 g, the contractility was approximately 20% greater than that before beginning gas perfusion.

In three other experiments, hearts were perfused with liquid for two hours, during which time isometric contractile force was recorded at constant diastolic tension (10g). Isotonic contractility was then recorded with a load of 10 g, following which perfusion with gas was begun. As shown in the example in Fig. 6, gas-perfusion caused a pronounced lengthening of the heart, with a concomitant increase in the contractile strength (30%). Fifteen minutes later perfusion was changed to liquid and the length of the preparation observed to diminish to the original.

C. Arrhythmias During Gas-Perfusion

Alternation in the contractile force was observed during gas-perfusion in 45 of 50 hearts. This usually began during the first 15 minutes of perfusion while contractile force was increasing, and was usually preceded by a short episode of ventricular extrasystoles. In many instances, a slight, transient ($\leq$ 5 minutes) decrease in contractility occurred during this period, but was followed by a continuing increase in contractility. The difference between alternate contractions, very small initially, increased during the next 45 minutes and remained constant thereafter. The transient decrease in force is not seen in the composite curve (Fig.4) because of the variation in starting time of the alternation.

Electrograms were obtained from 37 hearts with contractile alternans. No electrical alternation was seen in 27 of these (73%).

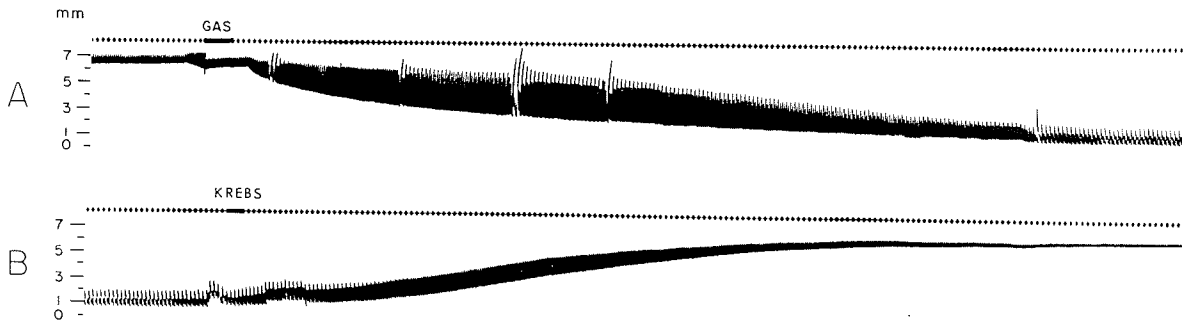

Figure 6, A: Effect of gas perfusion on isotonic contractility and diastolic "length" of a heart failed by prolonged perfusion with liquid. B: Same heart, liquid perfusion reinstated 15 minutes later.

The weaker beat in each of these hearts began after relaxation of the stronger beats was complete (Fig.7). In the remaining 10 hearts (27%) both contractile and electrical alternation was observed. In these instances, the weaker beat began before complete relaxation of the preceding stronger beat was complete. Alternation was not observed in the liquid-perfused hearts, although transient periods of ventricular extrasystoles were observed in the final ½ hour of perfusion in 4 (of 12) hearts.

Six hearts were subjected to increased perfusion pressure after 10 minutes of liquid perfusion. Electrograms and contractile force were recorded. The mean threshold for ventricular extrasystoles in this group was 187 ± 12 mm Hg. Fibrillation occurred in 2 instances. Following the return to the usual perfusion pressure (60 mm Hg) the contractile force in the remaining 4 hearts was 62 ± 10% of the pre-arrhythmic value and decreased rapidly thereafter.

Six hearts were perfused with gas for 20 minutes and the perfusion pressure then increased. The mean threshold for induction of the arrhythmia in this group was 124 ± 9 mm Hg which was significantly lower than that of the liquid perfused group ($P = <0.01$). Records from one experiment are shown in Fig. 8. The atrial beat appeared to remain normal during the arrhythmia. Upon return of the pressure to normal the pre-arrhythmic contractile force and electrograms were reestablished. The pressure at which the arrhythmia reverted to sinus rhythm when the pressure was lowered was identical to that at which the arrhythmia was initiated during the phase of rising pressure. The threshold pressure for arrhythmia in four gas-perfused hearts remained constant (± 5mm Hg)

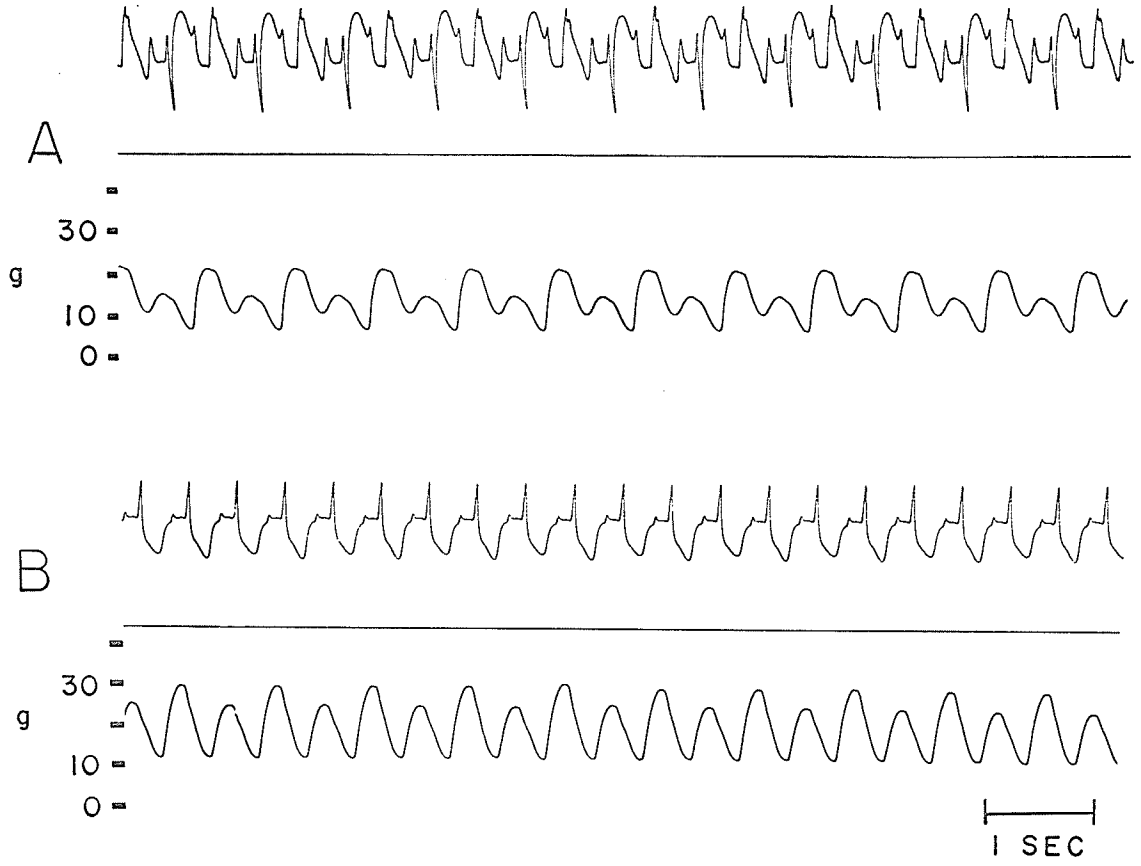

Figure 7. Contractile alternation during gas perfusion. Upper channel: electrogram, lower channel: isometric contractile force.
A: Electrical alternation accompanies mechanical alternation.
B: Mechanical alternation only.

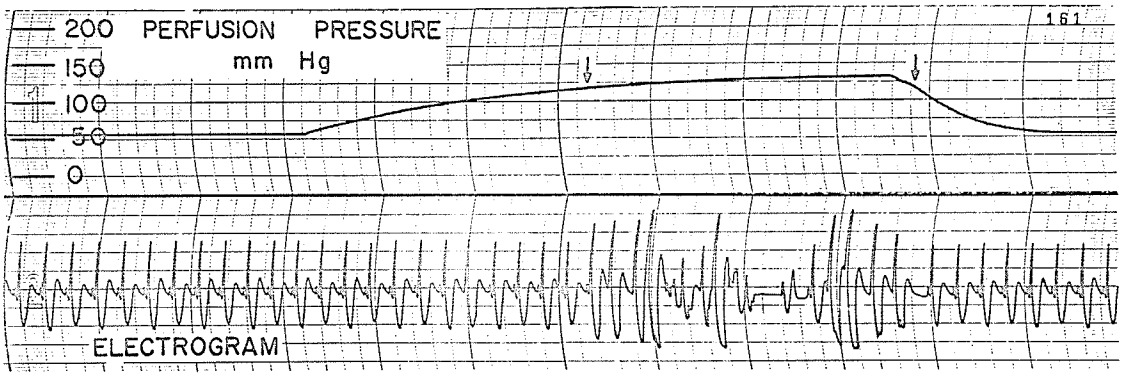

Figure 8. Response of a gas-perfused heart to increased perfusion pressure. Electrogram recorded with electrodes on right atrial appendage and on ventricular apex. The arrows indicate threshold pressure (120 mm Hg) for induction of arrhythmia and for return to sinus rhythm on decreasing the perfusion pressure.

when tested every 20 minutes for three hours.

D. Starling's Relationship

Five hearts electrically driven at 180 beats/min, were perfused with liquid for ten minutes. The effect of graded increases in resting tension on isometric developed tensions was then determined. Gas-perfusion was then started and the procedure repeated as soon as contractile force had become stable (approx. 15 minutes). The hearts were removed from the cannula and weighed. The relation of resting to developed tension, both expressed in terms of heart weight is shown in Fig. 9. For any resting tension, developed tension was greater during gas perfusion than during liquid perfusion. Imposed resting tensions were not allowed to exceed 35 g/g because it was found that excessive tensions caused irreversible changes in contractility.

Four hearts, driven at 180 beats/min were perfused initially with liquid containing glucose, 2 g/l. External work output was determined electronically (see Methods). Perfusion with substrate-free liquid was then initiated and the procedure repeated. Following this, the work determination was repeated during liquid + glucose-perfusion. This was done in order to ascertain that the short period of perfusion with substrate-free liquid had not changed the performance of the heart. Gas-perfusion was then begun, and the procedure repeated. Following this, the heart was removed from the cannula and weighed. The time required for applying the various loads during each perfusion period was less than ten minutes. Fig. 10 shows a characteristic series

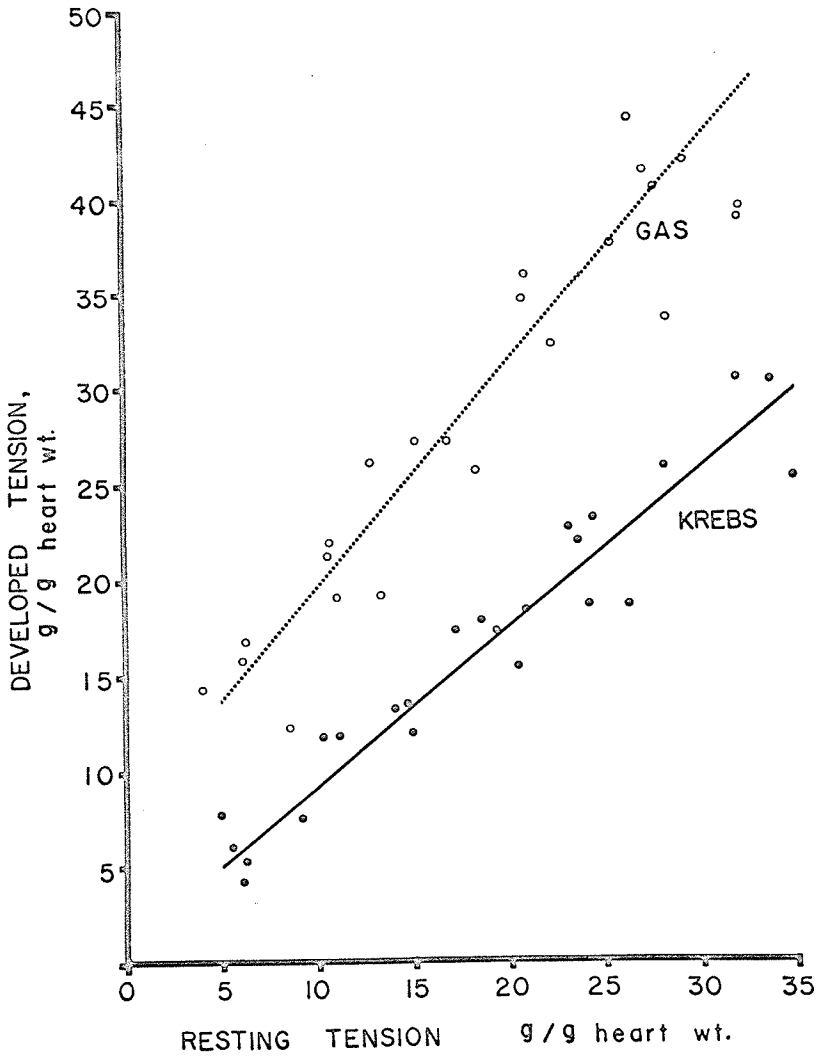

Figure 9. Relationship between resting tension and isometric developed tension in hearts perfused first with Krebs solution and then with gas. Rate: 180/min. Regression lines calculated by method of least squares.

of curves obtained in one heart. Except at the smallest and largest loads used, work output during gas-perfusion was approximately 1.5 times that observed during substrate-free liquid-perfusion whereas work output in the presence of glucose was three times that observed in its absence. The variation between hearts in work output at any given load made direct comparison between them difficult. It was found empirically that if work output of each heart during liquid, liquid + glucose, and gas-perfusion were expressed as a percentage of the maximum work obtained during perfusion with liquid + glucose, the data were normalized. Fig. 11 shows these normalized curves to have the same quantitative relationship to each other.

E. Pharmacological Studies

Injections of 1 ml of Krebs-Henseleit solution resulted in a transient (less than 20 seconds) 10% increase in contractile force. There was no change in heart rate. Injections of 1 ml of Krebs solution containing adrenaline, noradrenaline and isoproterenol (0.01 to 0.5 µg/ml) increased contractility to 10 - 200% of control. Dose-response curves of contractility were determined for isoproterenol and adrenaline in four hearts (Fig. 12). Changes in heart rates were not recorded. Isoproterenol was found to be approximately 10 times more potent than adrenaline. Other drugs such as acetylcholine and histamine (0.1 to 1.0 µg/ml) showed their usual negative and positive inotropic effects, respectively, on electrically driven preparations (Fig. 13).

