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A Comparison of the Effects of Carbon Dioxide and Hydrogen Ions on the Function of the Isolated Rat Heart

By

Ernest J. Belair, B.A., M.A.

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Department of Physiology
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Ottawa Canada

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G. N. Hainwood, Ph.D.
Associate Professor of Physiology
Research Supervisor

Ernest J. Belair
Ph.D. Candidate

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ABSTRACT

Isolated rat hearts were perfused by a modified Langendorff method to compare the effects of CO_2 and H^+ on their function.

Carbon dioxide had a rapid negative inotropic effect which was not dependent on the perfusate pH, or on the concurrent negative chronotropic effect and change in coronary resistance.

Carbon dioxide caused a negative chronotropic effect which was due, in part, to the decreased perfusate pH. This negative chronotropic effect was not due to the concurrent negative inotropic effect or to the changes in coronary resistance.

Intermediate concentrations of CO_2, between 6% and 20%, were found to have intermediate negative inotropic and chronotropic effects.

Carbon dioxide at concentrations of 10% and 20% caused coronary resistance to increase. This increase was independent of perfusate pH, rate or tension changes. Carbon dioxide concentrations between 6% and 10% caused coronary resistance to decrease.

Decreasing oxygen from 95% to 60% caused a positive inotropic effect.

Perfusates saturated with 20% CO_2 caused the release of some basic substances from the heart. This release was not dependent on decreased perfusate pH.

CHAPTER I

INTRODUCTION

I. GENERAL HISTORICAL INTRODUCTION

The presently known characteristics of carbon dioxide are in stark contrast to the name, "fixed air", applied to it by the eighteenth century chemists who discovered its presence in air. Our present knowledge of carbon dioxide causes us to characterize it as anything but a fixed entity. When one contemplates the physiological research on the function of carbon dioxide within living systems, it would seem more appropriate to call it "elusive air". One of the principal reasons that its nature is so elusive is that, in addition to dissolving in aqueous solutions, it also readily disappears as molecular carbon dioxide and reappears as another chemical entity, the hydrogen ion. This transformation occurs by way of what seems to be a simple chemical reaction involving carbonic acid as an intermediate. Were it completely transformed in solution to hydrogen ions and some anion, the study of carbon dioxide's effect on cell function would still be fairly simple. This is not the case, however. The closely related entities, carbon dioxide, carbonic acid, hydrogen ions, and bicarbonate

ions together participate in a complex relationship called a buffer system. The function of a buffer is to maintain the status quo of its environment with respect to the hydrogen ion concentration of the environment. The existence of this system adds to the elusive nature of carbon dioxide since carbon dioxide may either appear or disappear depending upon the density of the molecular population at the two extremes of the following chemical relationship:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-.$$

The complexity of these interactions is further increased by the knowledge gained from biochemical studies that the cells of living tissues may contribute both carbon dioxide and hydrogen ions from the reactions of intermediary metabolism. Thus it is possible to add functional entities to both ends of the system. From these simple considerations it is then readily apparent why attempts to separate the effects of carbon dioxide and hydrogen ions as possible controlling devices of various cells have been fraught with difficulty.

It seems that carbon dioxide was suspected of exerting a controlling effect on function very early in the history of physiology. Lavoisier's recognition of the necessity of removing carbon dioxide when breathing, as well as the necessity of bringing oxygen into the lungs, is an indication that he had at least a very broad appreciation of the potential controlling function of carbon dioxide. Lavoisier's

work pre-dated the popularization of the Cell Theory by about fifty years, and it is natural that he should think in terms of the total organism or, at best, in terms of organs. With the enunciation of the Cell Theory in the middle of the nineteenth century, the importance of the cell as the ultimate unit of organic function was stressed. There is evidence that physiological thinking gradually turned to the cellular level as the point where carbon dioxide might exert its controlling effect.

KEYS (1945) notes that as early as 1824 one Henry Hill Hickman wrote a letter to the Royal Society proposing that carbon dioxide be used as an anesthetic. This points to an appreciation that carbon dioxide could selectively control some cells of the body. MITSCHER (1865) considered that carbon dioxide could play a role in controlling body functions. BERT (1878) summarized his work on the effect of carbon dioxide on various organs, including skeletal muscle, by concluding that accumulation of carbon dioxide in the bodies of animals caused intra-organic oxidations to progressively decrease. This he visualized as occurring because of the interposition of a terminal obstacle which stops the whole series of chemical transformations. He also observed that the heart was the last to die in severe hypercapnia, and that this was in contradiction to the then common notion that carbon dioxide was a poison of the heart. From his observations of the depressive control exerted by

carbon dioxide on the organism, he cautiously suggested that carbon dioxide could be used as an anesthetic.

From the experiments which followed in the latter half of the nineteenth century, it seems logical to assume that carbon dioxide ceased to be thought of as a poison and that it was being considered more as a potential controlling agent of cell function. Scientific thinking was not sharpened to the distinction between the effect of the hydrogen ion and the effect of molecular carbon dioxide until the turn of the nineteenth century, when the work of Sørenson and Hasselbalch introduced the measurement of hydrogen ion concentration.

RINGER (1893) removed CO_2 from saline and found that frog ventricles would contract longer than if the CO_2 were left in the saline. However, he cautioned that "whether it has a specific effect or acts as an acid, these experiments do not decide". That he perhaps was thinking of CO_2 as a possible controlling device is indicated by the extension of his experiments to blood serum which also was observed not to sustain cardiac contractility when saturated with carbon dioxide.

WALLER and SOWTON (1896) did the same experiments with an isolated heart and also found that carbon dioxide halted the contractions, but observed that they were again resumed upon washing out the carbon dioxide with air-bubbled saline.

A further insight into the extent of carbon dioxide influence was provided by their observation that the heart's electrical activity stopped much later than the loss of contractility. The analysis was carried still a step further by their experiments to separate possible rate effects from the contractility effects. Stannius ligatures were applied to the heart and, while being electrically driven, a diminution of contractile activity was still observed.

The possible controlling function of carbon dioxide on nerve was investigated by WALLER (1895). His findings were that the "negative variation" (resting potential) was altered by carbon dioxide.

With the turn of the twentieth century, many different preparations were used to study the effect of carbon dioxide on the various organs containing the different types of muscle cells. These attempts to separate the effects of carbon dioxide and pH on muscle have not been as thorough nor as fruitful as the investigation into their role in the control of respiration. The efforts toward separating the effects of pH and CO_2 on muscle function were perhaps stimulated by the work of the respiratory physiologists. This trend may be reflected in the few efforts, in recent years, to compare the effect of pH and CO_2 on the isolated heart and on isolated skeletal muscle. A brief look at the efforts of respiratory physiologists to dissociate the

the effects of CO_2 and pH will serve to illustrate that one or both of these agents may be implicated in the control of cells, and that the isolation of their respective roles on cellular control is not always readily achieved.

The relative importance of carbon dioxide and the hydrogen ion on respiratory control was a matter which stimulated much controversy even shortly after HALDANE and PRIESTLY (1905) showed that carbon dioxide had an important role in the control of ventilation. They attributed this control, at that time, exclusively to the carbon dioxide pressure in the respiratory center. Shortly after this first paper, BOYCOTT and HALDANE (1908) concluded that arterial acidity, and not molecular carbon dioxide, was the stimulus for ventilatory control. The demonstration by JACOBS (1920) that CO_2 could penetrate cells and could then form an acid within the cells contributed to the concept that intracellular acidity was responsible for the control of respiration (GESSELL, 1923).

The Danish physiologists, represented by Krogh, Lindhard, and Nielsen, considered that the unique stimulus for ventilatory control was the molecular form of carbon dioxide. NIELSEN (1951) concluded that CO_2 was the unique stimulus for ventilatory control and that the hydrogen ion served as a potentiator of the CO_2 stimulus. SCHMIDT (1956) has since defined the role of hydrogen ions as being only slightly stimulatory and having mostly a toxic effect on

the respiratory center.

It would seem that the works of Jacobs and Gessell give the first hint that the study of the control of respiration, or the control of any physiological activity, could no longer be stimulated by a strict adherence to Claude Bernard's well-known generalization. According to this generalization, the regulation of function was accomplished by processes which tended to keep the internal environment - the blood plasma - constant despite changes in the external environment - everything outside of the body. However, if this concept is extended to include subdivisions of the internal environment, then the concept is as useful as it was when first postulated by Claude Bernard.

Since cells have been shown to be discrete entities within organs, it seems logical that they would have an important separation of their internal environment from their external environment by an inter-environmental barrier. This conceptual scheme emphasizes that the solution of the problem of control must take into account the behavior of the carbon dioxide-hydrogen ion combination toward the inter-environmental barrier, the cell membrane. Possibly their control functions are related to their effect on, or their mobility through, the cell membrane.

That the internal environment of the cell must, in turn, be divided into sub-units of internal and external environment, would seem to be a logical extension of this conceptual

scheme. Modern microscopy has shown that there are discrete organelles within the cell with barriers which effectively separate their environment into an external one and an internal one. Biochemistry has shown us that these particles are very close to the source of activities which control overall cellular function. The mitochondrion, with a complete membrane system of its own, is of primary importance to metabolic control of cellular activity. The endoplasmic reticulum, with a membranous organization, whose complete ramifications with respect to structure and function has just recently become apparent, also appears to be an important intracellular controlling device. The myofilaments of muscle cells, though without a membrane, effectively divide the intracellular space into subdivisions where control of activity may occur. To control the function of cells, it seems logical that the operation of controlling factors must be at the level of subcellular units. These subcellular units are reachable only through a sequence of internal-external environment complexes that possibly follow Claude Bernard's original dictum, but with infinitely greater complexity.

Speaking of respiratory physiology control, ROBIN (1963) has indicated that in order to understand its control processes, one must turn to the interior of the cell, because that is where the units, which can effect control, are located. What Robin has postulated as necessary for the

elucidation of the mechanism of control of breathing, is also applicable to the elucidation of the internal control of the heart.

That the carbon dioxide-hydrogen ion system, in all of its elusiveness, does have an effect on the heart, will be seen from the survey of the literature which follows shortly. Control of the heart immediately brings to mind control of the cardiac musculature, which certainly constitutes the bulk of the organ. However, there are two other functional units which may be controlled in conjunction with, or independently of, the myocardial cell mass. These two functional units are the coronary circulatory system and the rate determining system. These may be inter-related in some or all of the possible permutations, or they may not be at all inter-related. To study what controlling effects are exerted by the carbon dioxide-hydrogen ion complex, it seems logical to begin with the isolated heart. With the isolated heart, the cells may be kept in an environment that is as physiological as possible, and at the same time, extraneous factors may be kept at a minimum.

To study the heart isolated from all the usual in situ exogenous control mechanisms is not to deny the importance of these mechanisms. Rather it is an expression of what seems to be an inescapable conclusion that if the control of cells is to be understood, one must look to units where control is possible; this is the cell, or its intracellular

subdivisions. Controls from sources external to the heart must ultimately be effected at the cellular level in the heart. It may be exerted on the myocardial cells, on the modified myocardial cells of the impulse generating and conducting system, or on the smooth muscle cells of the coronary vasculature. Also, these controls must be imposed as supplementary or complimentary effects to those imposed by local conditions within the heart on the various controllable entities. It would seem that the first step toward understanding the function of cardiac controls is to determine whether or not cardiac controls exist locally, and if they do, what the relative contributions are for each of the various elements. The carbon dioxide-hydrogen ion combination is a likely candidate because of its ubiquitous nature as was previously discussed.

It would seem, from a first analysis, that the isolated heart is far removed from a study of function at the cellular level. It is, however, an almost completely homogeneous mass of cardiac muscle cells. It has the advantage of containing a complete and readily accessible distribution system for a physiological fluid. It also has the advantage of an indwelling physiological stimulator, the pacemaker.

Since the capillary system has been shown by KEARN (1936) to exist in some animals in close to a one to one ratio with the cardial muscle cells, utilization of the coronary vascular system permits reaching a maximum number

of cells with the perfusate. This eliminates the possibility of having a population of dying cells within an anoxic core as occurs in preparations which merely bathe the tissue in a physiological medium. With all of the cells of the myocardium theoretically equally exposed to the perfusate, the myocardium's behavior in response to various parameters is a model of the response of a single myocardial cell.

The behavior of the coronary vasculature, either directly to changes in one of the parameters or secondary to changes in myocardial cell function, may be assessed by the changes in flow through the coronary system. A change in coronary flow may be cautiously interpreted as the result of changes in smooth muscle tension. Changes in radius will cause the most marked flow changes through blood vessels, since, according to the Poisseuille-Hagen formula for flow, flow is directly proportional to the fourth power of the radius. Certainly, the arterioles, with their radius controlled by smooth muscle, give a most exact and exquisite control of flow. However, other factors may also change the radius of coronary vessels and, thereby, the flow through these vessels. A widespread swelling of capillary endothelium could alter flow markedly by effecting a change in capillary radius. Varying extramural pressure on the various parts of the vascular bed, by alterations of myocardial tension or by changes in the volume of extracellular fluid, could alter the radius of the vessels. This

again would be reflected by changes in flow. These indirect variants of vascular flow are not always separated from the effects of smooth muscle control in the works cited in the literature survey.

Therefore, the responses of the coronary vasculature may reflect smooth muscle response, but other cells may contribute to the total picture. A hypothesis relating vascular flow to smooth muscle cell function must only be made after careful examination of the results for contributions from the other potentially significant factors.

The function of the pacemaker may be analyzed in much the same fashion. However, since the pacemaker functions in conjunction with the other conductile tissues of the heart, a complete study of the effect of controlling factors on rate would have to take this into account.

Therefore, the isolated heart is proposed as a model system for the study of the response of the three cell types to the possible controlling action of the carbon dioxide-hydrogen ion system, and for the study of possible secondary interactions between the effects on the three cell types.

II. STATEMENT OF THE PROBLEM

The problem herein considered may be stated as follows: To study the responses of the three cell types found within the isolated heart to carbon dioxide, and to determine whether one or more of the three cell types respond to the influence of carbon dioxide in its molecular form or as the hydrogen ion.

III. A SURVEY OF THE LITERATURE

General. The literature survey is divided into three areas. The first area deals with the effect of carbon dioxide or hydrogen ions on the development of tension by cardiac muscle tissue. The second area considers the effect of carbon dioxide or hydrogen ions on the rate of contraction of the heart. The third area encompasses the alteration of smooth muscle tension by hydrogen ions, carbon dioxide, and oxygen concentration changes. Various kinds of smooth muscle preparations were surveyed on the assumption that evidence applicable to one of these other preparations may have some relevance to the smooth muscle of the coronary vasculature. Also, in the survey of this area of the literature, the effect of oxygen was included. The literature of vascular resistance links hypoxia and hypercapnia; and the experiments reported included situations in which hypoxia might be a factor in coronary resistance changes.

The effect of changing the carbon dioxide or the hydrogen ion concentration on the tension of the myocardium. GASKELL (1881) showed that when he perfused frog hearts with 0.75 sodium chloride solutions made acid by 1:10,000 or 1:20,000 lactic acid, the contractility of the ventricles

decreased.

RINGER (1893) studied the effect of CO_2 on myocardial tension in a qualitative manner by analyzing the effect of removing CO_2 from the distilled water used to make up the saline perfusion fluid. In these experiments, Ringer found that the solutions made with water which had been boiled to expel carbon dioxide would permit contractions to continue for longer periods of time than saline which did contain carbon dioxide. Ringer's experiments do not show whether this is a specific effect of carbon dioxide or one due to the increased acidity.

WALLER and SOMTON (1896) showed that by bubbling the perfusing saline with CO_2, the contractility of the isolated heart (species not stated) decreased and then disappeared. They also demonstrated that the electrical activity of the heart continued after the contractions disappeared. The effect of carbon dioxide was reversible when the saline was bubbled with air.

HENDERSON (1908) demonstrated that the presence of CO_2 in the blood was necessary for the maintenance of the proper activity of the mammalian heart.