The duration of action of these drugs (3 - 5 min) did not differ from that observed upon injection of equally effective doses

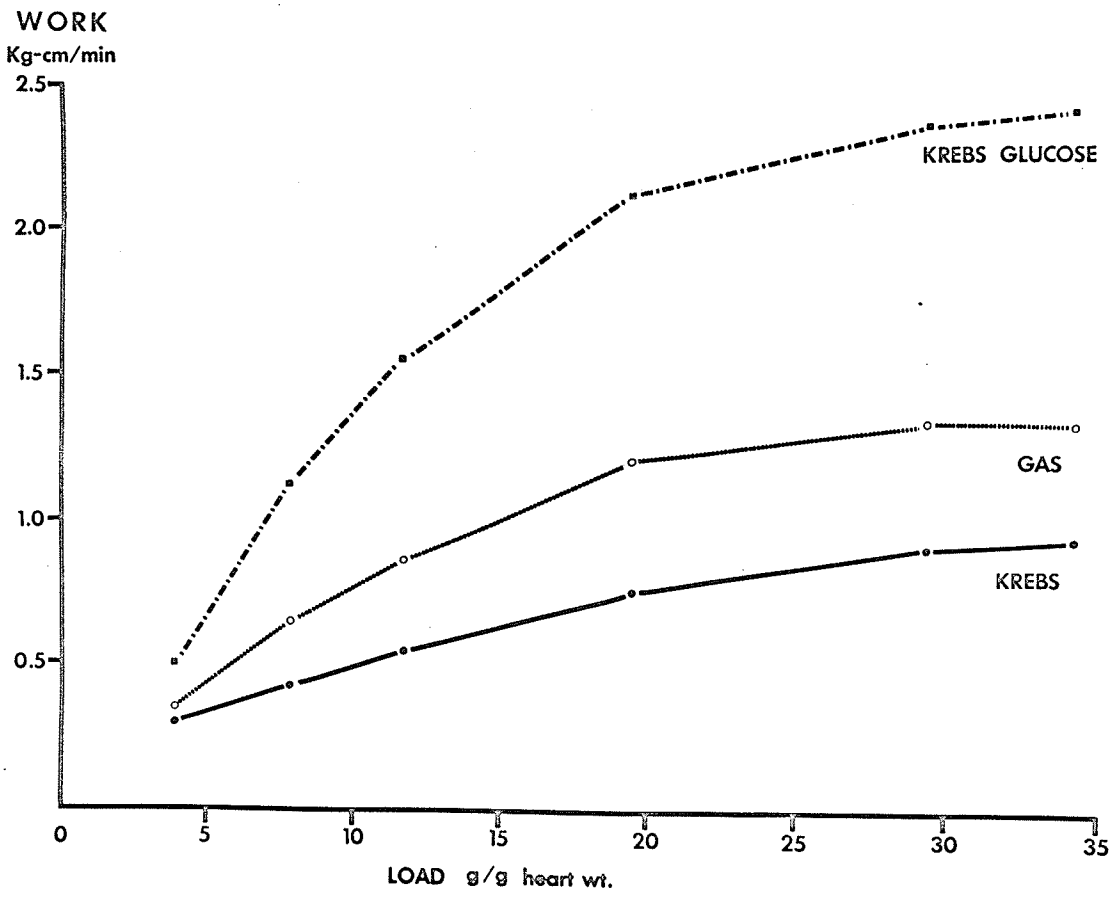

Figure 10. Effect of various loads on the work output of one heart perfused with Krebs plus glucose, substrate-free Krebs solution, and gas. Heart rate 180/min.

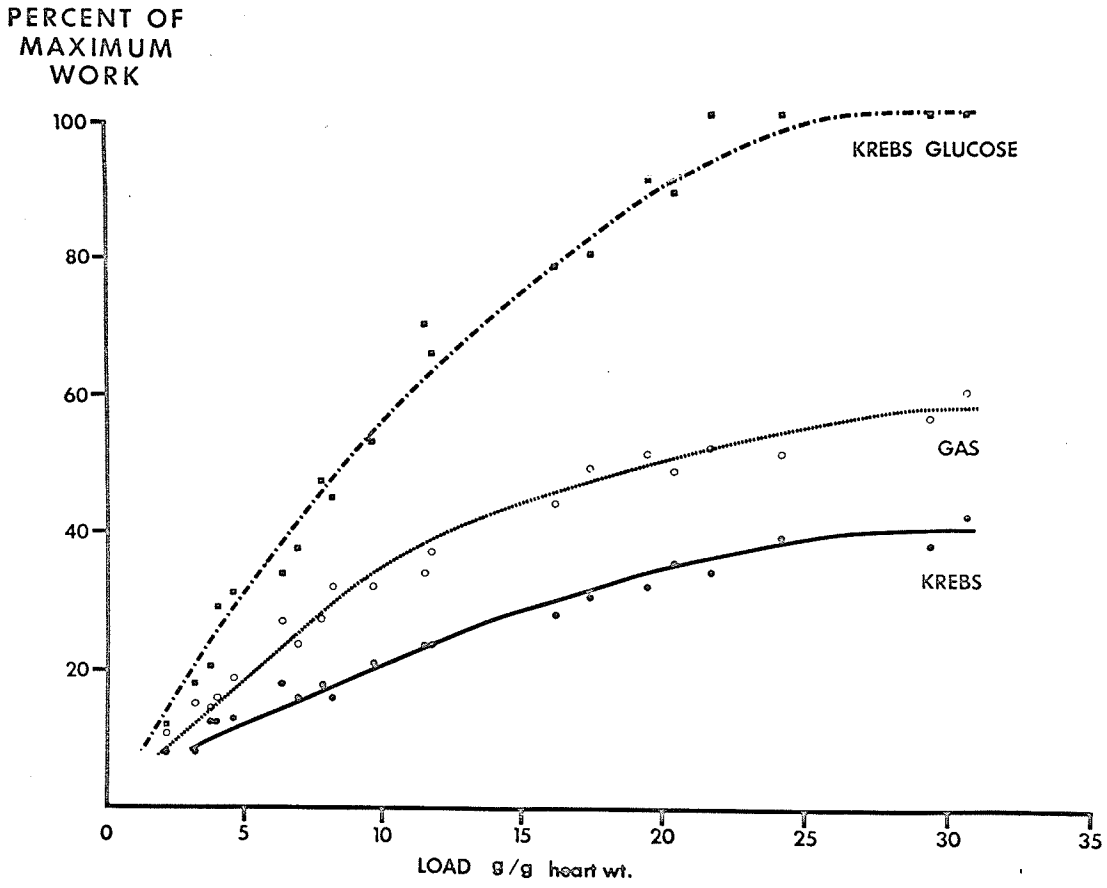

Figure 11. "Normalized" curves for 4 hearts treated as in Fig. 10, work output of each heart is expressed as percent of maximum obtained during perfusion with glucose-containing solution.

into liquid-perfused controls.

In 4 experiments the stimulus voltage of driven preparations was increased to 10 X threshold in order to determine the response of the gas-perfused heart to the release of endogenous catecholamines. As can be seen in Fig. 14, the positive inotropic response to this procedure as well as its duration following return to normal voltage levels are increased by prior administration of 1.0 ml of cocaine hydrochloride solution (1.0 μg/ml). The potentiation due to cocaine administration could be abolished by a brief (3 - 5 minutes) period of liquid-perfusion. Administration of 1.0 ml of Pronethalol, (1 μg/ml) did not significantly alter contractile force, but prevented the increase in contractility due to increased stimulus voltage.

Three hearts were obtained from animals which had received reserpine, 2 mg/kg IP, 24 hours prior to sacrifice. They were unresponsive to increases in stimulus voltage.

Injections of ouabain (1×10^{-9} to 1×10^{-6} g/ml) were made at irregular intervals between the fourth and tenth hours of gas perfusion. No positive inotropic effects were seen in 7 experiments of this type, nor was there any modification of the rate of spontaneous failure of these hearts. The addition of ouabain (1×10^{-9} g/ml) to the pre-perfusion fluid also did not affect the time course of contractile force changes during gas perfusion (see Section IV-C).

It has been pointed out that 90% of gas-perfused hearts develop contractile alternans which is only occasionally associated with electrical alternation. Only 2 of 4 hearts pretreated with 1×10^{-10} g/ml ouabain developed alternans, and none of 8 hearts treated with 3×10^{-10} or with 1×10^{-9} g/ml developed contractile alternans.

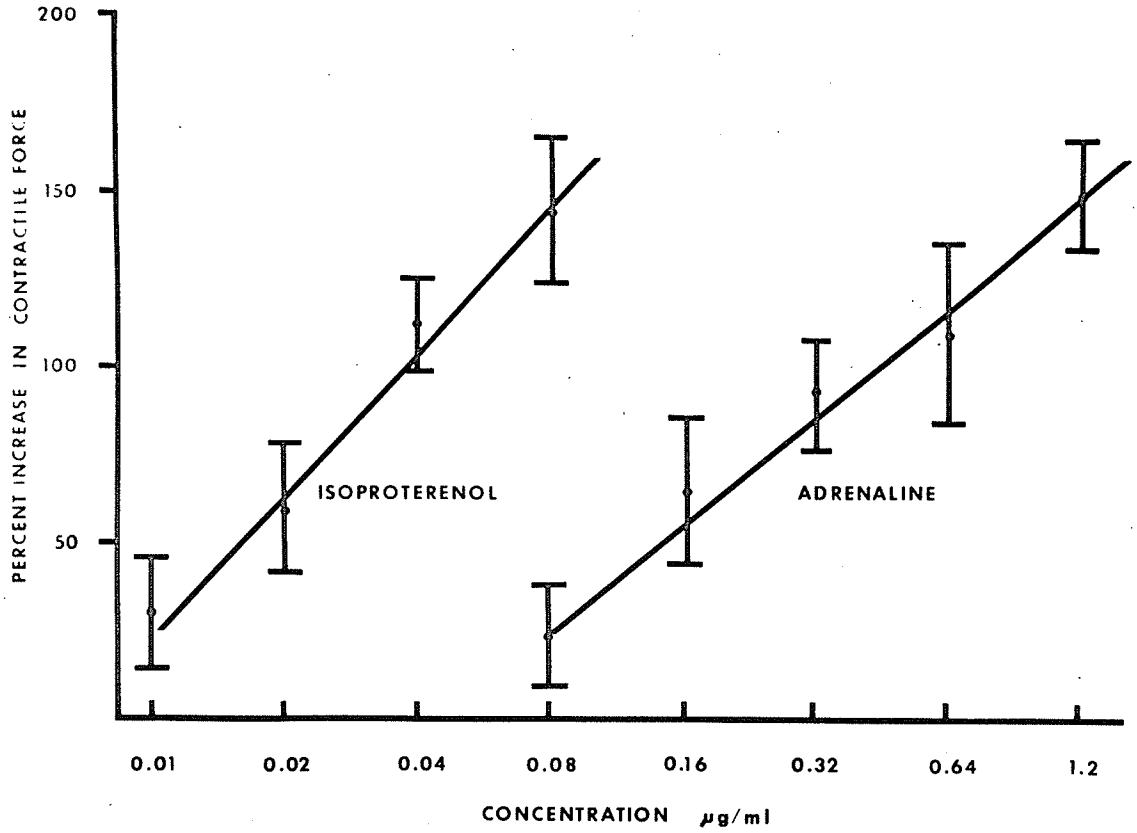

Figure 12. Effect of catecholamine administration on contractile force during gas perfusion. Bars show S.E. (four hearts).

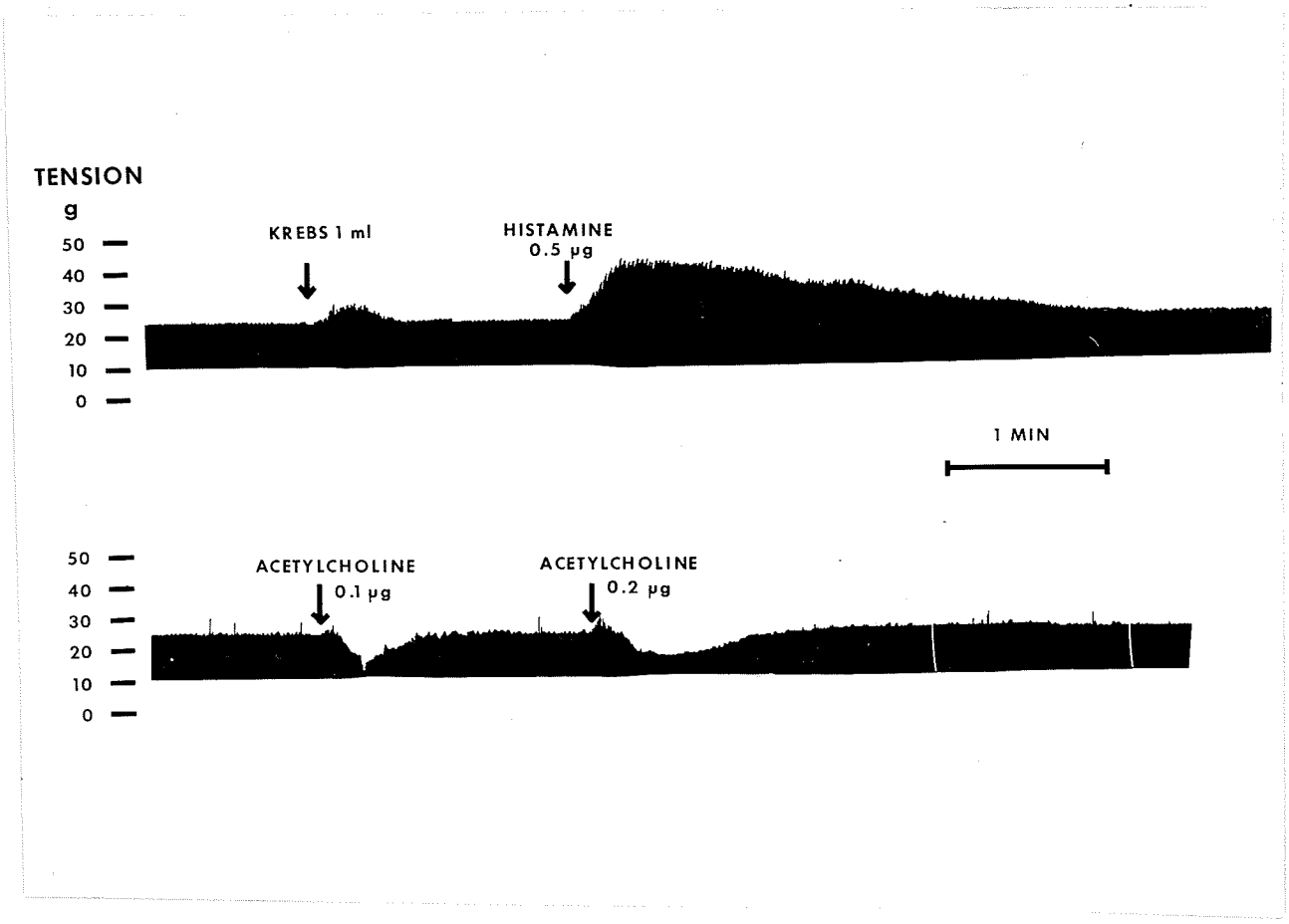

Figure 13. Effect of histamine and acetylcholine administration on the contractile force of a gas-perfused heart. Heart electrically driven at 180/min.