JERUSALEM and STARLING (1910), using frog and turtle hearts perfused with Ringer's solution bubbled with oxygen and carbon dioxide, found no augmentation of contractile strength by carbon dioxide, even at the 5% level. Depression of the size of contractions began at the 7%

carbon dioxide level and increased with increasing carbon dioxide tension. The curves relating the effect of CO_2 to contractile tension resembled those of GASKELL (1881) for the effect of lactic acid on the contractility of the frog heart. Jerusalem and Starling also reported on heart-lung preparations in the same paper. Using five heart-lung preparations from cats and one from a dog, they found that when the lungs were aerated with gas mixtures containing 2% to 8% carbon dioxide, the contractility of the ventricles was enhanced. With aeration of the lungs by gas mixtures containing 12% to 20% CO_2, the tension at systole was decreased markedly and the tension at diastole was decreased to a lesser degree. From these experiments they concluded that there must be an optimal concentration of CO_2 at which cardiac contractility is maximum.

BURRIDGE (1912) observed a decrease in contractile strength of the frog's ventricle when the hydrogen ion concentration was increased by weak inorganic acids. Organic acids and strong inorganic acids had no effect on the contractility of the ventricles.

MARKWALDER and STARLING (1913), using the dog heart-lung preparation, with the blood protected from clotting by hirudin, found that 12% CO_2 in the gas mixture aerating the lungs caused the myocardium to relax, as shown by a dilation or increase in circumference. They also noted that asphyxia caused a more severe effect than was observed

with just an increase in carbon dioxide. They reasoned from this that some metabolites other than CO_2 must be exerting their influence.

MINES (1913) used a solution of NaCl, KCl, and $CaCl_2$ buffered by a borate-acetate buffer to perfuse the frog heart. When HCl was added to make the pH equal to 2, or when NaOH was added to increase the pH to 12, the heart stopped in a strongly contracted state. When small changes in hydrogen ion concentrations were made, there was a slow decrease in contractility.

CLARK (1913) observed a decrease in the force of contraction of the isolated frog ventricle perfused with Ringer's solution when the pH reached 6.5. This occurred whether the pH was reduced by the addition of strong mineral acids or by increase of the CO_2 concentration bubbling the saline. Increased acidity had a beneficial effect on contractility between pH 8.3 and pH 6.7. He concluded that the beneficial effect of acidity was only partly due to the effect of the hydrogen ions, and that there must also be some direct effect of CO_2 on the muscle fibers. Indicators were used to follow the pH changes.

PATTERSON (1914) confirmed the findings of MARKWALDER and STARLING (1913).

DALE and THATCHER (1914) perfused the frog's heart with saline buffered with a borate-acetate buffer and found that the sinus venosus stopped beating at pH 4.0, the auricles at

pH 5.4, and the ventricles at pH 6.5. This indicates that all parts of the heart have a tolerance to acidity well beyond physiological levels which is much greater than had been believed previously.

SALANT and JOHNSTON (1924) found that only a small decrease in the force of contraction occurred in the isolated frog heart when the pH was decreased from 7.2 to 6.5. They also observed that the decrease in force of contraction was of the same order of magnitude as the decrease in rate.

GREMELS and STARLING (1926) demonstrated that the changes in pH produced by CO_2 had a greater effect on the volume of the dog heart than the pH changes produced by adding HCl. As additional evidence that CO_2 was more effective in altering myocardial contractility, it was observed that the changes in function in response to CO_2 occurred several minutes before the blood pH had reached equilibrium.

FEIGEN et al (1952) found that some CO_2 was required for the maintenance of the stability of tension development of the isolated rat ventricle strips. They also reported that the absolute magnitude of contraction tension decreased with the addition of any carbon dioxide.

BONIFACE and BROWN (1953) studied the effect of carbon dioxide on contraction tension in the open chest dog. They proposed the hypothesis that CO_2 was the cause of depressed myocardial contractility. However, no attempt was made to separate the possible effects of decreased pH from those of

increased carbon dioxide concentration. The effect of decreased oxygen concentration arising from the introduction of larger partial pressures of carbon dioxide was not evaluated either. Despite the depressing effect of CO_2, the heart tended to recover its control contractility after exposure to high CO_2 tensions for 15 to 30 minutes.

VAUGHAN WILLIAMS (1955) designed a series of experiments to analyze the relative effects of carbon dioxide and pH changes on contraction tension, rate of contraction, and conduction velocity. These experiments were done on isolated rabbit auricles perfused with a modified Locke's solution. The changes in pH were accomplished by manipulating the concentrations of bicarbonate or carbon dioxide according to the Henderson-Hasselbalch relationship. Changes in bicarbonate concentration were made by altering equivalent amounts of the chloride concentration. The cation in both cases was sodium. The force of contraction was greater in alkaline solutions and less in acid solutions. This was true except when the bicarbonate concentration was increased beyond the 20-30 millimolar range, and then even increases in alkalinity produced depressions of contractility. Upon analyzing the relative effects of pH changes versus changes in carbon dioxide concentration on auricular contractility, Vaughan Williams concluded that the force of contraction did not change unless the pH of the bathing solution was changed. In other words, the increases in

carbon dioxide concentration were without effect on auricular contractility unless these increases were allowed to increase the hydrogen ion concentration.

PRICE and HELRICH (1955), using the dog heart-lung preparation, changed the pH by changing the hydrogen ion and the carbon dioxide concentration. They concluded that increases in hydrogen ion concentration were responsible for the decreased contractile strength, and that changes in CO_2 concentration had no direct effect on the myocardium.

McELROY et al (1958) perfused seven isolated guinea pig hearts by the Langendorff method. Tension changes were recorded from the apex of the heart by an ink-writing lever. They attribute the decrease in contractility to the increase in hydrogen ion concentration only. They further concluded that changes in CO_2 concentration, or bicarbonate concentration have no direct effect on the contractility of the myocardium.

DARBY et al (1960) studied the effect of increased CO_2 concentration in the air respired by the open chest dog preparation under forced ventilation. Increased CO_2 caused a marked reduction of contractility of the ventricular musculature. THAM was used as a buffer in an attempt to separate pH effects from the carbon dioxide effects. Because control experiments with THAM indicated that it had an inherent positive inotropic effect, it was difficult to evaluate the contribution of the acid-base balance

correction. However, the authors were of the opinion that CO_2 had a pronounced effect on the energy production by myocardial cells. This, in turn, would affect the degree of contractility produced by the cells. They further suggested that CO_2 may have a depressing influence on energy production by a mass action effect which inhibits aerobic or anaerobic energy metabolism.

DOWNING et al (1965) used the auto-supported cat heart preparation to assay the effect of metabolic acidosis on myocardial contractility. Infusions of lactic or hydrochloric acids, which lowered the pH to 6.80, caused little or no reduction in the contractility of the myocardium.

The effect of changing the carbon dioxide or the hydrogen ion concentration on the rate of contraction of the myocardium. JERUSALEM and STARLING (1910) showed that carbon dioxide in excess of 8% supplied to the lungs of the cat or the dog heart-lung preparation, caused a slowing of contraction rate.

MARKWALDER and STARLING (1913), using the dog heart-lung preparation, demonstrated a decrease in the rate of contraction with increasing carbon dioxide concentration.

MINES (1913) demonstrated that solutions acidified with strong acids slowed the excised frog heart. The saline in which the heart was immersed was buffered by borate-acetate

buffers.

DALE and THATCHER (1914) noted that the rate decreased to zero at different pH values for the various parts of the heart as cited for the cessation of contractility.

ANDRUS and CARTER (1922) studied the effect of acidosis on the rate of contraction of the turtle heart. Their buffer system was of doubtful stability since they used Ringer's solution with 0.015% $NaHCO_3$ without equilibration with CO_2. The desired pH was reached by titration with HCl. They found that acid solutions decreased the heart rate and alkaline solutions increased the heart rate.

ANDRUS and CARTER (1924), again using the bicarbonate buffer without CO_2, found that acidosis caused a decreased heart rate in perfused dog hearts. When the pH was made less than 7.0, the function of the heart was impaired. Alkaline solutions restored the rate.

Using isolated rabbit auricles bathed with a solution buffered by bicarbonate in equilibrium with various concentrations of CO_2, ANDRUS (1924) found that alkaline solutions did not increase the rate of contraction. It was observed that the rate remained stable until the pH fell below 7.2, and then the rate decreased.

SALANT and JOHNSTON (1924) found only a small decrease in the rate of contraction of the frog heart when the pH was decreased from 7.2 to 6.5. The time lag of this effect was approximately twelve minutes.

EDWARDS (1930) removed the heart from frogs anesthetized with urethane. The rate first increased and then decreased when exposed to new pH values from 8.2 to 6.8. Ringer's saline was used and the pH was changed by additions of NH_4HCO_3.

A study of the acid tolerance of the dog heart by GESELER et al (1946) showed that the arterial pH had to fall to 7.00 before cardiac rate was affected. A further fall to pH 6.85 was required before the heart was arrested. This also demonstrates a tolerance by the heart to much lower pH values than previously believed. There was no evidence of partial or complete heart blocks. The authors concluded that metabolic acidosis does not cause the decrease in rate, but that it is due to an increase in CO_2 concentration.

WHITEHORN and BEAN (1952) used the decerebrated dog preparation to study the effect of various gases on heart rate. Carbon dioxide (11.5%) in oxygen caused slowing of the heart.

BROWN and MILLER (1953) caused dogs to respire CO_2 concentrations as high as 80% in experiments designed to study the tolerance of the dog heart to high CO_2. At these higher concentrations, CO_2 caused cardiac arrest without any evidence of partial or complete blocks on the ECG. No distinction between the effect of high carbon dioxide concentration and the effect of low oxygen concentration was made, however. The authors concluded that CO_2 appeared

to have a greater depressive effect on heart rate than is produced by acids at equivalent pH levels.

BONIFACE and BROWN (1953) observed a marked bradycardia when open chest dogs were ventilated with increasing CO_2 concentrations. Again, these authors did not separate the effects of decreased pH and decreased oxygen concentration from the postulated effects of high carbon dioxide.

STONE et al (1955) found no constant change in rate, or arterial blood pH in whole dogs breathing 30% carbon dioxide in oxygen.

VAUGHAN WILLIAMS (1955) studied rate changes in his isolated rabbit auricle preparation and reported that rate remained stable over a wide range of carbon dioxide concentrations, pH changes, and bicarbonate concentrations. The rate was often decreased at pH values of less than 7.2, but it was not decreased at alkaline pH values.

NAHAS (1956) showed that CO_2 produced bradycardia in the whole curarized dog. He could not determine whether this was an effect due to carbon dioxide or to the increased hydrogen ion concentration. He also stated that he was unable to determine whether the effect was exerted on the myocardium directly, on the sympathetic tone, or on the chemoreceptors.

McELROY et al (1958) concluded, from experiments on isolated guinea pig hearts, that an increase in hydrogen ion concentration was the factor which caused bradycardia

and not the change in carbon dioxide or bicarbonate concentration.

SALZANO and HALL (1960) reported that CO_2 produced bradycardia in dogs. No separation was made of the pH and CO_2 effects.

MITHOEFER and KAZEMI (1964) used whole dogs to study the effect of CO_2 and metabolic acidosis on rate. They concluded that the initial control heart rate determined whether increased carbon dioxide concentration produced bradycardia or tachycardia. In their opinion, CO_2 had a direct effect upon the heart to cause bradycardia. They postulated that such an effect could be upon undegenerated vagal endings or upon other "peripheral mechanisms". The dogs were decapitated to separate the central from the peripheral mechanisms. Metabolic acidosis, produced by infusions of hydrochloric or lactic acids, caused a decrease in blood pH comparable to that produced by carbon dioxide. The pH decrease caused by the acids, did not, however, alter the rate of myocardial contraction.

The effect of changing the carbon dioxide, oxygen, or the hydrogen ion concentration on vascular resistance and on smooth muscle tension. GASKELL (1881) studied the effect of weak lactic acid and weak sodium hydroxide on the flow through the vascular bed of the frog's mylohyoid muscle.

By measuring the vessels' diameters with a microscope and by counting the rate of saline drops effluxing from the venous end of the bed, he concluded that acidic solutions caused vasodilation. He also observed that basic solutions caused vasoconstriction.

BAYLISS (1900) used the skinned hind legs of frogs, perfused through the aorta, to study the effect of CO_2 and lactic acid on the circulation of the hind legs. Carbon dioxide and lactic acid had the same vasodilating effect when they were dissolved in Ringer's fluid.

DIXON (1902) exposed the frog's stomach to a wide range of pH changes. Weak lactic acid (1:10,000) caused the isolated frog stomach to relax, and increasing the strength of the acid accelerated the relaxation. Strong lactic acid solutions (1:500), when applied to the serosal side of the stomach, caused a rapid increase in tonus with a subsequent slow relaxation. He called attention to the similarity of the known responses of blood vessels and the myocardium to these agents with the response of the isolated stomach.

FARMER (1908) used the uteri of cats or rabbits as models of a mass of smooth muscle cells to analyze the response of smooth muscle to pH changes. Using oxygenated Ringer's solution, he demonstrated that increased alkalinity augmented the tone of the uterus, and that decreased alkalinity decreased the tone. Strongly acid solutions

arrested the uterus in a contracted state.

With the heart-lung preparation of dogs that had received injections of hirudin to prevent clotting, MARKWALDER and STARLING (1913) studied the effect of CO_2 on the coronary circulation. A Moreavitz cannula was inserted into the coronary sinus and the outflow measured. It was found that 12% CO_2 caused dilation of the coronary blood vessels. The observation that asphyxia produced a more marked effect than the increased CO_2 alone, caused them to postulate that some non-volatile metabolites must be present in addition to CO_2. They reasoned that, in this manner, the heart could increase the circulation through itself whenever increased demands were made on its functional capacity.

FLEISCH (1918) perfused the vasculature of the hind legs of frogs, in situ, with oxygenated Locke's solution. Oxygen lack caused a rapid and intense vasoconstriction. Carbon dioxide varied according to the concentration used. Low concentration (3%) caused dilation of the blood vessels, and higher concentrations caused vasoconstrictions which increased in intensity with increasing CO_2 concentrations. The results obtained when HCl was used to increase the acidity, were similar to those obtained with CO_2. Hydrochloric acid produced dilation at a normality of 0.001 and constriction at 0.004. The smooth musculature of the vascular bed was not paralyzed since it readily responded to

other constrictor agents. He concluded that the dilator effect was mediated through the nervous elements, and that the constrictor effect was a direct one on the muscles of the arterioles.

ATZLER and LEHMANN (1921) found that when frog vessels were perfused with Ringer's solution, there was no change in perfusion rate between pH 5 and pH 7.1. Vasoconstriction occurred below pH 5 and above pH 7.1. The presence or absence of the central nervous system made no difference in these responses. Because of the direct relationship between vascular flow rate and pH, they suggested that this was due to a physico-chemical reaction, probably with the contractile proteins. ATZLER and LEHMANN (1923) found that the zone where no vascular reaction occurred varied with the buffering capacity of the buffer in the perfusate--the greater the buffering capacity, the more narrow the zone.

LEAKE et al (1923) reported that buffered solutions of various pH values affected the blood vessels of the frog in various ways. The blood vessels were dilated maximally at pH 7.2, and were constricted when the pH was less than 7.2 or more than 7.4.

EVANS and UNDERHILL (1924) studied the effect of various hydrogen ion concentrations on an assortment of smooth muscle containing organs, including the retractor penis of the bull, uterus of the guinea pig, uteri of the cat and rabbit, various small intestine sections, and the

iris of the cat. They reported that the effect of increasing the hydrogen ion concentration was to cause relaxation of smooth muscle. However, if a preparation had been in an alkaline medium previously, a subsequent exposure to acid produced an increase in tone.

HILTON and EICHHOLTZ (1925) found only a modest increase in coronary blood flow when the vessels were exposed to concentrations of 5% to 8% CO_2. They disagreed with MARKWALDER and STARLING (1913) in that they claimed that non-volatile vasodilators were not involved in the control of coronary blood flow. They were of the opinion that the reduced pO_2 had a direct effect on the vessel wall and thus caused vasodilation.

McSWINNEY and MENTON (1927) perfused various sections of the stomach from rabbits, cats, and rats with Ringer-Tyrode solution whose pH was altered by changing the CO_2 concentration. They found that a moderate change to the acid side of pH 7.5 caused relaxation of the isolated organs, and a change to the alkaline side caused contraction. Large changes of pH to values between 5.9 and 2.1 caused contraction of the organs. They suggested that the reaction of smooth muscle to pH change was of a physico-chemical nature.