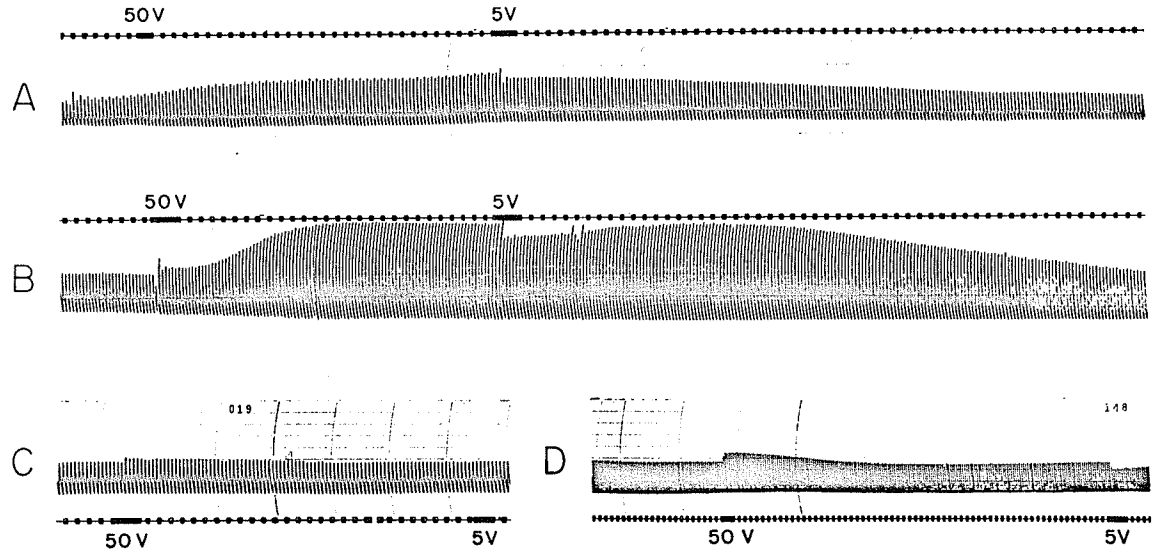

Figure 14. Effect of drugs on the contractile response of gas-perfused hearts to catecholamines released by supramaximal electrical stimulation. Heart rate 180/min. A: Control; B: after cocaine 1 µg; C: cocaine washed out, treated with pronethalol 1 µg; D: as in A, but heart obtained from an animal treated with reserpine 24 hours previously.

F. Biochemical Studies

Isolated hearts are rapidly depleted of their glycogen stores when perfused with substrate-free liquid (27,96,97,99). It has also been demonstrated that the oxygen consumption of such tissue falls by no more than 25%, indicating that some endogenous source of energy is utilized under such conditions (3,27,103). Contractility increases when the heart is challenged with catecholamines (32), and lactate is produced during hypoxia as in glucose-supplied hearts (96,97).

Shipp et al (99) have demonstrated that isolated rat hearts subjected to one hour's perfusion with substrate-free liquid utilize their endogenous lipid (especially phospholipid) as sources of energy.

Gas-perfused hearts beat for considerably longer periods of time than did their liquid-perfused controls. Was this because of metabolic differences? It was therefore of interest to examine the gas-perfused heart and its liquid-perfused control in regard to these aspects of their metabolism.

Hearts were removed from four kittens, the ventricles frozen and powdered and later analyzed. The values for various myocardial fractions found in this group served as control for the other groups. These were perfused as follows:

GROUP	PERFUSATE	PERIOD OF PERFUSION	HEART RATE	RESTING TENSION
I	Liquid	5 Min.	Sinus	10 g
II	Liquid	2 Hrs.	Sinus	10 g
III	Gas	5 Min.	Sinus	10 g
IV	Gas	3 Hrs.	Sinus	10 g
V	Gas	3 Hrs.	168/Min*	10 g
VI	Gas	3 Hrs.	168/Min*	25 g
VII	Gas	3 Hrs.	180/Min*	10 g
VIII	Gas	6 Hrs.	Sinus	10 g
IX	Gas	10 Hrs.	Sinus	10 g

* Electrically Driven

Groups I, II, III, IV and IX were analyzed for water, glycogen, total carbohydrate, lactate and pyruvate content. The results are shown in Table I. Water content was less than control in all groups except in those perfused with liquid for 2 hours (Group II). This may be explained by the fact that the control group was not perfused with gas before freezing, whereas the vasculature of all groups had been cleared of liquid by gas perfusion. The difference in water content between the control group and the 5 minute liquid-perfused group (Group I) or the 5 minute gas-perfused group (Group III) represents the water content of the coronary vessels, and agrees with published accounts of the volume of this compartment (268). The absence of any change in water content during 10 hours of gas-perfusion (Group III vs. IX) indicates that adequate humidification of the perfusate and of the environment was achieved in the perfusion system. The larger water content of

TABLE I: EFFECT OF PERFUSIONS ON WATER AND CARBOHYDRATE CONTENTS

	Group and Treatment	Water content percent	Glycogen µmoles/g	Other carbohydrates µmoles/g	Total carbohydrates µmoles/g	Lactate µmoles/g	Pyruvate µmoles/g
	Control (0 time)	80.9 ± 1.5	72.4 ± 3.8	47.9 ± 4.6	121.3 ± 8.2	95.7 ± 7.6	0.16 ± 0.02
I	Liquid 5 min.	76.6 ± 0.4*	23.8 ± 2.0*	19.3 ± 1.9*	44.6 ± 5.8*	33.2 ± 4.1*	0.08 ± 0.01*
III	Gas 5 min.	76.1 ± 0.3	13.8 ± 0.1*	15.6 ± 1.5	29.2 ± 2.6*	10.6 ± 2.5*	< 0.03**
IV	Gas 3 hours	76.3 ± 0.4	13.4 ± 0.5	9.2 ± 0.9*	22.8 ± 1.8	< 0.1**	< 0.03**
IX	Gas 10 hours	76.8 ± 0.5	13.6 ± 0.1	10.0 ± 0.6	23.9 ± 0.9	---	< 0.03**
II	Liquid 2 hours	84.3 ± 1.9*	19.6 ± 0.7*	17.4 ± 1.8*	38.2 ± 3.1*	9.2 ± 1.9*	< 0.03**

means ± standard error, four hearts per group; results calculated per g/dry wt. of tissue.

as glucose equivalents.

* significantly different ($P < 0.05$) from preceding group and control.

** pooled sample

isolated tissues after prolonged perfusion with liquid media (Group II) has often been reported (170,172).

Tissue glycogen content decreased precipitously during the early stages of both liquid and gas-perfusion (Groups I and III). Prolonged perfusion with either perfusate produced only negligible decreases thereafter. Changes in non-glycogen and total carbohydrate contents closely paralleled those of the glycogen levels. Lactate and pyruvate contents also declined rapidly during the early periods of perfusion, and fell to values so low that pooling of tissue in some groups became necessary.

Tissues from Groups II, IV, VIII and IX (liquid 2 hours, and gas 3, 6 and 10 hours) were analysed for the following: Total lipid, phospholipid, triglyceride, free fatty acid and total and esterified cholesterol. The results are shown in Table II.

In agreement with the observations of Shipp et al (99) it was found that liquid-perfused hearts utilized lipid to supply their energy, and that this energy came primarily from the metabolism of the phospholipid fraction. Small decreases without statistical significance ($P > 0.05$) in the triglyceride and esterified cholesterol fractions were noted. The free fatty acid and total cholesterol fractions were unchanged.

Gas-perfused hearts also utilized lipids to supply their energy. Phospholipids were calculated to represent 93% of the total loss of lipid material lost during the first three hours of perfusion. This calculation was made on the basis of the combined theoretical total fatty acid content of the various fractions. It was also calculated that phospholipids comprised 83% of the total lipid used during 10 hours

TABLE II: EFFECT OF PERFUSIONS ON LIPID CONSTITUENTS

	Group and treatment	Total lipid mg/g	Phospholipids µmoles P/g	Triglycerides µmoles/g	Free fatty acids µmoles/g	Total cholesterol µmoles/g	Esterified cholesterol, as % total cholesterol
	Control (0 time)	155.9 ± 2.3	156.3 ± 2.8	11.5 ± 1.5	51.4 ± 3.3	23.2 ± 0.9	57.5 ± 1.6
IV	Gas 3 hours	147.9 ± 3.4*	131.8 ± 2.1*	11.5 ± 1.4	49.1 ± 2.4	23.7 ± 0.9	49.8 ± 0.7*
VIII	Gas 6 hours	142.1 ± 1.9**	122.3 ± 5.7**	10.3 ± 0.8	45.4 ± 3.0	24.9 ± 0.6	51.8 ± 0.3*
IX	Gas 10 hours	131.8 ± 3.6**	112.4 ± 3.8**	9.3 ± 0.5*	40.8 ± 2.3*	24.3 ± 0.5	49.7 ± 2.2*
II	Liquid 2 hours	145.0 ± 2.7*	134.3 ± 3.9*	10.9 ± 1.4	50.8 ± 3.7	25.1 ± 1.0	53.7 ± 1.9

means ± standard error, four hearts per group; results calculated per g dry wt. of tissue.

* significantly different ($P < 0.05$) from control.

** significantly different ($P < 0.05$) from preceding group.

of gas perfusion. Decreases in the triglyceride and free fatty acid fractions became statistically significant ($P < 0.05$) only at the end of 10 hours of gas-perfusion.

Hearts perfused with liquid for 2 hours contained the same amount of phospholipid as hearts perfused with gas for 3 hours. The former group, it should be noted, had failed by the end of the perfusion period, whereas the contractile force of the gas-perfused group did not differ from control. Since the heart rates of these preparations differed considerably, it became of interest to correlate the total number of heart beats with the total consumption of phospholipids. The mean number of beats in each group were obtained by counting from the polygraph record. Three additional groups were perfused with gas for three hours with electrical control of heart rate (Groups V, VI and VII) and diastolic tension was increased in one group from the usual 10 g to 25 g (Group VI).

The results, expressed as phospholipid utilization and number of beats during the perfusion periods are shown in Table III. They are also shown graphically in Fig. 15. It can be seen that in all groups in which resting tension was maintained at 10 g, a clear correlation exists between phospholipid utilization and the total number of beats, independent of the period of perfusion or the time course of contractile force. Group VI was perfused for three hours and had the same number of beats as Group V (30,240 beats/3 hrs.) but differed from it in that resting tension was maintained at 25 g. The increased resting tension caused a 30% increase in phospholipid consumption over the three hour period.

TABLE III

PHOSPHOLIPID CONSUMPTION AS A FUNCTION OF RESTING TENSION AND TOTAL NUMBER OF BEATS

Group and Treatment	Resting tension (g)	Total No. of beats	Phospholipid content μmoles P/g dry wt.	Phospholipid consumption μmoles P/g dry wt.
Control	-	-	156.3 ± 2.8	-
II Liquid 2 hours	10	15,962 ± 843	134.3 ± 3.9	22.0 ± 4.8
IV Gas 3 hours	10	18,903 ± 931	131.8 ± 2.1	24.5 ± 3.5
V Gas 3 hours*	10	30,240	119.8 ± 3.1	36.5 ± 4.2
VI Gas 3 hours*	25	30,240	108.5 ± 2.6	47.8 ± 3.9
VII Gas 3 hours*	10	32,400	117.8 ± 3.6	38.5 ± 4.6
VIII Gas 6 hours	10	27,918 ± 1,202	122.3 ± 5.7	34.0 ± 6.4
IX Gas 10 hours	10	37,808 ± 1,921	112.4 ± 3.8	43.9 ± 4.7

* Electrically driven

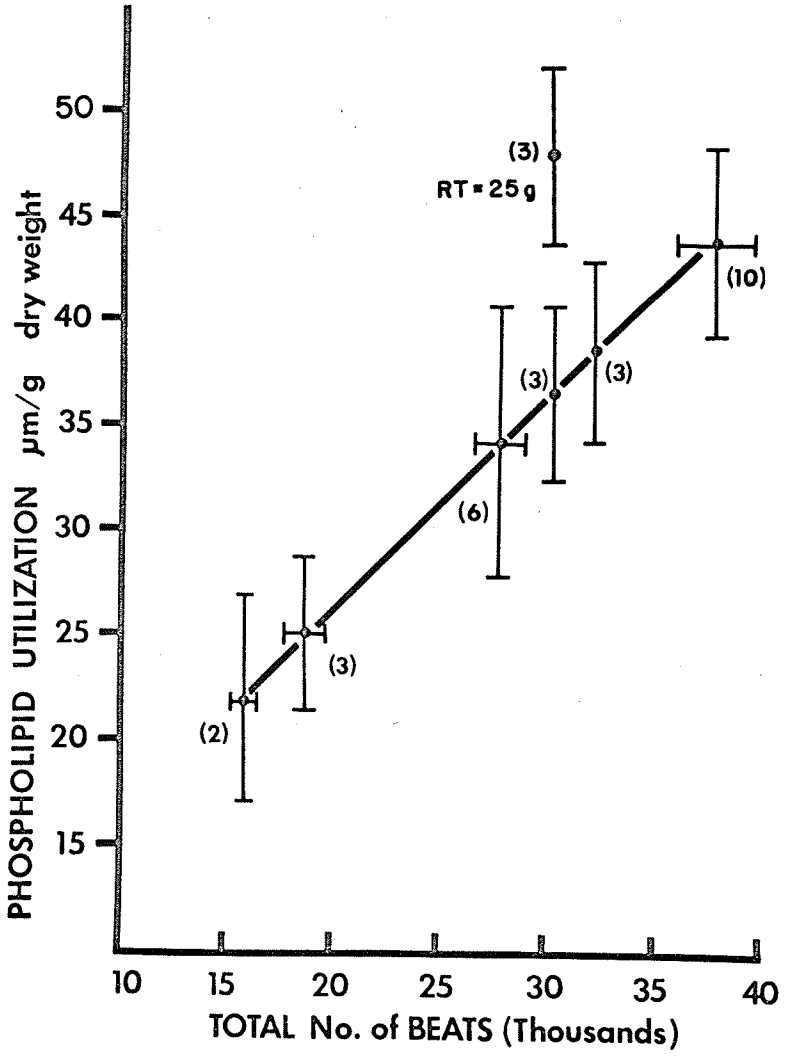

Figure 15. Relationship between phospholipid utilization and number of heart beats. The numbers in parentheses refer to hours of perfusion. The 2-hour group was liquid-perfused, all others were gas-perfused. Resting tension was 10 g except in group shown upper center. Horizontal bars: S.E. of heart rates in spontaneously beating preparations. Vertical bars: S.E. of phospholipid consumption. Refer to Table III for calculations.

The data from Table 3 and Figure 15 were used to calculate the basal or "resting" phospholipid consumption and the consumption per heart beat using the equation:

$$y = Ax + bt$$

WHERE:

y = total phospholipid consumption (μ moles/g)

a = total number of beats

x = phospholipid consumption (μ moles/g/beat)

b = resting phospholipid consumption (μ moles/g/hour)

t = time in hours.

By substitution of the appropriate values, the resting consumption was found to be 2.1 μ moles/g/hour. The consumption per heart beat was 0.00102 μ moles/g. The confidence limits of these values were not estimated.

An interesting calculation can be made based upon the assumptions that if (a) the phospholipid contains 2 fatty acid moieties, (b) these fatty acids are structurally similar to oleic acid, and (c) that they are completely oxidized. Upon these assumptions, the QO_2 may be calculated, since 1 μ mole phospholipid would theoretically require 1209 ml of oxygen for complete oxidation. The QO_2 in ml/mg dry wt./hr is 13.3 for the liquid perfused group, and ranges from 9.8 to 15.5 for groups IV, V and VII (perfused for 3 hours). These figures, whose validity is questionable because of the previously stated assumption, nevertheless are similar to the published values of 6-39 μl/mg/hr for cat heart under somewhat similar conditions of work and rate (27,33,269).