McDOWALL (1928) found that when the vessels of the hind limb of the cat were perfused at pH levels below 7.4, the vessels would dilate. As the pH continued to decrease farther below 7.4, the dilation was converted to a

constriction at some point which varied with each animal.

GREEN et al (1941) found that CO_2 had no effect on coronary blood flow in the open chest dog. Anoxia or ischemia did, however, cause an increase. No attempt was made to isolate the heart from the influence of the other organs of the body.

STEIN and WEINSTEIN (1942) showed that bathing in carbon dioxide saturated water baths produced vasodilation of the human skin vessels.

ECKENHOFF et al (1947) found no significant CO_2 effect on coronary blood flow in the open chest dog preparation. Anoxia and low pH (7.08) did, however, cause an increase in coronary flow.

GALLWITZER-MEIER (1950) measured the pH of the venous blood of the stimulated dog's gastrocnemius muscle, and he concluded that blood pH was not the sole, or even the most important, factor responsible for the vascular adjustments occurring during muscular activity.

The blood flow of the normally innervated hind leg of the dog was increased by changing the pH from a control value of 7.4 to either the acid or the basic side (KESTER et al, 1952). The cutaneous vessels were an exception in that they constricted at pH values more basic than the control. These authors established the desired pH values of their solutions by HCl, NaOH, or complex buffers which introduced several new ions into the circulation. DEAL

and GREEN (1954) using the same preparation but changing the pH with only HCl or NaOH, obtained the same results.

The oxygen content of the coronary sinus gave the best correlation with coronary resistance according to the report by KATZ et al (1955).

HACKEL and CLOWES (1956) found that the vasodilation, produced by low oxygen content of the blood, was not mediated by either the sympathetic nervous system or the adrenal glands. They considered that this observation proved that the effect of low O_2 concentration was due to a direct action on the smooth muscle of the arterioles or to the release of vasodilator substances from the myocardium.

FLEISHMAN et al (1957) found that 20% CO_2 had very little effect on the resistance to flow in the forelimb. Their conclusions do not agree with their data presented in their graphs. EMANUEL et al (1957) came to a similar conclusion with respect to the effect of CO_2 on renal blood flow. They observed that 20% CO_2 had very little effect on blood flow through the kidney.

BERNE et al (1957) concluded that myocardial pO_2, as reflected in the coronary sinus blood, was the critical factor in the vasodilation seen in myocardial hypoxemia. The oxygen lack at the tissues was postulated to be responsible for vasodilator metabolites being released from the heart muscle, and they, in turn, affected the coronary vessels. This response was demonstrated to be free of the

control of the sympathetic nerves or of the adrenal gland.

McELROY et al (1956) used the isolated guinea pig heart to study the effects of low pH versus high pCO_2 on the coronary circulation, as well as for studying the effects on rate and tension. They concluded that the increased flow rate through the coronary system, with increasing concentrations of CO_2, was due to the decrease in pH and not to the increased carbon dioxide.

Using the isolated heart of the rat, at 28°C., TAYLOR and YOUNG (1959) studied the effect of increased hydrogen ion concentration versus increased carbon dioxide concentration. They found that increased hydrogen ion concentration increased coronary resistance whether the hydrogen ion concentration change was effected by increased pCO_2, HCl, or by lactic acid. The perfusate, Kreb's-Hinger bicarbonate solution, did have phenol red added to it as a pH indicator, however.

CRAWFORD et al (1959) questioned that CO_2 excess had any vasoactive function after their experiments with the dog hind limb. They concluded that oxygen lack was the cause of vasodilation, possibly acting through some released metabolites.

WEST and GUZMAN (1959) used angiocardiography to demonstrate that inhalation of 15% CO_2 for 30 seconds caused marked vasodilation in dogs.

DIJI and GREENFIELD (1960) approached the problem of a possible local CO_2 effect controlling vascular resistance by experiments on peripheral circulation. They injected 12.5% to 100% CO_2 under the skin of the human forearm. They noted, in a qualitative fashion, that this always caused vasodilation. The possible effect of trauma was eliminated by control injections of air or nitrous oxide which was without effect.

FEINBERG et al (1960) used the isolated rabbit heart preparation and perfused the coronary vessels with Ringer-Locke solution containing hemoglobin. Varying the hemoglobin concentration allowed them to vary the oxygen delivered to the myocardial cells. Decreasing the oxygen content of the solution caused an increase in coronary blood flow. Intravascular oxygen tension did not fall until the perfusion fluid passed the resistance vessels. This suggested to them that the vasodilator response was secondary to tissue hypoxia.

ZBOTEK et al (1961) studied the flow changes in the hind leg of the dog by plethysmography following blood pH changes by respiratory changes or by infusion of buffers. They found that both acid and basic pH levels caused flow to increase. It should be noted, however, that there was no nerve blockade, and that no attempt was made to separate the pH effect from the effect of high CO_2.

MOLNAR et al (1962) reported that various acids, producing a slight increase in hydrogen ion concentration, caused vasodilation in the forelimb and the coronary arteries of the dog. Acids were found to produce a much greater effect than inhalation of carbon dioxide.

CARLILE et al (1964 a) used isolated small femoral arterial branches lying on the surfuce of the muscles of the hind leg of the dog, to study flow changes resulting from pH changes. Tyrode's solution, titrated with various acids, provided the required pH changes. Decreasing the pH from 7.40 to 7.15 caused an increase in flow of 87%. Further decreasing the pH to 6.80 caused a progressive decrease in flow until the flow leveled off to a point just above the control value. Increasing the pH to 7.65 caused a 50% increase in flow. CO_2 and O_2 were kept at the same level at all times, 5% and 95% respectively. They considered that the most likely mechanism for the operation of this demonstrated hydrogen ion effect was the inhibition of some enzyme by a less than optimum pH environment.

KIRYUSHINA (1964) measured the flow in the left coronary artery of dogs breathing 3% to 30% CO_2. With high CO_2 concentrations, he observed an initial period of vasoconstriction followed by vasodilation.

WANG and KATZ (1965) produced an acidosis localized to the coronary system by a special heart preparation in the open chest dog. DMO (5,5-dimethyl-2, 4-oxazolidinedione) was

used to decrease the pH to a mean value of 7.18. At this pH coronary sinus flow varied biphasically; increasing slightly (6%) for 15 seconds and then decreasing gradually to 15% below the control level in 15 seconds.

LOVE and TYLER (1965) measured coronary vessel flow by Rb^{86} uptake. Hypercapnia in dogs caused an increased isotope uptake, which indicated increased coronary flow, in the inner portion of the left ventricle; but there was decreased uptake in the outer portions of the left ventricle, an indication of decreased flow. The authors concluded that the patterns of regional flow changes in the coronary system were very complex.

Conclusions from the literature. The most striking characteristic of the results reported by the various authors, is the lack of uniformity of results. Recent reports show no improvement in the degree of uniformity over the earlier reports; hence, the lack of uniformity is probably not due to the degree of sophistication of equipment.

There are relatively few reports of experiments which attempt to assay the difference between pH effects and CO_2 effects with minimal changes in the ionic environment. Often preparations were used which included complex buffers or indicators in the perfusate. Titration of perfusates

with strong acids markedly changes the anionic concentrations. This, in turn, could alter membrane concentration and electrochemical gradients.

While a few experiments were reported which analyzed the effect of CO_2 and pH on the three parameters (contraction tension, rate, and coronary flow rate), there is a paucity of experiments which reported control situations checking the effect of the alteration of one parameter on another.

Therefore, it seems that the results reported in the literature give evidence of the need for investigating the cellular responses of the heart's three cell types to carbon dioxide and/or pH. Even recently, WANG et al (1965) concluded that "previous attempts to determine the effect of pH on circulation have given non-significant results". It also seems evident that attention should be directed to the tissue changes that could occur between immobilizing the animal and placing the isolated organ in an artificial environment for study, to the possible effect of extraneous ions introduced into the perfusate, and to any interaction of these parameters.

IV. GENERAL EXPERIMENTAL CONSIDERATIONS

The isolated heart preparation. The effect of shifts in the various ionic and molecular components of muscle cells and their environments, have been studied mostly by using the muscles of cold blooded animals, particularly isolated skeletal muscle. The behavior of cardiac muscle tissue was investigated most frequently by using the isolated heart of a poikilotherm. There have been relatively few attempts to study the effect of ionic variations on the isolated intact mammalian heart (McELROY et al, 1958).