G. Electrolyte Studies

Groups of hearts were gas-perfused for 5, 60, 120 or 180 minutes, 35.0 ml of iso-osmotic sucrose solution were then perfused, and the effluent collected and analyzed for sodium and potassium in fractions. Fig. 16 shows the results of sucrose perfusion on a heart perfused with gas for five minutes. It can be seen that the sodium content of the sucrose solution, high in the first few fractions, decreased sharply thereafter. Fig. 17 shows the same data expressed as cumulative milli-equivalents of sodium collected vs. the sucrose volume collected. As can be seen, in Fig. 16 approximately 90% of the total cumulative sodium collected was in the first 10 ml of sucrose perfusion. This was also true for the potassium collected in the perfusate. It is to be expected that the sucrose solution would remove a certain amount of intracellular electrolytes from the heart. This could not be estimated with any accuracy but is apparent from the low slope of the electrolyte content of the late samples obtained. For the purpose of calculations, it was assumed that the first 10 ml of sucrose perfusate had removed effectively all the extracellular electrolytes. It is this value which is given in Table IV.

No estimation of the extracellular space was made in these experiments. However, it is reasonable to assume that this space was in equilibrium with the liquid perfusate which contained 144 mEq/l of sodium ion. It was further assumed that no change in the extracellular sodium concentration occurred during 5 minutes of gas perfusion. From Table IV, line 1, the extracellular space was then calculated as $\left[\frac{3.025}{144} \times 1000\right]$ or 21.0 ml/100 g tissue. This figure was used to calcu-

TABLE IV

SODIUM AND POTASSIUM REMOVED BY SUCROSE PERFUSION

	Group and treatment	Na^+ mEq/100 g wet wt.	K^+ mEq/100 g wet wt.
I	Control, Gas 5 min.	3.025 ± 0.286	0.126 ± 0.011
II	Gas, 1 hour	2.694 ± 0.281*	0.185 ± 0.22*
III	Gas, 2 hours	2.101 ± 0.253*	0.211 ± 0.030*
IV	Gas, 3 hours	2.023 ± 0.181**	0.203 ± 0.019**
V	Ouabain, 1×10^{-9} g/ml pre-perfusion, Gas 3 hours.	2.001 ± 0.187**	0.216 ± 0.022**

means ± standard error, four hearts per group.

* significantly different from control group ($P < 0.05$).

** not significantly different from previous group ($P > 0.05$).

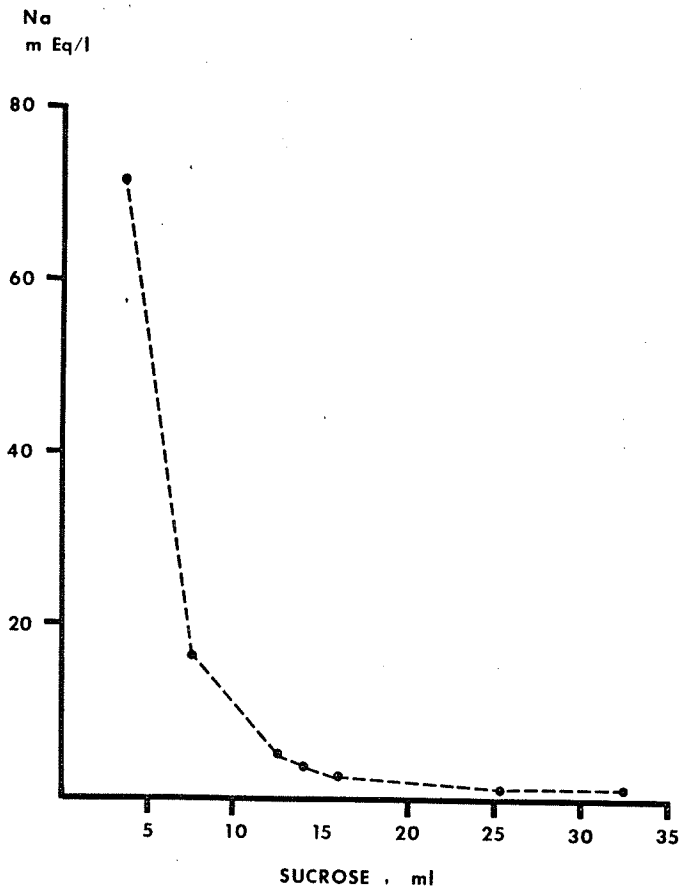

Figure 16. Concentration of Na^+ in sucrose perfusate after 5 minutes of gas perfusion. Heart weight (wet) = 10.46 g.

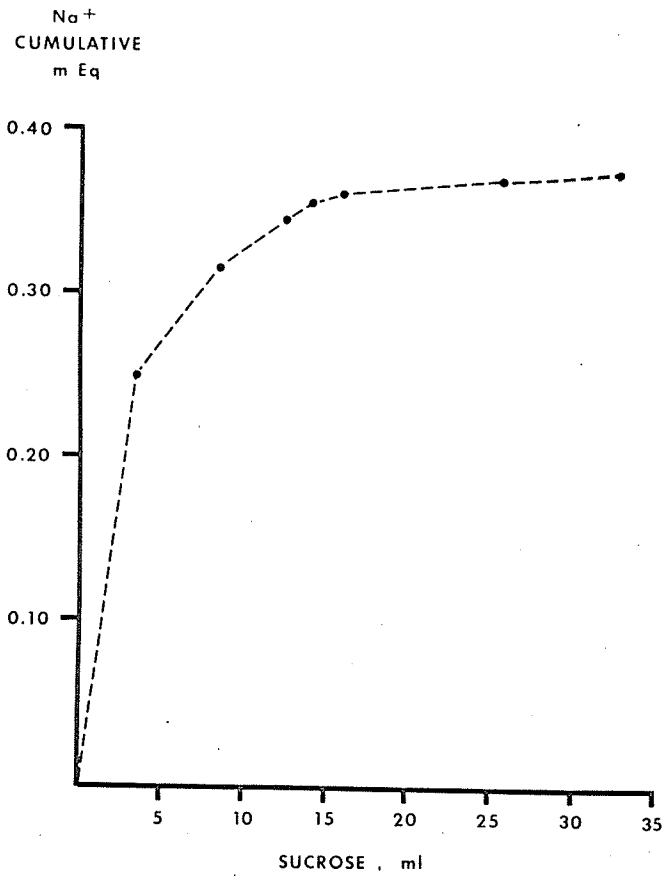

Figure 17. Same data as in Fig. 16, but expressed as cumulative or total sodium collected during sucrose perfusion.

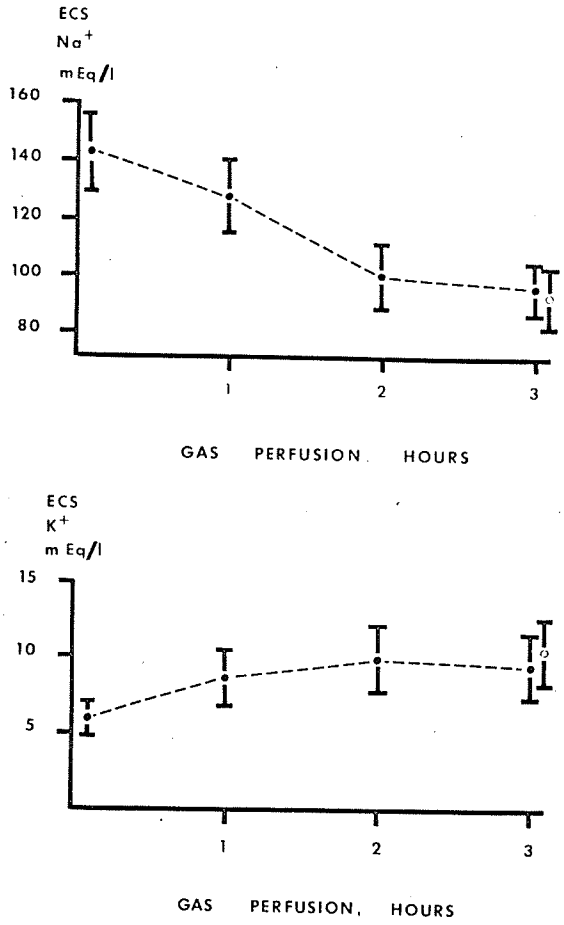

Figure 18. Calculated extracellular space electrolyte content during gas-perfusion. See text for method of calculation. Bars show S.E. Open circles: hearts pre-perfused with ouabain, 1 x 10^{-9} g/ml.

late the extracellular sodium and potassium concentrations of the extracellular space shown in Fig. 18. It should be stated that this further calculation makes the additional assumption that no change in extracellular fluid volume occurred during the perfusions, an assumption which finds only minimal justification in the total water content values shown in Table I.

Fig. 18 shows that net shifts in sodium and potassium ions occurred only during the first two hours of gas perfusion. It would appear that under the conditions of these experiments, in which there is no equilibration between the extracellular fluid compartment and a third (vascular) compartment of constant composition, an 'equilibrium extracellular concentration' of ions is reached. Ouabain, in a concentration which will later be shown to be "therapeutic" in this preparation (see Section IV), did not affect this "equilibrium concentration".

SECTION IV

RESULTS

A MECHANISM FOR FAILURE OF LIQUID-PERFUSED HEARTS AND FOR THE INOTROPIC EFFECT OF DIGITALIS GLYCOSIDES

The most important difference between substrate-free liquid- and gas-perfused hearts is the time course of changes in contractile force. The results shown in the previous section indicated that this could not have been due to major differences in their metabolism. The changes in intracellular sodium and potassium ion concentrations during gas perfusion did not appear to be sufficiently different from those observed by others during equilibration with a liquid medium (169,170,171) to account for the differences in contractility.

The most important technical difference between the two types of preparations is the inability of the gaseous perfusate to remove substances other than carbon dioxide from the preparation. The hypothesis was made that this technical difference was causative to the maintainance of stable contractile force by the gas-perfused heart. Early work concerned with this aspect of failure in isolated hearts was reviewed in Section I. It should be pointed out that only A.J. Clark considered the removal from the heart by the perfusate of an inotropic substance, whereas all others considered failure to be due to the lack of an essential substance in the perfusate. It seemed possible that some material which is removed from the heart by the liquid perfusate was accumulating in the extracellular space of the gas-perfused heart. If this material were essential for normal contractility, its removal from the preparation by small volumes of liquid should cause abrupt decreases in contractile force.

A. The Effect of Intermittent Perfusion with Small Volumes of Liquid on the Contractility of Gas-Perfused Hearts

Hearts, electrically driven at 168/min, were gas-perfused in the usual manner at a constant resting tension of 10 g. Five ml of substrate-

free Krebs-Henseleit solution were administered every 30 minutes, beginning after 60 minutes of gas perfusion. Each of these administrations of liquid will hereafter be referred to as a "wash", the liquid collected after passage through the heart as a "washing", and a series of four washes as a "wash-out".

The immediate effect of each wash was an increase in contractile force to 192 ± 21% of the pre-wash value. This increase was of only 1-minute duration and was followed by a decrease in contractile force. A typical record obtained during one wash is shown in Fig. 19. The negative inotropic effects of successive washes differed from one another. The absolute decrease in contractile force was always greater as a result of the last two washes than after the first two. Fig. 20 shows the time course of contractility during wash-out in a group of 4 hearts. The initial increase in contractility was ignored in preparing this figure. The contractile force 30 minutes after the last wash, expressed as percent of the initial (liquid pre-perfusion) value, was taken as an indicator of wash-out failure. In Fig. 20, therefore, wash-out failure proceded to 34 ± 6% of initial. Spontaneous failure of unwashed hearts under otherwise identical conditions had reached 85 ± 6% of initial at that time.

A constant observation was an increase in the diastolic tension resulting from each wash, which made it necessary to readjust the tension during the five minutes following each wash. Wash-out had no effect on the contractile alternans which was present in 3 of the 4 preparations.

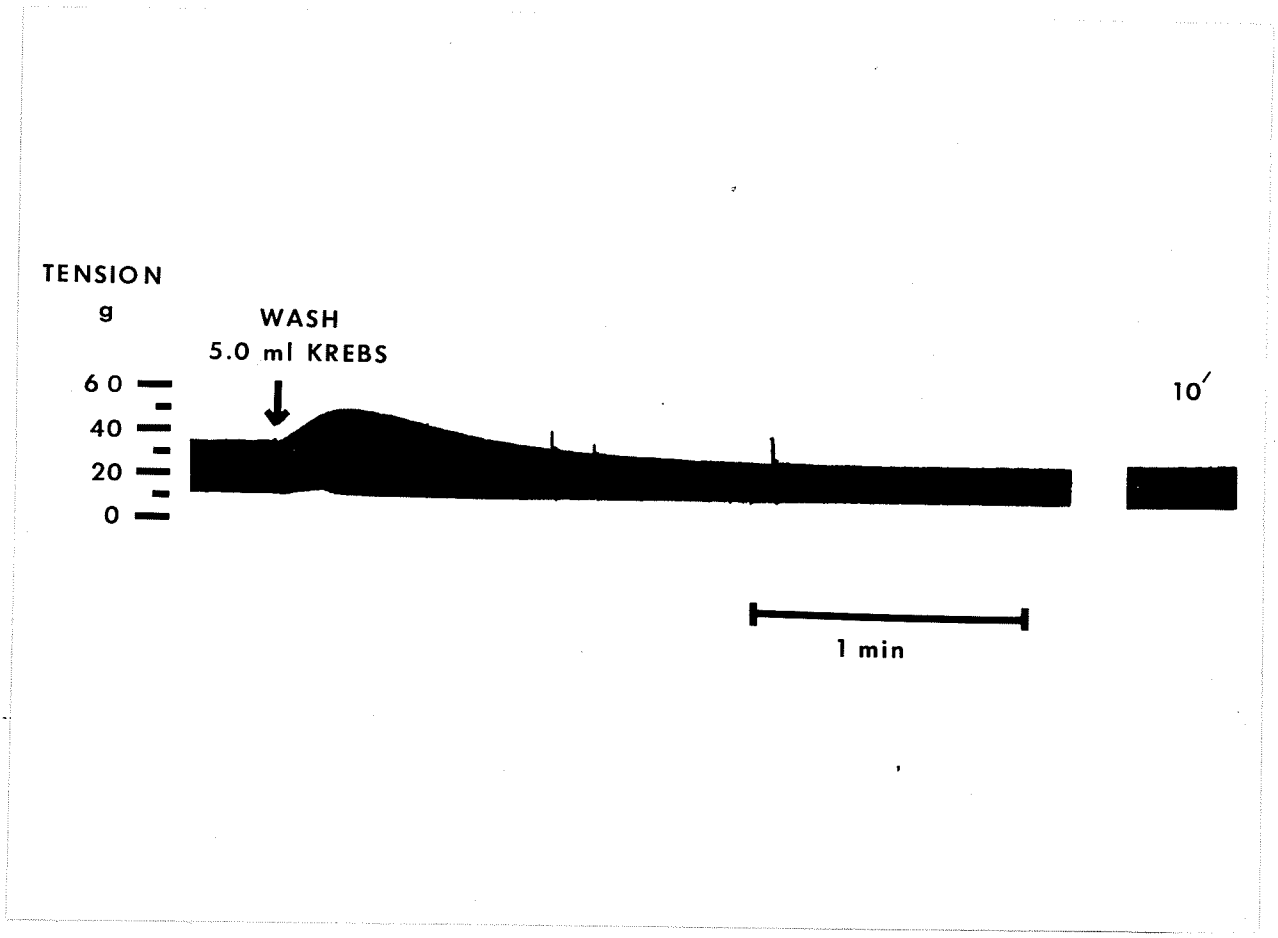

Figure 19. Effect of a single 5.0 ml wash on the isometric contractile force of a gas-perfused heart. Resting tension maintained manually at 10 g. Heart rate 168/min. Extreme right: contractile force 10 minutes after wash.