The isolated heart preparation has the advantage that variations in function resulting from alterations of a controlled number of variables, may be studied without the complicating influence of factors external to the heart. Many exogenous controls influence the heart in situ. These are very important for the proper function of the intact organism. In initial studies of cellular function, they merely cloud the already complex series of relationships. For example, nervous control of the heart, from the sympathetic or parasympathetic subdivisions of the nervous system, can influence the function of the intact heart. Various reflex patterns originating in the central nervous system can alter the functional pattern of the heart. Endocrine controls are present though not clearly defined.

Erythrocytic ionic shifts, resulting from oxygen and carbon dioxide shifts, are present in every vascular bed and complicate the characterization of what is happening at the level of the cells being studied.

The function of the smooth muscle cells of the coronary arterioles can best be done using isolated hearts perfused with saline, when the study involves the effect of acidic environments. Acid has a swelling effect on red blood cells which could alter the viscosity of blood and complicate the results (CARRIER et al, 1964b).

CARRIER et al (1964a) considered that removal of the influences of the nervous and endocrine system was essential to their study of the pH effects on vascular resistance and, consequently, used the isolated artery segment.

Another advantage of the isolated heart is that changes in function may be allowed to reach a steady state. This is enhanced by the absence of external compensatory mechanisms. The achievement of a steady state permits measurement of variables which otherwise might be transient only. In many cases, the rate of achievement of the steady state may be altered merely by manipulating the temperature in the isolated organ preparation. This may permit an otherwise impossible analysis, thereby yielding further information about cellular processes without damaging cellular mechanisms.

A disadvantage of an isolated organ is that it can readily be labelled a failing organ, since the only obvious fate of that organ is complete failure of function and death of the constituent cells. This perhaps places a stigma on the results from isolated organs. However, is an organ which is failing so non-physiological? Are not all organs of the adult animal body in some stage of failure?

If the isolated heart is used to study cell function, then the conclusions, resulting from these studies, should be valid as long as at least a majority of the cells studied are living. Although the question of the nature of life is a philosophical one, every biologist learns that living cells have certain characteristics. As enumerated in general biology, the characteristics of cells which are living are: Metabolism, excitability, growth, or regeneration. Some cells, depending on the degree of their specialization, may possess more than one of these characteristics. It is fortuitous that the heart's myocardial cells possess a visible indicator of the presence of these characteristics, since contraction expresses the presence of metabolism and excitability. Thus, visual examination of the heart is all that is required to ascertain that the myocardial cells are alive. The pacemaker cells readily indicate that they are alive by the presence of a coordinated wave of contraction. The smooth muscle cells of the

blood vessels are more difficult to check. However, if evidence can be obtained that changes in vascular diameter occur, then the smooth muscle cells may be contracting and, therefore, may be living still. When the presence of these characteristics, indicating that living cells are predominantly present, can no longer be detected, then no information can be gained about the function of living cells.

By following the lead of the workers with tissue cultures, it is quite possible that perfusing media could be developed which would support isolated hearts for long periods of time. This is hardly necessary, however, since acute experiments, lasting two or three hours, already yield much information about the function of the various cell types. The characteristics which indicate that the cells are alive, are still vigorously manifested at the end of such experiments.

Another disadvantage of the isolated organ is that it may gain extracellular fluid and become edematous, due to the inexact duplication of the proper osmotic balance by the perfusate. Edema is not completely unphysiological since it can be observed, to various degrees, in various organs of the animal during normal physiological situations. With respect to experiments on cells, increase in extracellular water will increase the path length over which diffusion must occur. This retards the flow of ionic information to the cell or to the observer. Extracellular

water may cause an increase in the intracellular water. This becomes a problem, in these studies, when intracellular characteristics change so that normal patterns of contractility or irritability are altered. The time required for this to happen seems to far exceed the normal duration of an experiment.

Inclusion of colloidal material has been used to retard edema. However, since the perfusate used permitted reproducible and reversible changes in cell function, the addition of these agents was not deemed necessary in these experiments. Further, since the cycles of experimental perfusion were of short duration, the changes observed were rapid compared with the changes which might occur from slow deterioration of the preparation. Evidence that the deterioration of these preparations is very slow will be discussed in a later section.

Therefore, it was concluded that the advantages of the isolated heart, resulting from removal of external variables, were greater than the disadvantages. In the study of the control of cell groups within the heart, the threat to clarity of results was greater from the externally applied, and as yet poorly characterized, controls than from the consequences of isolating the heart. However, it should be stressed that isolation of the organ must occur under conditions which permit minimum metabolic damage to the tissues, if one is to obtain a valid sample of the function

of the living cell population.

Factors in isolating a heart with a maximum number of living cells. The damage to the cells of the heart during the preparation for in vitro perfusion is most likely to occur as a result of the continued metabolism in the anoxic open chest condition. FURCHTGOTT et al (1958) reported that diminuition of the oxygen supply caused a rapid loss of the working ability of heart muscle. KARDESH et al (1958) found that there was an early change following the onset of anoxia, as manifested by alterations of the intracellular action potentials within five minutes.

That the beating heart represented an organ under the stress of continued metabolic requirements for oxygen, has been appreciated for some time. It is clear, if even only intuitively, that the slowing of contraction rate, or complete stoppage, would be advantageous to anyone desiring to preserve the heart with a maximum number of living cells. Early efforts to slow or stop the heart were based on the work of RINGER (1883) who demonstrated that different cations altered the heart rate. HOOKER (1929) showed that excess KCl could be used to stop the heart due to potassium's inhibitory effect. $CaCl_2$ could then be used to restart the heart.

MONTGOMERY et al (1954) revived Hooker's work, and used his method to stop a heart in fibrillation.

MELROSE et al (1955) reported that potassium citrate allowed the isolated heart to be stopped for as long as fifteen minutes with no apparent damage upon recovery. One heart was arrested for as long as 55 minutes and made a fair recovery. Hearts stopped by asphyxia recovered slowly and only partially to their control contractility after only five minutes of arrest, however. In whole dogs, if a proper dose of potassium citrate was used, the heart could be arrested for as long as 15 minutes and would restart immediately upon replacement of the citrate solution with normal Locke's solution. This was also true for the hypothermic animal. It should be noted that potassium citrate also inhibits clotting of the blood, and, as will be discussed below, perhaps part of its beneficial effect was due to this inhibition.

The agent used for slowing the heart would preferably require less severe ionic environmental changes than is necessary with high doses of KCL. Also, the recovery should be achieved quickly and without such agents as $CaCl_2$ or adrenaline. Hypothermia was considered because of its widespread use in reducing the rate of metabolism in various organs. HEGNAUER et al (1950), BERNE (1954), and COVINO et al (1957) showed that hypothermia decreased the conductivity, excitability, and rhythmicity of the heart.

EBERT et al (1964) also showed that at 25 C, the heart rate of the dog decreased by an average of 67% of its control value. WHITTOW et al (1965) demonstrated that in the chicken, heart rate decreased to less than 1/3 of its control value when the animal's temperature was dropped by immersion in a cold water bath.

That cooling was beneficial to the survival of the kidney, was demonstrated by MURRAY and HOLLEN (1954). By using a cooled, well-oxygenated perfusate, the survival time of an isolated kidney was doubled.

If the heart could be slowed by hypothermia which cools the deep tissues of the heart as well as the surface tissues, then there should be uniform preservation of the tissues in a uniformly undamaged condition. Theoretically, this should occur with the infusion of cold saline by way of the heart's own extensive distribution system, the coronary system. Damage may be further minimized if the saline is aerated with a mixture of 95% oxygen and 5% carbon dioxide. In addition to providing oxygen, the proper acid-base balance will be maintained by the saline's buffer system.

There is considerable evidence that various disease states, conditions producing shock, or acidosis cause sludging of the blood. This sludging then forms micro-clots which persist to cause irreparable cellular damage. Crowell and his coworkers laid the experimental basis for prevention of this occurrence. CROWELL and READ (1955)

demonstrated that heparin injections provided 100% recovery from severe hypotensive periods, while control dogs without heparin showed only 7% recovery from hypotension. By reverse perfusion, they demonstrated that the control dogs' blood had formed minute emboli that were barely visible to the unaided eye. They also demonstrated that the rate of blood clotting was increased by hemorrhage, sympathetic stimulation, or epinephrine injections. This underlines the need to keep an experimental animal, which is to furnish an isolated heart, under the most optimal conditions possible until the heart can be protected from the consequences of micro-emboli. It also makes suspect, the results of workers who decapitate an animal and then isolate the heart, even though the thoracic cavity may be cooled with ice.