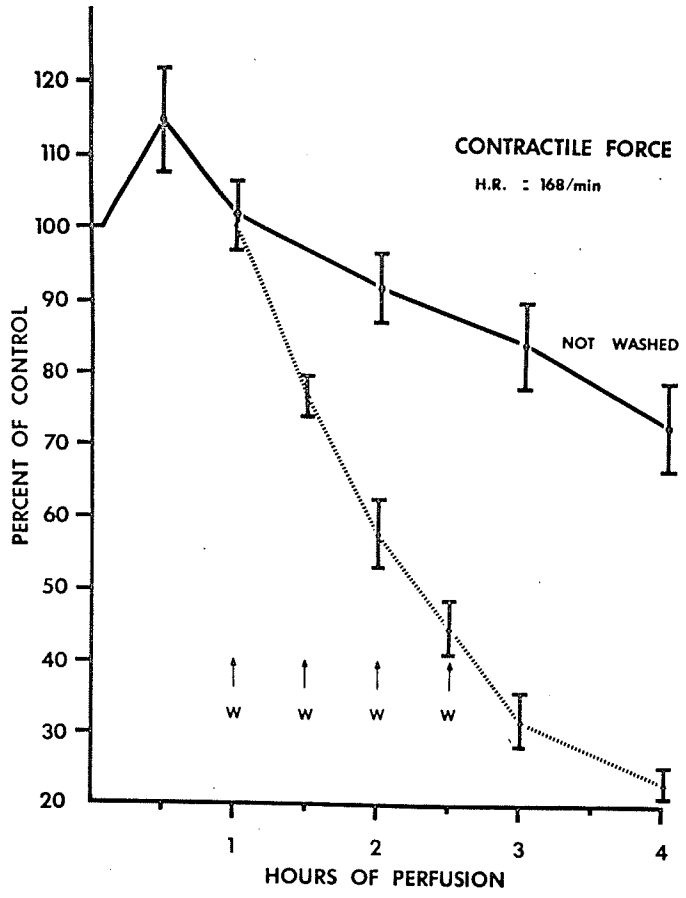

Figure 20. Dotted line: Effect of four washes on contractile force during gas-perfusion. Solid line: Hearts, gas-perfused, but not washed. Bars show S.E.

B. Preliminary Chemical Characterization and Determination of Biological Activity of Washings

A number of early observations indicated that the washings contained a material possessing some inotropic activity. For example, the degree of wash-out failure of two hearts which were washed with washings from two other hearts appeared to be slightly less than the degree of failure of the hearts receiving fresh solution. These differences were far from statistical significance and this line of investigation was abandoned. It was also found that the washings exerted a slight positive inotropic effect on cat atria failed by prolonged incubation in Krebs solution. Prolonged standing of washings at room temperature or at 0.5°C appeared to decrease significantly this inotropic activity.

Extraction of the washings with equal volumes of 1:1 chloroform-petroleum ether resulted in absence of activity both in the aqueous medium (tested after removal of solvents by nitrogen gas) and the residue obtained by evaporation of the organic phase.

It was found that the inotropic activity could be concentrated in a precipitate obtained by addition of ammonium sulfate to the washings. Washings were saturated to 25% and the resultant buoyant precipitate collected on 8.0 μ Millipore® filters. Addition of ammonium sulfate to 50, 75 and 100% saturation resulted in precipitation of flocculent precipitates which were also filtered through Millipore® filters. Bioassay of these precipitates on cat atria failed by prolonged driving (see Methods) showed that the inotropic activity resided only in the precipitate obtained at 25% saturation. This fraction was therefore the only one isolated in later experiments.

A single experiment was done to compare the activity in the washing with that in the precipitate obtained from that washing. This indicated that the precipitated material, which had been redissolved in an equivalent volume of Krebs solution, had appreciably more inotropic activity than the washing.

Analysis of washings indicated the presence of substantial quantities of protein and adenylates (see below, subsection D). The precipitated material gave positive tests for protein and trace reactions for lipid (hydroxylamine method). It also appeared to contain phosphate, but(despite the fact that it had been washed with an aqueous solution of ammonium sulfate) this may have been due to residual contamination from the Krebs solution.

It was found that the addition of 1 mg/ml glutathione to the solution in which the precipitated material was dissolved would preserve its biological activity for periods exceeding 4 hours. Glutathione alone had no effect on the assay at the concentrations obtained after dilution in the tissue bath.

Pooled samples of the material precipitated at 25% ammonium sulphate saturation were examined for inotropic activity in the presence of various pharmacological agents. The results of 6 experiments indicated that the inotropic effects of 4-12 μg/ml (as protein) of the material were not altered by the presence of atropine, 3×10^{-8} g/ml, or pronethalol, 3×10^{-7} g/ml. The concentrations of these blocking agents were observed to prevent the inotropic effects of acetylcholine 1×10^{-6} g/ml, and of adrenaline 5×10^{-8} g/ml, respectively.

The maximal effect of the material on isolated atria was determined in 4 experiments. The contractile force returned from the failed

value of approximately 30% of initial to approximately 80% of initial. Ten-fold increases in concentration were used to ascertain that this was the maximal inotropic activity which could be obtained. Submaximal effects of the substance could be reversed by a single change of the bath fluid. Several changes were needed to reverse maximally effective concentrations. Each of these changes resulted in a new, apparently steady-state level of contractile force. This is illustrated in Fig. 21.

C. The Effect of Digitalis Glycosides on Wash-Out Failure

Ouabain, 1×10^{-9} g/ml, was added to the pre-perfusion fluid of 4 hearts. These preparations were electrically driven at 168/min and gas-perfused for 5 hours. Ouabain had no effect on the initial strength of contraction. The time course of changes in contractility also remained unaffected by the drug ($P > 0.10$ tested at 30-min interval of the curve shown in Fig. 4).

Ouabain, 1×10^{-10}, 3×10^{-10} and 1×10^{-9} g/ml, was added to the pre-perfusion fluids of three groups of 4 hearts. These preparations were electrically driven at 168/min and gas-perfused for 3 hours. The wash-out procedure was begun one hour after starting gas perfusion. The washes did not contain the drug. The washings were collected and analyzed as detailed below.

Fig. 22 shows the effect of these treatments on wash-out failure. It is apparent that failure is completely prevented by the highest concentration used. The degree of failure after pre-perfusion with 1×10^{-9} g/ml ouabain was 81 ± 4% which was not significantly different from control ($P > 0.5$). The prevention of wash-out failure appeared to be related to the concentration of the drug.

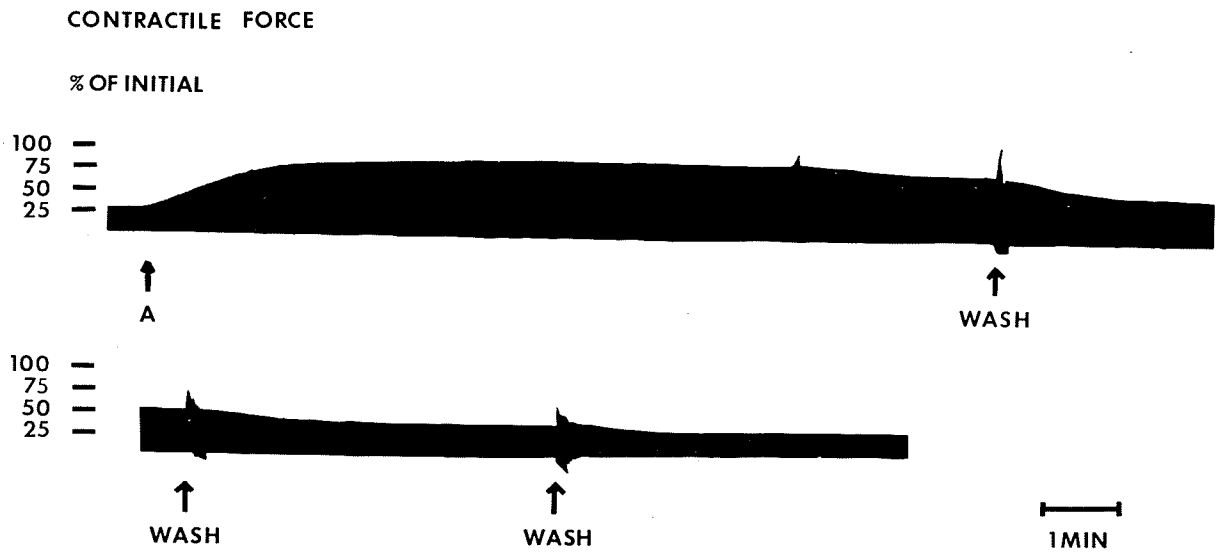

Figure 21. Effect of precipitate obtained from washings on the contractile force of a failed cat atrium. At A, material added to bath (48.1 μg/ml as protein). Note necessity for several changes of bath solution (wash) to return contractile force to pre-administration level.

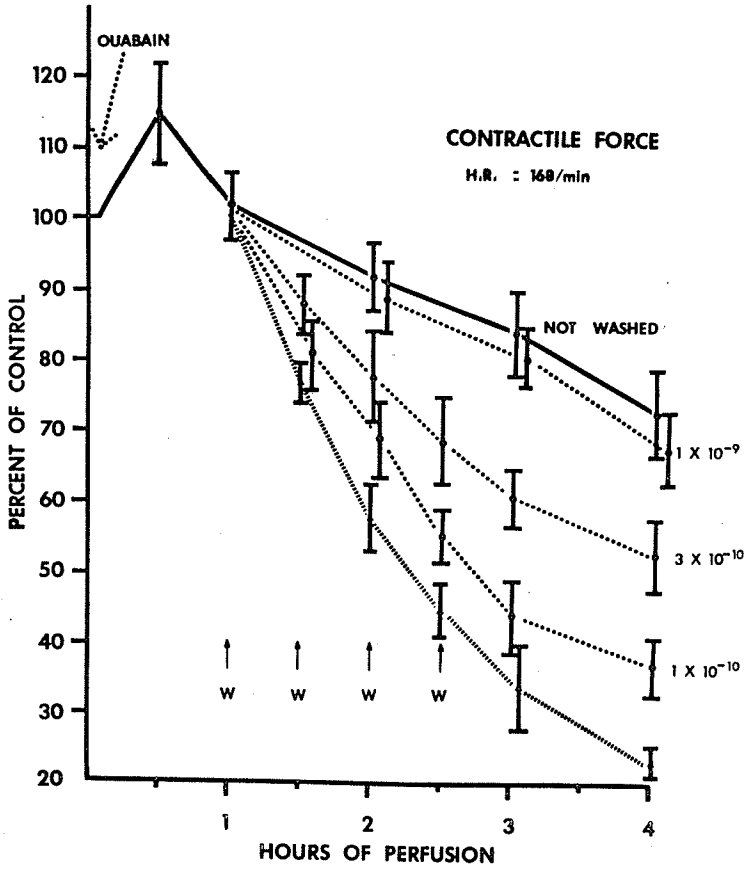

Figure 22. Effect of addition of ouabain to pre-perfusate on wash-out failure. Solid line and lowest dotted line same as in Fig. 20. Central dotted lines: Pre-treated with ouabain, concentrations (g/ml) shown on extreme right. Bars show S.E.

The maximally effective concentration in these experiments is at least 10 times less than those usually employed for maximal inotropic action on ventricular muscle in vitro (2). It is conceivable therefore that the effect of ouabain in these experiments was not linked to its inotropic effect, but related rather to the presence of a non-specific structural feature such as detergency or a steroid nucleus. The glycoside digitoxin and its analogue dihydrodigitoxin were chosen to test this hypothesis. The parent compound is 10-25 times more potent than its dihydro derivative (190). If a given concentration of digitoxin protects against wash-out failure due to its inotropic effect, then even a 10-fold greater concentration of dihydrodigitoxin should be less effective. This relationship would not hold true if protection were related simply to the general chemical structure.

It was decided to conduct these experiments under circumstances in which the drug used in each experiment was unknown to the worker. Arrangements were made for another student to add either digitoxin, 1×10^{-9} g/ml, or dihydrodigitoxin, 1×10^{-8} g/ml to the solution which was used for pre-perfusion of the hearts. Two hearts were perfused simultaneously. The identity of the "unknown" drug was diagnosed on the basis of protection against wash-out failure. The total protein content of the washings was also used to distinguish the two compounds; this will be described below. Table V A shows the results obtained. It is evident that 4 hearts were maximally protected by the pre-perfusion. These were diagnosed as digitoxin hearts and this was confirmed by examining the key. Two hearts were partially protected, and the diagnosis of dihydrodigitoxin pretreatment also was confirmed. Two hearts

(from a single experiment) did not fit into either group because there was evidence for only minimal protection. The questionable diagnosis of dihydrodigitoxin was not confirmed for these hearts which had received the parent compound according to the key. As this difference could not be resolved by a close check on the records made by all concerned, an additional 8 hearts were treated with the two compounds, but not under "blind" conditions. Table V B shows the results of these experiments. The activity of the two compounds is differentiated clearly and the data fit closely those obtained in 6 of the 8 "blind" experiments. It was considered probable therefore that an error had occurred in the addition of drug to the pre-perfusate on one of the days of the blind experiment and the data for this experiment were ignored in calculating the protections offered by the two compounds (Table V). It should be stated that the differences between digitoxin and dihydrodigitoxin is still statistically significant if the two hearts in question are included in the digitoxin group ($P < 0.05$).

The contractile force remaining after wash-out in the digitoxin pretreated hearts was not significantly different from that remaining after pretreatment with the same concentration of ouabain (1×10^{-9} g/ml). It was significantly greater than that remaining after pretreatment with ouabain, 3×10^{-10} g/ml. The contractile force after wash-out in hearts pretreated with dihydrodigitoxin 1×10^{-8} g/ml was significantly less than that remaining after pretreatment with 1×10^{-9} g/ml of the other two glycosides but was not significantly different from the ouabain 3×10^{-10} g/ml group. There is therefore a 30-fold difference in potency between digitoxin and its dihydrogenated derivative. This is in agreement with the literature.

TABLE V

THE EFFECT OF PRETREATMENT WITH DIGITOXIN AND DIHYDRODIGITOXIN ON WASH-OUT FAILURE

A. Blind experiment

Heart No.	Contractile force as percent of initial remaining after wash-out	
	Pretreatment with Digitoxin 1×10^{-9} g/ml	Dihydrodigitoxin 1×10^{-8} g/ml
1	81	
2	93	
3		63
4		57
5	88	
6	89	
7	47*	
8	46*	

B. Additional experiment

1		54
2		64
3	94	
4	88	
5		50
6		53
7		66
8		57
Mean ± S.E.	88 ± 5	58 ± 2

* not included in calculation of mean

It was pointed out in Section III E that pre-perfusion with ouabain, 1 x 10^{-9} g/ml effectively prevented the development of contractile alternans in gas-perfused hearts. The experiments reported here allowed observation of the effect of other glycosides, at least during the first hour of gas perfusion. Digitoxin, 1 x 10^{-9} g/ml, also gave complete protection against contractile alternans. Dihydrodigitoxin, on the other hand, appeared to be less effective than ouabain when compared at the concentrations affording equivalent protection against wash-out failure (1 x 10^{-8} vs. 3 x 10^{-10} g/ml). These data are given in Table VI.

D. Analysis of Washings for Protein and Adenylic Acid Derivatives (adenylates)

Aliquots of each washing were analyzed for protein and for adenylates (see Methods). The results are shown in Tables VI and VII. All results are given as μg/g wet wt. heart. The protein content of the washings appeared to decrease throughout the wash-out procedure, but the differences between successive washings were not statistically significant. The last washing contained significantly less protein than did the first in all instances. The protein content of the first washings was considerably more variable than that of subsequent washings. Pretreatment with ouabain, 1 x 10^{-9} g/ml, caused significantly less protein to be lost from the heart to each of the washings (Table VII). The other concentrations of ouabain also decreased the loss of protein to each washing, but these decreases were not statistically significant.

The total protein lost during wash-out indicates the effect of drugs more clearly than does the protein content of individual washings.

Table VI and Fig. 23 compare these totals. Except for the effect of ouabain 1 x 10^{-10}, the quantity of protein lost during wash-out of control hearts is significantly greater than that lost by hearts which had been pretreated with the glycosides.

Losses of protein were clearly correlated with protection from wash-out failure. As was shown in sub-section C above, protection was the same after pretreatment with digitoxin 1 x 10^{-9} g/ml and ouabain at the same concentration. The total loss of protein in these groups was also identical. Similarly, dihydrodigitoxin, 1 x 10^{-8} g/ml was very similar in effect to ouabain, 3 x 10^{-10} g/ml. Again, the total loss of protein by these groups was not different from one another, but both differed significantly from control.

Perfused hearts lose adenylates (218). Loss of adenylates by hearts was used as a rough estimate of non-specific cell damage during gas perfusion, since excessive loss of these compounds should serve as a rough indication of cell damage. Table VII shows that the first washings, obtained after one hour of gas perfusion, contained almost exactly twice as much adenylates than did subsequent washings which had been obtained after only $\frac{1}{2}$ hour of gas perfusion. The data show clearly, however, that there was no excessive loss of these substances during the wash-out procedure. Table VI and Fig. 23 show that none of the digitalis glycosides, at any concentration, affected this loss of adenylates to the washings.

TABLE VI

RESUME: EFFECTS OF DIGITALIS GLYCOSIDES ON WASH-OUT FAILURE, TOTAL PROTEIN AND ADENYLATE CONTENT OF WASHINGS AND INCIDENCE OF CONTRACTILE ALTERNATION

	per cent contractile force after washout	total protein in washings	total adenylate in washings	number of hearts with contractile alternation
not pretreated	34 ± 6	664 ± 55	140 ± 10	3/4
ouabain 1 x 10^{-10} g/ml	44 ± 5*	563 ± 28*	154 ± 17*	2/4
dihydrodigitoxin 1 x 10^{-8} g/ml	58 ± 2	512 ± 9	158 ± 12*	3/8
ouabain 3 x 10^{-10} g/ml	61 ± 4	486 ± 18	142 ± 14*	0/4
ouabain 1 x 10^{-9} g/ml	81 ± 4	378 ± 18	147 ± 17*	0/4
digitoxin 1 x 10^{-9} g/ml	88 ± 5**	389 ± 15**	149 ± 11*	0/6
not washed	85 ± 6**	—	—	4/4

as µg/g wet wt., means ± standard error

* not significantly different from untreated group ($P > 0.05$)

** not significantly different from ouabain 1 x 10^{-9} g/ml group ($P > 0.05$)

TABLE VII

PROTEIN AND ADENYLATE CONTENTS OF WASHINGS

Protein in washings*

wash	control	ouabain 1×10^{-10} g/ml	ouabain 3×10^{-10} g/ml	ouabain 1×10^{-9} g/ml
1	219 ± 49	183 ± 38	172 ± 31	137 ± 27
2	165 ± 119	146 ± 15	138 ± 15	120 ± 23
3	146 ± 16	122 ± 13	106 ± 12	66 ± 8
4	134 ± 17	112 ± 14	70 ± 7	55 ± 7

Adenylates in washings*

wash				
1	58 ± 5	63 ± 7	57 ± 7	61 ± 7
2	31 ± 4	26 ± 3	30 ± 3	30 ± 4
3	24 ± 3	32 ± 3	24 ± 3	31 ± 4
4	27 ± 3	33 ± 4	31 ± 4	25 ± 4

* as µg/g wet wt., means ± standard error

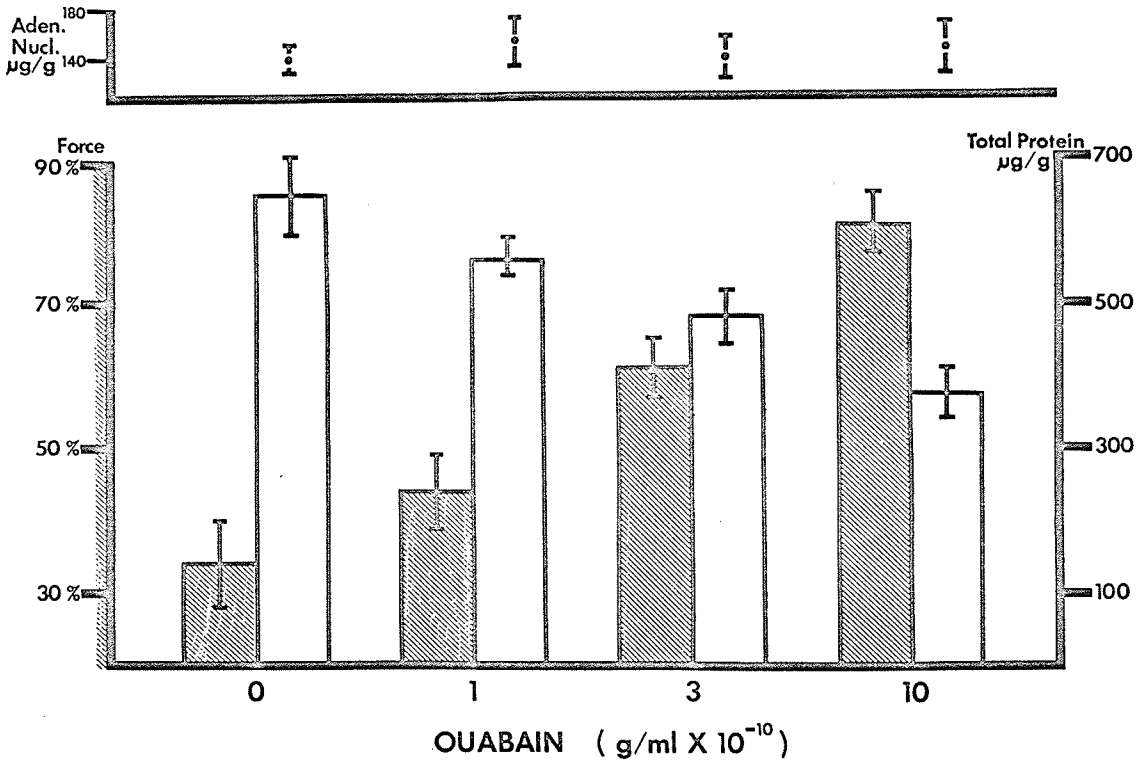

Figure 23. Influence of ouabain pre-perfusion on contractile force and total protein and adenylate content of washings. Abscissa: concentration of ouabain in pre-perfusate. Ordinate: Upper left total adenylate in washings; lower left, remaining contractile force; right, total protein in washings. Shaded columns - contractile force; open columns - protein. Bars show S.E.

E. Bioassay, and Protein and Lipid Content of the Material Precipitated at 25% Saturation with Ammonium Sulphate

The dried precipitates obtained from the individual washings were stored at 20°C. The Millipore® filter was then macerated in 2.0 ml of 0.9% NaCl which contained 1 x 10^{-3} g/ml glutathione. Two 0.5 ml aliquots were removed for protein and lipid analysis and the remainder used for bioassay. The two aliquots were frozen for analysis at a later time.

The protein content of the material, expressed as μg/g wet weight of heart, is shown in Table VIII. The amount of material appeared to decrease as the wash-out procedure proceded, but statistically significant differences in protein content were observed only when the material obtained from the first washing was compared with that obtained from the third and fourth. Ouabain, 1 x 10^{-10} g/ml, did not change the protein content of the material significantly, but the two higher concentrations caused a significant decrease in the protein content of the material from each of the washings. This also holds true when the total protein contents of precipitates are compared.

Unfortunately, it proved impossible to make any reliable determinations of the lipid content of the precipitated material. No trace of lipid was found in any of the samples from hearts which had been pretreated with any concentration of ouabain. However, samples from untreated hearts contained identifiable traces of lipid as shown by a minimal absorption at the absorption peak (520 μ) of the hydroxylamine complex. None of these samples showed absorption sufficient to bring them on to the standard curve which had been obtained using triolein.

TABLE VIII

EFFECT OF OUABAIN PRE-PREFUSION ON THE QUANTITY OF PROTEIN PRECIPITATED FROM WASHINGS BY AMMONIUM SULPHATE

		After pretreatment with ouabain g/ml		
washing	control	1×10^{-10}	3×10^{-10}	1×10^{-9}
1	122 ± 14	103 ± 12	89 ± 13	53 ± 6
2	97 ± 10	84 ± 8	68 ± 9	55 ± 5
3	83 ± 9*	53 ± 5*	47 ± 5*	46 ± 4
4	70 ± 5*	58 ± 3*	50 ± 4*	41 ± 4*
means ± S.E.	372 ± 33	298 ± 27	254 ± 28**	195 ± 24**

as μg/g heart wet wt.

* statistically different from first washing ($P < 0.05$)

** statistically different from control ($P < 0.05$)

These negative results were obtained despite the fact that care was taken to remove glutathione from the solutions used for lipid analysis. Glutathione interferes with the hydroxylamine reaction. Oxidation at room temperature by bubbling with gaseous oxygen for 10 minutes was found to remove the interference.

The bioassay of the precipitated material was severely hampered by the extremely limited amount available and by the variability of the preparations used. The isolated, failed atrium was used because preliminary experiments had shown that the precipitated material appeared to be much more active in failed than in unfailed preparations. Although it proved to be relatively easy to fail the atria to approximately 30% of initial by driving them for 2-3 hours at rates of 4-5/sec, this level of failure did not remain constant thereafter. Decisions concerning contractility were often made difficult because extrasystoles or failure to follow the 1/sec standard driving rate caused interval-dependent changes. The volume of solution containing the material was small and the activity to be expected was only slight (5 ml washing - 1 ml protein and adenylate analysis = 4 ml precipitated, of which redissolved material 50% was used for chemical analysis and 20% was not recoverable from the container). It must also be remembered that the concentration of protein in the solution was not known at the time of bioassay. Accordingly, a standard dose of 0.5 ml of redissolved material was added to the 10-ml bath containing the failed atrium and the resultant changes in contractile force were classified as : - = no response; $\pm$ = slight increase in contractile force; + = return of contractile force to approximately 50% of initial (unfailed); ++ = return to greater than 50% of initial. The responses were later matched with

the protein determined for the same volume of material. Table IX shows the data obtained.

It is apparent that there is considerable variability in the final protein concentration of the assay fluid as well as in the response obtained at equivalent protein concentrations. In general, the greater responses to material from control hearts appears to be associated with a greater protein content. There was considerable overlap between the various groups, and it was found useful to summarize the data relating activity to protein concentration. Table X A summarizes the responses listed in Table IX without regard to the protein concentration causing these responses. Table X B shows the relation between activity and the treatment of the gas-perfused hearts in the mid-range of protein concentrations in the assay bath (7 to 14 μg/ml).

Arrangement of the data in the manner shown in Table X gave a strong indication that the amount of protein in the precipitated material was not clearly related to the inotropic activity. Another experiment was done to clarify this point and to obtain information on the lipid content of the precipitated material.

Two gas-perfused hearts were subjected to wash-out failure and the ammonium sulfate precipitable material from all the washings of both hearts was filtered through a single Millipore® filter. Two other hearts were pretreated with ouabain, 1×10^{-9} g/ml, and were treated in the same manner. The materials were dissolved in volumes of 0.9% NaCl calculated to yield approximately equal protein concentrations. This calculation was based on the weight of the hearts used and the total protein recovered in the previous experiment (Table VIII, i.e. 10 ml for the material from untreated hearts, 6 ml for that from ouabain pre-

TABLE IX

RESPONSE OF ISOLATED FAILED ATRIA
TO MATERIAL PRECIPITATED FROM WASHINGS

	group and treatment							
			ouabain					
washing	untreated		1×10^{-10} g/ml		3×10^{-10} g/ml		1×10^{-9} g/ml	
#1	++	20*	+	18	+	16	+	8
	++	13	+	14	+	10	+	7
	+	21	+	13	+	10	-	8
	+	14	+	11	±	12	-	5
#2	++	18	+	16	+	13	-	11
	++	13	+	13	+	7	-	9
	++	10	±	9	-	11	-	7
	±	11	-	16	-	9	-	5
#3	++	15	+	8	±	9	+	8
	++	9	+	7	±	9	-	7
	+	10	-	9	±	6	-	5
	+	6	-	4	-	4	-	4
#4	++	13	±	10	+	8	-	8
	+	12	±	10	±	7	-	7
	++	8	±	8	-	8	-	5
	+	7	±	4	-	5	-	4

* figures refer to final bath concentration as protein (μg/ml). For explanation, see text.

TABLE X

A: Summary of responses at all protein concentrations

		ouabain g/ml		
	untreated	1×10^{-10}	3×10^{-10}	1×10^{-9}
++	9	0	0	0
+	6	8	6	3
±	1	5	5	0
-	0	3	5	13

B: Summary of responses at both protein concentrations of 7-14 μg/ml

		ouabain g/ml		
	untreated	1×10^{-10}	3×10^{-10}	1×10^{-9}
++	6	0	0	0
+	4	6	5	3
±	1	4	4	0
-	0	1	3	7

treated hearts. Aliquots were taken for protein and lipid analysis, and the remainder of the solutions used for bioassay on three failed cat atria.

Table XI summarizes the results of this experiment. The changes in contractile force due to wash-out were within the limits of the experiments reported in subsection C above. The protein found in the precipitates was very close to the protein calculated as 'expected' on the basis of the previous experiments. The ammonium sulfate precipitated material from the washings contained a significant amount of lipid (0.07 μ moles/g wet wt. heart). Pretreatment with ouabain, 1 x 10^{-9} g/ml, caused the expected approximately 2-fold decrease in the total precipitable protein. It had a much more pronounced effect on the total lipid content of the material. The protein : lipid ratio of the material was therefore affected considerably.

There was sufficient material to test several concentrations of each sample on each of three atria. The material from the ouabain-pretreated hearts did not suffice, however, to obtain a maximum response. The responses in Fig. 24 are expressed as the percent of contractile force before the preparations were failed by prolonged driving, i.e. the mean level of contractile force before adding the material was 32%. Each point on the graphs is the mean of the determinations on the three preparations.