Crowell and Read further concluded that no clot formation occurred if enough heparin was given. They also reasoned that irreversible shock, produced by hemorrhage, may be due to the widespread formation of such small blood clots as a result of excessive stimulation of the coagulation mechanisms.

CROWELL and SMITH (1956) found that although heparin did improve recovery of brain function after circulatory arrest, further improvement could be made with additional precautions. Based on the hypothesis that loss of function was due to the formation of micro-clots, a streptokinase

was infused to dissolve these micro-clots. As a further protective measure, the brain was perfused with Locke's solution to wash away the micro-clots. These precautions permitted stoppage of cerebral circulation for 20-24 minutes with complete and immediate restoration of function upon reinstatement of circulation. Crowell and Smith reasoned that the sequence of damaging events might be as follows: 1. Circulation stopped but metabolism continued. 2. Since no oxygen was being supplied, anaerobic metabolism predominated so that lactic acid and carbonic acid accumulated. This resulted in a decreased pH as was observed by STONE et al (1941). 3. The decreased pH probably caused coagulation of blood in the capillaries adjacent to the cells. 4. When flow was resumed, most of the capillaries were plugged by thrombi and the adjacent cells died subsequant to resumed flow.

That sludged blood occurred in varying degrees of severity was demonstrated by KNISELY (1951). He showed that anoxia caused by sludged blood resulted in marked changes of the endothelium surrounding the anoxic area.

A combination of anticoagulant measures and immediate removal of blood was shown by WEBB et al (1957) to permit the dog heart to be without coronary circulation for up to 90 minutes with minimal apparent damage. When coronary circulation was resumed, the heart could maintain its circulatory function for longer periods of time than the

control dogs. They concluded that perfusion of the coronary vessels to remove all blood, extends the period of time during which the heart may remain viable. It was concluded that the time limits of viability of the unoxygenated myocardium are related, in large part, to the preservation of a patent capillary bed and not only to the duration of acute ischemia.

MUIRHEAD et al (1956) obtained the same beneficial effects upon perfusing the kidney with heparinized saline solution. Although the kidney had been ischemic for 20 to 40 minutes, a successful homotransplant was made.

Therefore, there is sufficient evidence to conclude that a protocol, whereby an excess of heparin was injected intravenously into a well anesthetized rat, should produce a heart minimally damaged by micro-clots. To further protect the heart, it seems a sound practice to infuse cooled oxygenated saline immediately following the injection of heparin. Cooling would decrease the rate of myocardial metabolism. The saline would flush out the blood, and its oxygenated condition would further protect the myocardial, smooth muscle, and the endothelial cells from damage.

The general plan of experimental procedure. The coronary system was perfused by way of the aorta. All experimental changes were made from the control condition in which the heart was perfused with saline containing 28 mEq bicarbonate, bubbled by 95% oxygen - 5% carbon dioxide. This yields a solution with a pH of approximately 7.40. Return to the control perfusate followed each experimental perfusion. All control values refer to the magnitude of a parameter during the control perfusion immediately preceding the experimental perfusion being examined.

The experimental situation for which the response of the three cell types was thoroughly characterized, resulted from perfusion with saline again containing 28 millimolar bicarbonate, but bubbled with 80% oxygen and 20% carbon dioxide. This produced a pH of approximately 6.80. These levels of pH and pCO_2 were chosen even though they are beyond commonly accepted physiological levels for cells because they occur in states of disturbed function. Characterization of the behavior of the three cell types, under these conditions, might be useful. Conditions resulting in low pH or high pCO_2 will be described in a subsequent section.

Further experimental procedures were carried out to assess the effect of changes in rate, tension, coronary flow rate, and reduced oxygen tension on any of the other parameters.

After a thorough characterization of the responses to a decrease in pH accompanied by an increase in CO_2 concentration, the effect of decreasing pH without increasing carbon dioxide was studied. This was done by reducing the bicarbonate concentration in the perfusate, thus decreasing pH to approximately 6.80 without increasing CO_2 concentration beyond the control 5% level. Thus the aim of changing pH with a minimum of ionic change was followed.

Bicarbonate has been characterized as not permeating membranes, except those of red blood cells, by the following workers: JACOBS (1920), FENN (1936), BOYLE and CONWAY (1941), TOBIN (1956), CALDWELL (1958), WADDELL and BUTLER (1959), ROBIN (1961), KOSTYUK and SOROKINA (1961), and KIBLER et al (1964). Therefore, a compound with the same cation, sodium, and a nonpermeating, nontoxic anion was used to replace sodium bicarbonate in the solution of decreased pH.

Control perfusion for experiments with saline in which only the pH was changed, was the same saline with 28 mEq bicarbonate, 95% O_2 - 5% CO_2 as described for the experiments in which both pH and pCO_2 are changed. Control experiments for the effect of the compound replacing sodium bicarbonate were also analyzed.

After establishing the dependence of the characteristic responses on pH or pCO_2, or on both, a few experiments were performed using intermediate concentrations to analyze the possible linearity of the influence of the controlling factor.

V. SPECIFIC LIMITS OF THE PROBLEM

The problem is to determine whether increased carbon dioxide concentration or increased hydrogen ion concentration causes the characteristic effects on the three cell types found in the isolated heart; the myocardial cells, the cells of the pacemaker, and the smooth muscle cells of the vasculature. The study of this problem requires the study of the effect of decreased oxygen tension, the effect of a filler gas, and the effect of a compound used to replace sodium bicarbonate. These effects were studied only as necessary controls to the elucidation of the principal problem.

The study of the possible interaction of rate changes, contraction tension changes, and coronary resistance changes was pursued only as far as was necessary to ascertain whether the effect of pH or pCO_2 was a direct one, or was operating indirectly through another parameter.

VI. POSSIBLE SIGNIFICANCE OF THE PROBLEM

CROWELL and KAUFMAN (1961) measured the pH change in the various organs of dogs following circulatory arrest. The pH in all organs was found to drop very rapidly. In approximately 14 minutes the pH of heart tissue had dropped to 6.80, and continued to decrease until it reached a pH of approximately 6.30 in 56 minutes.

LILLEHEI et al (1956) stated that clinical situations occasionally occur in which the pCO_2 exceeds 100 mm Hg, and it may even exceed 200 mm Hg in some instances. BOAK and [illegible]RTH (1938) also reported clinical situations with pCO_2 values at the same high level.

BEAIRSLEE and DICKEN (1953) reported that anesthesiologists were becoming more aware of the clinical significance of carbon dioxide as a factor in cardiac arrest and anesthetic convulsions.

PHIPPS (1947) showed that cardiovascular changes during and following anesthesia were related to hypercapnia.

Therefore, the behavior of the three cell types under conditions of severe hypercapnia, and the resulting pH changes, have pertinent clinical significance.

CHAPTER II

METHODS

I. GENERAL CONSIDERATIONS

The purpose and the advantage of using the isolated perfused heart, to study the function of its component cells, is that this study is possible without the complicating effect of such factors as hormonal influence, erythrocyte ionic shifts, peripheral acid-base changes, or peripheral resistance variations. The advantage gained by the removal of exogenous variables must not be negated, however, by a pre-perfusion procedure which alters the cells structurally, biochemically, or physiologically. As previously discussed in Chapter I, some measures could be taken to enhance the quality of the preparation. Although these procedures may seem to complicate the preparation, the basis for their inclusion was the production of a higher quality preparation.

The perfusion apparatus may seem unnecessarily complex. The reason for its development was that it assisted in the maintenance of controlled environment, or that it permitted more exact recording of a variable.

II. MATERIALS, ANESTHESIA AND GENERAL OPERATIVE PROCEDURES

The hearts from 57 male, Wistar Strain rats were used for this study. The rats weighed between 150 and 300 grams at the time of the experiments. The anesthetic used was Nembutal injected intraperitoneally in a dose of 5 milligrams per hundred grams of animal weight. Nembutal Sodium powder, dissolved in physiological saline, was used in the early experiments, while Nembutal Sodium Veterinary Sterile Solution (Abbott Laboratories) was used in Experiments 23 to 57. There was no difference in the response to the two types of Nembutal solutions.