This experiment confirmed the previous observation that the maximal effect of the material was to return contractile force to approximately 85% of the initial. In addition, it showed clearly that the "specific activity" of the protein was considerably less in material from ouabain-pretreated hearts than from the control hearts (Fig. 24 A).

TABLE XI

CHEMICAL ANALYSIS AND BIOASSAY OF POOLED WASHING PRECIPITATES

	control	ouabain pretreatment 1×10^{-9} g/ml
wet wt. of hearts	6.51, 5.14 g	6.82, 6.21 g
contractile force after 4 washes	41%, 33%	81%, 74%
total combined protein expected in precipitate	4333 μg	2345 μg
protein found	4450 μg	2940 μg
lipid found in combined precipitate	83.2 μmoles	3.5 μmoles
Protein: lipid ratio	1: 0.018	1: 0.0012
Bath concentration at approx. 50% effective concentration (as protein)	1.3 μg/ml	25.0 μg/ml

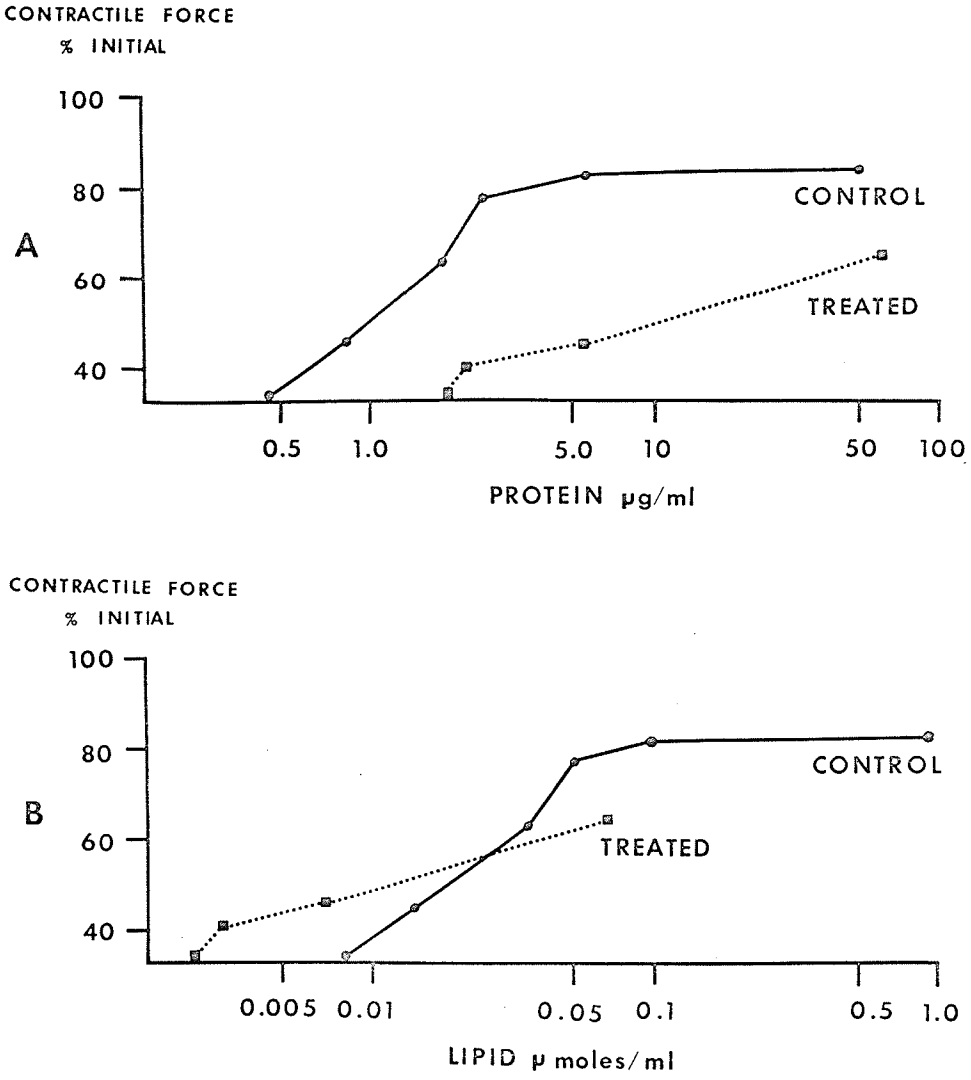

Figure 24. Effect of pooled wash-out material on the contractile force of failed isolated atria.

A: concentration plotted as µg/ml protein.

B: concentration plotted as µ mole/ml lipid. Control = material obtained from untreated hearts. Treated = material obtained from hearts pretreated with ouabain, 1 x 10^{-9} g/ml. See text for further details.

Plotting the same data in terms of lipid content (Fig. 24 B) leads to the very puzzling conclusion that the material obtained from ouabain-pretreated hearts, although containing almost 30 times less lipid, has greater "specific activity" than the material obtained from control hearts. Unfortunately, this experiment has not been repeated and one must therefore be most cautious in interpreting the very limited data.

SECTION V

DISCUSSION OF RESULTS

Gas-perfused hearts beat more strongly, perform more work and fail much more slowly than do hearts perfused with substrate-free Krebs solution. There are several possible reasons for this. Lentini (270) reported that the optimum oxygen tension for isolated heart tissue is 700 mm Hg, a partial pressure exceeded in the liquid perfusate used in the present studies. The adequacy of oxygen supply by liquid perfusates is considered sufficient in preparations which are not doing external work (see introduction). However, they may not supply sufficient oxygen to support work.

We showed (Fig. 11) that hearts produced more work during gas perfusion than during periods of substrate-free liquid perfusion. Glycogen could not have been the source of energy under the conditions of these experiments. If it is assumed that phospholipid utilization was not a limiting factor to performance of work, these results would tend to support the contention of Blinks and Koch-Weser (24) that the supply of oxygen is a determinant of work production. However, if oxygen supply were the only determinant of capacity to perform work, one would have expected the difference between gas-perfused and liquid-perfused hearts to be much greater than that which was obtained. The total oxygen delivered at 20 ml/g/min gas flow is obviously far in excess of that at 2 ml/g/min liquid flow. This difference can in part be due to the fact that the Langendorff preparation is able to increase its oxygen supply by vasodilatation and thus perform relatively more work than the papillary muscle preparation discussed by Blinks and Koch-Weser.

There is much evidence, summarized in Section I, that oxygen is not the only determinant of work capacity. Whalen and Faugman (39) reported that when the oxygen supply to working papillary muscles was

curtailed, oxygen consumption fell by 50% but work production decreased by only 7%. Neely, Liebermeister and Morgan (82) showed that exogenous carbohydrate is necessary for maximum work production. Our results (Fig. 11) clearly support their conclusion by showing that hearts perfused with liquid containing glucose can work approximately twice as much as do gas-perfused hearts. We are inclined to conclude that oxygen supply becomes a major determinant of work capacity when exogenous carbohydrate is present, but is only a minor determinant of this capacity when exogenous carbohydrate is lacking. The differences in contractility, work capacity and rate of spontaneous failure between substrate-free liquid-perfused hearts and their gas-perfused counterparts must therefore be due to some factor other than the availability of oxygen.

The biochemical results indicate that the patterns of utilization of endogenous substrates were similar in liquid-and gas-perfused hearts. Myocardial glycogen and other carbohydrate materials decline rapidly in both preparations. The low glycogen levels which were reached, and the fact that these values remained constant while lipids were being utilized, would indicate that the residual glycogen represented a carbohydrate fraction which was metabolically unavailable. The absolute values for glycogen in Table I are subject to some error. The hearts were rapidly cut from the cannula in these experiments, and small amounts of both right and left ventricular tissue were lost. Since these two muscle masses differ in their glycogen content (271), minor errors maybe due to differences in the loss of right vs. left ventricle. The extremely low values of lactate and pyruvate would indicate that both liquid-and gas-perfused hearts were capable of utilizing these substances, but that glycogen depletion decreased their production.

Our results agree with those of Shipp et al (99) who showed that intracellular lipids constituted the primary source of energy of rat hearts following glycogen depletion. We found that phospholipids represented over 85% of the lipid material which disappeared. The results also indicate that the rate of phospholipid utilization could best be correlated with the number of heart beats occurring during the perfusion periods. The data shown in Fig. 15 would indicate that the phospholipid utilization per beat was independent of duration of perfusion, heart rate, or contractile status when diastolic tension was maintained at 10 g. Thus, our results strongly support the hypothesis of Wollenberger (44) and Furchgott and Lee (81) that cardiac failure cannot be due to changes in the utilization of substrates but must be related to a defect in the conversion of energy into contractile force. An increase in diastolic resting tension of 15 g was observed to cause a 30% increase in substrate utilization. This result would indicate that the quantity of phospholipid utilized per beat was dependent only upon the diastolic tension. This observation strongly supports the hypothesis of Whalen (33) and Lee (272) concerning the dependence of oxygen consumption in isolated ventricle on the initial resting length or tension. Cranefield and Greenspan (26) have challenged the findings of Whalen (33) and Lee (272) on the basis of the thickness of the papillary muscles used. They felt that the lengthening of the tissues when diastolic tension was increased could cause a decrease in the radius of the papillary column. This presumably would shorten the diffusion path for oxygen to the core of the muscle and hence increase the oxygen consumption therein. In our hearts, however, perfusion was via the normal vasculature and this should have eliminated any problems of gaseous diffusion.

The heart rate of gas-perfused preparations decreased sharply during the first hour and remained relatively stable thereafter. This is in contrast to liquid-perfused hearts whose rate decreased gradually throughout the two-hour perfusion. Webb (90) suggested that the energy for the maintainance of heart rate was derived from glycolysis rather than oxidation. Glucose appears to be active in the maintainance of membrane potential, for its presence protects the membrane from the effect of short periods of anoxia (63). However, the profound bradycardia in gas-perfused hearts cannot be ascribed to decreased glycolysis because glycogen depletion was as rapid and as great in the faster-beating liquid-perfused controls. It is possible that the accumulation of adenylates in the extracellular space which we have shown to occur is responsible for the bradycardia. Relatively low concentrations of these compounds have a strong negative chronotropic action (273). The total adenylates contained in the first washing from gas-perfused hearts accumulated during the hour during which the greatest change in heart rate occurred in undriven hearts. Given the assumptions used for the calculation of the extracellular space as described in Section III-G and the probability that AMP is the largest constituents of the mixture, the concentration was calculated to be similar to that reported by others to cause bradycardia. However, evidence not reported in this thesis is beginning to accumulate that substances injected into the perfusate do not reach the atria (it is now considered unfortunate that no attempt was made to observe the effects of sympathomimetic amines on the heart rate). The concentrations calculated for ventricular tissue are not necessarily similar to those occurring at the sinus node.

Several questions arise from the demonstration of high sensitivity of the gas-perfused heart to arrhythmias induced by increases in the perfusion pressure. One of these concerns the stimulus for arrhythmia. The ventricular walls were slit and the pulmonary artery and vein were patent in our preparations so that increases in pressure in the atria and ventricles cannot have acted as stimuli. Increased perfusion pressure must therefore have acted at the level of the coronary vasculature, causing distention of the vessels and the surrounding myocardial tissue. This confirms the hypothesis of Keating (274) Mainwood (275) and Panniset et al (276). Stretching of cardiac tissue has been shown to decrease conduction velocity in ventricular muscle and the atrioventricular node (274,277), and to increase the rate of slow depolarization and the duration of the action potential (278,279). Any of these factors may lead to ventricular arrhythmias similar to those occurring in our experiments, in which increased pressure did not appear to affect the atrial rhythm.

Another point of interest is the difference in pressure threshold of gas- vs. liquid-perfused hearts. Although the absence of glucose has been shown to increase the susceptibility of perfused hearts to experimental arrhythmias (101,102,280), this does not account for the differential between our preparations for the reasons given earlier in this discussion. It is possible that the ionic changes occurring in the gas-perfused heart account for the difference, for it has been shown (281) that decreased extracellular sodium content predisposes isolated hearts to experimental arrhythmias. A decrease in extracellular sodium content occurs in gas-, but not in liquid-perfused hearts.

The pressure-induced arrhythmias could be induced repeatedly in gas-perfused hearts without affecting contractility at normal pressures. Induction of arrhythmia in liquid-perfused hearts resulted in irreversible changes in contractility. This confirms earlier reports that increases in perfusion pressure of liquid-perfused preparations causes "metabolic disarrangement", decreased contractile force and the early onset of failure (77,282). It is possible that the difference is a purely mechanical one, i.e. that the low pressure levels causing arrhythmia in gas-perfused hearts are below threshold for effects on contractility. However, it must be pointed out that the levels affecting contractility in liquid-perfused hearts are well within the range which are known not to have similar effects in vivo.

Contractile alternans occurred in gas- but not in liquid-perfused hearts. It was usually preceded by several skipped beats, an observation confirming those made in vivo (283,284). It was accompanied by electrical alternans in 27% of the observations which is closely similar to the incidence of electrical alternation during contractile alternans induced by methoxamine in vivo (285) and by anoxia in vitro (286). It is easy to explain contractile alternans during electrical alternation as a combination of the well-known decreased contractile force accompanying an extrasystole and post-extrasystolic potentiation.

It is more difficult to explain the more common occurrence of contractile alternans in the absence of electrical changes. Kruta (140) reported that alternation occurs at supra-optimal heart rates. We showed that it may also occur during the severe bradycardia of gas-perfused hearts. Braveny (286) concluded, on the basis of studies with metabolic

inhibitors, that alternation represents an unstable form of contractility due to an inadequate supply of energy. Alternation occurred during a general negative inotropic action of his inhibitors. It occurred in our experiments during the phase of positive inotropic action of gas perfusion. Although alternans appeared to coincide with the exhaustion of glycogen, the fact that equivalent loss of glycogen in liquid-perfused hearts did not cause alternans would negate the importance of carbohydrates. It appears possible that alternans is a pharmacological effect of the inotropic material which we have shown to accumulate in the extracellular fluid of gas-perfused hearts.

Pretreatment of the preparation with low concentration of ouabain decreased the incidence of contractile alternans in a dose-related manner without affecting the time course of contractile force. Injected into the gas-perfused heart during spontaneous failure, it did not affect either the strength of contraction or the alternans present at that time. We have shown ouabain pretreatment to prevent the loss of the inotropic material from gas-perfused hearts, i.e. to prevent its accumulation in the extracellular fluid. The effects of ouabain on contractile alternans would therefore also fit the suggestion that this phenomenon is due to the inotropic material.

The early increase in contractile force and the slow rate of spontaneous failure of gas-perfused hearts may be due to one or to several mechanisms. They can probably not be accounted for by changes in the energy metabolism of these preparations because lipid consumption per heart beat was not different from that of liquid-perfused hearts. An increase in contractility was noted by Lentini (270) in papillary

muscles exposed to a liquid medium equilibrated with 100% oxygen. He suggested that this was a sign of "oxygen toxicity" which increases metabolism in liver slices. He did not show any such increase, nor did he eliminate the absence of carbon dioxide as a causative factor.

Continuous release of catecholamines is not a factor. Injection of pronethalol in doses sufficient to block the effects of catecholamines released by increasing the strength of stimulation did not decrease the contractility of the gas-perfused heart. This would have been expected if catecholamines played a role. A single heart, gas-perfused for 5 hours, contained the same concentration of catecholamines as its unperfused control when analyzed chemically.

The absence of any overt influence by ouabain on the time course of contractile force was expected because the glycosides are known to exert little action on preparations which are maintained in the absence of carbohydrate (4,118,119,120). Failure in the gas-perfused preparation might be similar to the clinically-occurring states of high-output failure, which also respond poorly to the glycosides. Based upon an early suggestion by A.J. Clark (146), we hypothesized that failure was due to the loss of an inotropic factor whose removal was impossible during gas-perfusion. If digitalis acted to prevent this loss in liquid-perfused preparations, then it could not be expected to exert a similar effect in a preparation in which loss did not occur.

Hadju and Leonard (195) examined the perfusates of their liquid-perfused preparation for an inotropic substance without success. This is not surprising in view of our finding that the material is very unstable. We hypothesized that if an inotropic material were accumulating in the extracellular space of gas-perfused hearts, it would be

obtained in a relatively concentrated form from the small quantities of liquid perfusate injected intermittently during the wash-out procedure. The results indicated that this was indeed the case, and that ouabain pretreatment decreased the quantity and specific inotropic activity (in terms of protein) of the material collected in this manner.

The rate of fall of contractile force during the wash-out procedure was similar to the rate of failure previously observed in liquid-perfused hearts. Yet the gas-perfused preparations received a total volume of liquid during wash-out failure equal to that received in one minute of liquid perfusion.

The washings possessed a slight positive inotropic action when administered to the failed, isolated atrial preparation. Fractionation by serial precipitation with ammonium sulphate indicated that the inotropic activity resided in that fraction obtained at 25% of ammonium sulphate saturation. The positive inotropic effects of this fraction were not altered in the presence of atropine and pronethalol. The material was unstable, but its inotropic action could be preserved by the addition of glutathione. The material also contained lipid. The quantities of material precipitated from the washings of both the control and the ouabain-pretreated groups was half that of the total protein contents.

The question was raised that the material so obtained might be the result of cellular death and disintegration. Had the smaller quantities of material which appeared in the washings of ouabain-pretreated hearts been a consequence of some life-prolonging effect of digitalis, then this should have been reflected by variations in the quantities of other cellular constituents (such as adenylates) in the washings. This was not the case.

The ability of ouabain to diminish the total amount of protein and the precipitable protein recovered in the washings was shown to be directly related to its concentration in the liquid pre-perfusate. This effect was also correlated with the protection afforded by this glycoside against wash-out failure. Pre-treatment of gas-perfused hearts with a concentration of digitoxin equal to that of ouabain resulted in equal protection against wash-out failure and a similar decrease in the quantity of protein in the washings. A ten-fold greater concentration of dihydrodigitoxin exerted less protection against wash-out failure, indicating that these actions of the digitalis glycosides were probably linked to their inotropic effect.

Because of the small quantities which were obtained from individual hearts and the crudity of the assay, we were unable to determine in the early experiments whether ouabain simply decreased the amount of material precipitated, or whether it had decreased both the amount and the specific inotropic activity. To resolve this, the combined precipitates from 2 untreated hearts were compared with material obtained from 2 hearts which had received ouabain. Since the relative proportions of material from these groups was predictable, direct comparisons for inotropic activity of equivalent microgram amounts (as protein) were made. It was found that equal concentrations of material did not possess parity in inotropic activity. This experiment did little to clarify the possible role of the lipid present in the wash-out material. The data presented in Table XI indicate that ouabain exerted an even greater lipid-sparing than protein-sparing effect on the hearts. The lipid concentration activity curve (Fig. 24B) indicates that the lipid which was obtained from the ouabain pretreated hearts was of <u>greater</u> molar potency

than the lipid material of the control precipitate. These results can be interpreted in several ways. Inotropic activity could have resided primarily with some component of the protein fraction, with the lipid fraction representing a non-specific activity shared by a host of commonly-occurring fatty acids often present in cellular debris (15). On the other hand, if inotropic activity is essentially related to the lipid content, with the protein fraction acting as a "carrier", then the depression of lipid wash-out by digitalis would have more significance than the relatively smaller inhibition of protein wash-out. Interpretation is made impossible by the finding of greater inotropic activity of the lipid obtained from digitalis-protected hearts. A third possibility is that we are dealing not with one but two cardio-tonic systems, either one of which may be affected by the cardiac glycosides. Clearly, further work is necessary to resolve this problem.

SECTION VI

GENERAL DISCUSSION

When hearts or ventricular tissues are maintained in liquid which contains carbohydrate, the contractile force, oxygen utilization, and ATP and CP levels remain normal for several hours before a gradual contractile failure becomes evident. In the absence of substrates, failure rapidly ensues within the first hour of treatment, but with only small concommitant decreases in oxygen utilization, and ATP and CP levels. A problem posed at this point is whether the failure observed during these two methods of liquid-perfusion and that which occurs during gas-perfusion share any common etiology. Wollenberger (44) considered the spontaneous failure of hearts perfused under the best of circumstances (presence of blood, adequate substrate, and oxygen) and concluded that a defect in the ability to utilize available energy stores existed. An explanation for the appearance of this defect has not, however, been forthcoming. Spontaneous failure in the absence of substrate has not received a great degree of attention, perhaps because this state does not occur clinically. Yet, we may be able to refer to data obtained from such experiments to add additional light to Wollenberger's hypothesis. The small decreases in myocardial ATP attendent to substrate-free perfusion cannot alone account for either the rapidity of onset or the relatively severe rate of spontaneous failure. Additional confusion is added by reports that some species can recover contractility upon supply of exogenous substrates, whereas others cannot (see introduction).

The isolated cat heart perfused without exogenous substrates can regain contractility only if substrate is added to the perfusate before the onset of spontaneous failure. This "permissive" period lasts approximately as long as do the myocardial glycogen stores, which are

gradually depleted during "substrate-free" periods (Gabel, unpublished). We may then consider glucose as an important factor in studies of experimental failure. Its presence delays the onset of spontaneous failure, and, as seen in our data permits maximum work output when work loads are imposed. Webb (90) suggested that glycolysis may support some extra-mitochondrial source of ATP which, although small, acts as a "permissive" factor in the utilization of existing larger stores. Lehninger concluded that the intra-mitochondrial ATP pool is unexchangeable with the sarcoplasmic pool, and that a process of high energy phosphate transport takes place without physical carriage of the nucleotide moeity across the mitochondrial wall (89). The normally slow loss of NAD, NADP, and adenosine from isolated hearts is also hastened by the absence of glucose (93,94,223). Could glucose be exerting its failure-delaying effects by, in some way, preserving the intracellular contents of materials necessary for the efficient utilization of its high energy stores? The possible relationship between these losses and spontaneous failure has received only rudimentary consideration in the literature. A slightly different viewpoint, however, is evident in the literature concerning the beneficial effects of the addition of serum or plasma to the perfusate of isolated hearts. The question which I should like to raise is whether these two considerations can be reconciled in view of the present state of knowledge.

Consider that saline perfusates cause contractile failure because they either fail to supply a necessary factor or because they remove a necessary factor. Perfusate glucose could conceivably be acting to delay failure by either permitting constant cellular synthesis of these factors or by affecting cellular permeability and thereby prevent

their loss. The presence of glucose was stated to maintain myocardial cellular "integrity" during prolonged anoxia, and to permit restitution of contractility upon the return to the aerobic state only if it was present during the anoxic period (59,63). From this, and the considerations presented previously concerning the presence of perfusate substrate (see introduction), a hypothesis concerning glucose may be forwarded: the presence of glucose in the perfusate prevents the loss of certain necessary factors from isolated hearts and thereby delays the onset of spontaneous failure.

The next question is: What is the factor which is lost into the perfusate during substrate-free conditions, and what is its original source? The second part of this question is not meant to be facetious. The factor could be synthesized in the myocardium or blood-borne to the heart from another organ. The possibility that the material is synthesized in the heart cannot be completely dismissed since a slow loss of NAD, NADP, adenosine and other water soluble intermediates might take such synthesis eventually unachievable. The ability of serum or plasma to restore the failed isolated heart would suggest that it contained the factor which previous perfusion had removed. This need not be true, since it might have contained a factor which normally served some physiological purpose other than the regulation of myocardial contractility but which could at least partially substitute for the material in question during a compromised state.

The work of Hadju and Leonard (193,196,199,200,201) indicates that a triumvirate of serum globulins interact to exert a very specific inotropic action on myocardial contractility. They also demonstrated that the serum levels of one such globulin could be correlated with

clinical heart disease. Although data such as this is encouraging, reports concerning other plasma factors also bear close examination. That plasma lipid fractions can exert inotropic action has often been reported. The addition of chloroform extracts of serum to heart perfusates delays the onset of spontaneous failure much the same as does serum (4). The inotropic actions on failed myocardial tissue by various fatty acids and phospholipids has also been reported (187,192-196).

To our knowledge, the experiments with gas-perfused hearts represent the first proof that an inotropic substance can be obtained directly from beating, isolated hearts. The "Muscle-Activity-Factor" reported by Wertheimer and coworkers (231,232,233), although obtained from the perfusate, has been described only in terms of its effect on muscle metabolism. Indeed, it is yet to be established whether our material, Hadju and Leonard's Cardioglobulin, and Wertheimer's MAF truly represent different myocardial systems or whether these are indeed one and the same thing. It would be interesting to determine if MAF possesses any inotropic activity, and whether the Cardioglobulins and our material affect muscle metabolism.

There is as yet much work which must be done before the significance of our data can become clear. Of prime importance would be the application of techniques to separate the active substance from the cellular debris which undoubtably occurs in the washings. Once this is accomplished, the importance of the lipid moiety could then be determined. It is apparent from the results of the experiments in Section IV-E that digitalis pretreatment decreased the quantity and specific activity of the material as judged on the basis of protein content. The results of the lipid analysis indicated that digitalis exerted a "sparing" effect

on this fraction as well. The activity-concentration curve derived from this information, however, indicated that what little lipid did wash out was of greater molar potency than the lipid obtained in washings from untreated hearts. This was taken as meaning that the lipid fraction simply represents a non-specific mixture of cell lipids whose appearance in the washings is coincidental to, but not related to the protein content. We are not prepared at present to accept this as being the actual state of affairs. Experiments described in Section IV-B indicated that removal of lipid by treatment with organic solvents abolished the inotropic activity of the material. This would indicate that lipid was essential to activity, and that results of the experiments just cited were influenced by the presence of both active and inactive lipid.

Is the loss of inotropic material into the washings truly causal to failure, or is it simply secondary to failure? Let us assume that the isolated heart begins its perfusion period with a certain amount of this material either in or bound to the myocardial cells. This assumption is made because any hypothetical myocardial synthesis of the material does not appear to be sufficient to replenish such stores after washing the gas perfused heart or after the administration of glucose to the heart failed by prolonged perfusion with substrate-free liquid. This is further supported by the work of Hadju and Leonard on Cardioglobulins. They showed that the "bound" Cardioglobulins were released with each heart beat. It is also possible that our material acted in the same manner. If some of the material was released with each beat, it might be expected to have accumulated in the extracellular space, thereby being "available" for removal by the wash solution. If the material which accumulated extracellularly was pharmacologically inac-

tive, then its loss during gas perfusion by washing should not have affected the contractile force or rate of failure. The material possessed inotropic activity on isolated atria, so it may be safely assumed that it exerted essentially the same effect on the isolated heart.

If this material, and the degree of its loss from the myocardium is an important factor in the failure of isolated hearts, what then is its physiological function? On the premise that failure is a defect in the utilization of otherwise sufficient energy stores, the wash-out material might be acting in the process by which ATP is transferred from the mitochondrion to the contractile apparatus. The presence of lipid in the material would lend credence to such a hypothesis, since a host of mitochondrial and sarcoplasmic factors which are suspected to take part in this process contain lipid ~~moieties~~ moieties which are essential to their activity (21,89).

A second hypothesis which might be proposed is that the material functions as part of the sarcoplasmic reticular apparatus which regulates calcium movement. The "relaxing effect" of sarcoplasmic reticulum granules has been suggested to involve the binding of intracellular calcium and thereby cause the inhibition of myosin ATP-ase (287). To test this, measurement of the calcium binding and ATP-ase properties of the material might be fruitful. It would also be of interest to examine the effect of this material on myocardial calcium content and exchangeability, since ouabain has been shown to affect the latter but not the former parameter (156). The possible lipo-protein nature of the material is an important consideration, since Koketsu et al (288) have shown that calcium is strongly bound by lipo-proteins obtained from muscle cell membranes, and that extraction of the lipid

~~moiety~~ moiety inhibits this activity. Recently, Fujimoto and Harary (289) indicated that rat heart cell grown in vitro required the presence of serum in order to beat and contain the normal compliment of calcium-activated ATP-ase. They further showed that lipid extracts of serum were equally effective in promoting normal metabolic activity in their cultures.

In our studies, it was noted that liquid-perfused hearts and washed gas-perfused hearts underwent a definite physical shortening or "partial rigor" during failure. Although no attempt was made to quantitate this effect, its occurrence suggested that the loss of the inotropic material may have been related to it.

The ability of ouabain and digitoxin to decrease both the quantity and the specific activity of the recovered inotropic material would seem a convenient explanation for the mechanism of digitalis action. This hypothesis at first glance appears plausible since the data demonstrated an inverse relationship between ouabain concentration and "wash-out" inotropic material. Several possibilities first come to mind in view of some of the admittedly sparse data which was presented. Ouabain could have acted to prevent failure by either changing cell membrane permeability so as to prevent the loss, or indirectly by affecting the material at some intracellular (binding) site. On the other hand, there is no way at present to deny the possibility that the material "sparing" effect of ouabain is not purely secondary to some other effect of the glycoside.

The adage that some experiments may not answer questions, but simply introduce new ones applies here. In retrospect, many more studies on points raised by our results can be suggested. The metabolic studies

indicated that the gas-perfused preparation lent itself well to investigations of lipid metabolism. The effects of several isometric diastolic tensions were described. It would be of interest to compare the rate of endogenous substrate utilization between periods of isometric tension development and those of isotonic work. Furthermore, what was the cellular source of the endogenous phospholipid which was utilized? We did not measure myocardial ATP and CP contents, but data of this kind might reveal whether or not failure during prolonged gas-perfusion involves a decrease of these energy stores as the substrate stores become severely depleted.

Measurement of the cardiac action potential during gas perfusion might shed some light on the genesis of the prominent contractile alternans which was observed, as well as to the lowered threshold to pressure-induced arrhythmias. The effect of injected anti-arrhythmic agents should also be considered.

In our studies, the removal of the wash-out material was performed under conditions of constant isometric diastolic tension and heart rate. If the wash-out material is indeed intimately involved with the regulation of cardiac contractility, then the amounts obtained by washing may differ in preparations in which greater isometric contractile force is evoked by the use of inotropic agents such as the catecholamines and xanthine derivitives. The heart rate as well as the performance of external work may also influence the rate of failure and/or the amount of material obtained by wash-out. We washed the hearts every 30 minutes. Would another washing schedule have caused a different rate of failure?

Since the activity of the wash-out material can be preserved by rapid precipitation with ammonium sulphate, the hypothesis presented

for gas-perfused hearts should be extended to liquid perfused preparations. It is implicit in our hypothesis that essentially similar results will be obtained concerning the "sparing" action of the glycosides. If such determinations prove feasible, then a study of wash-out in the presence and absence of glucose should prove fruitful.

SECTION VII

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