When anesthetized, the rats were placed on a rat board with the ventral side upward. The jaws and the four limbs were clamped in a stationary position. Pin type electrocardiographic electrodes were then inserted into the four limbs to permit monitoring heart rate changes during the operative procedures.

The skin was removed from the neck region, exposing the external carotids and the clavicles. Heparin was injected into the left external jugular vein using a 25 gauge hypodermic needle inserted under the left clavicle into the left jugular vein. The dosage of Heparin Sodium (Mann Research Laboratories) used was 2.24 milligrams (300 Units) per 100 grams of animal weight. The injected 1% solution was made using the physiological saline as solvent. Following the

heparin injection, the right jugular vein was carefully exposed for approximately two centimeters cephalad from the clavicle in preparation for the cold infusion.

The cold infusion fluid was prepared well in advance of the operation, according to the formula described later. The infusate was cooled, and kept cold, by placing it in a 250 ml aspirator bottle surrounded by crushed ice. The solution was placed in a stand 180 cm above the level of the anesthetized rat. The solution reached the rat through Tygon tubing with an internal diameter of 1/8 inches and an external diameter of 3/16 inches. The solution in the tubing was kept cold by ice cold water circulating within a 3/4 inch Plexiglass cylinder surrounding it. Ice cold water was continuously circulated back to the ice bath by a Roll-Flex Laboratory Pump (Cole-Parmer Instrument Company). Using appropriate reducers, the infusate was conducted by the shortest possible length of tubing to the polyethylene cannula with an internal diameter of 0.560 mm and an external diameter of 0.965 mm (PE 50). The infusate was bubbled with a 95% O_2 - 5% CO_2 gas mixture for a minimum of thirty minutes prior to the infusion. Flow was controlled by an adjustable clamp applied to the tubing. A schematic of this apparatus is shown in Figure 1.

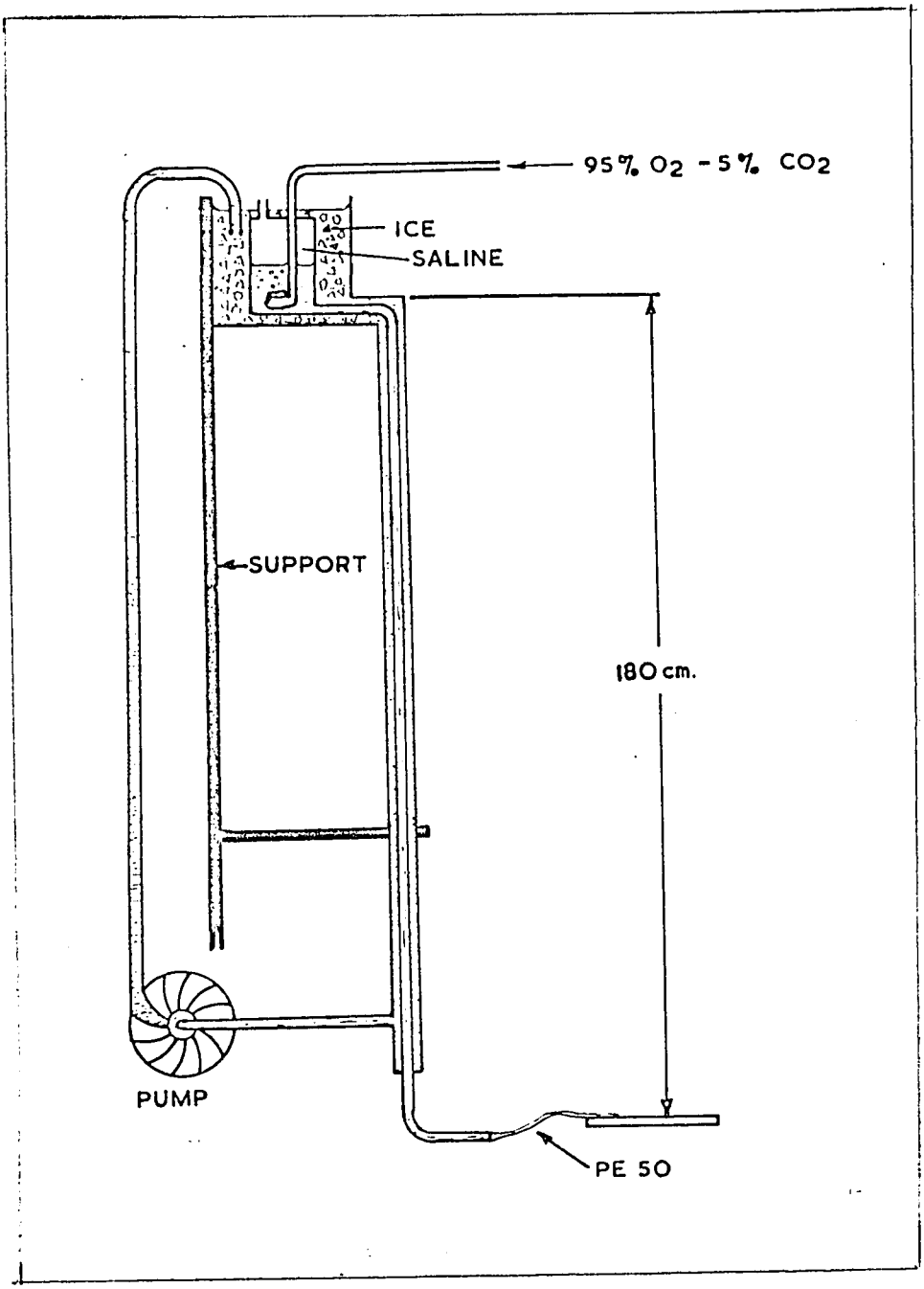

FIGURE 1. The Cold Saline Infusion Apparatus

Cannulation of the right external jugular was begun approximately two minutes following the injection of the heparin so as to permit the complete circulation and effect of the heparin. Before inserting the cannula, saline was flushed through until the coldest possible saline was available. After inserting the cannula, the appropriate ties having been made, the right external jugular was cut cephalad to the cannula in order to allow drainage of the blood as it was replaced by the ice-cold saline. In four experiments, the chest temperature change was monitored by a surface temperature probe which read out by means of a Yellow Springs Instrument Company, Model 46 TUC Multi-Range, Multipurpose Thermistor Thermometer.

When the heart rate had reached a minimal equilibrium level, the ECG electrodes were removed and the heart was exposed as quickly as possible. To expose the heart, the thoracic cage was cut around its periphery and removed completely. This was done rapidly by cutting the abdomen transversely below the diaphragm, freeing the ventral border of the diaphragm, cutting anteriorly through the thoracic wall to the axillary region, and then freeing the thoracic cage by a transverse cut at the level of the clavicles. As soon as the heart was exposed, the ice-cold saline, previously used for perfusion, was used to bathe the exterior of the heart. The continued hypothermia kept the cardiac contractions at a minimum rate. While the heart was thus

bathed, the common carotids and the subclavians were tied off, and the aorta was cannulated at the level of the aortic arch using a polyethylene cannula (PE 280) with an internal diameter of 2.15 mm and an external diameter of 3.25 mm. The heart was freed from all surrounding or attached tissues, blood vessels, and nerves, by careful dissection. Then the heart was placed in a petri dish containing cold saline for the final preparations before perfusion.

While in cold saline, the final preparations of checking the position of the cannula, attaching a silver wire, and attaching the cannula to a section of the perfusion system, were completed as rapidly as possible. The position of the cannula relative to the semilunar valves was checked by observing the extent of the rise and fall of the liquid in the cannula. If the valves had been violated, the preparation was discarded. The cannula was cut to a length of about 1 centimeter and attached to the tubing which, in turn, served to attach the complete perfusion train to the total perfusion apparatus. See Figure 2. All branches of the tubing and the cannula were filled with saline and clamped off to prevent emptying. One end of an approximately 15 centimeter length of 0.008 inch diameter silver wire was pushed through the apex of the myocardium, looped, and tied securely. This wire was used later to activate the tension transducer and also as

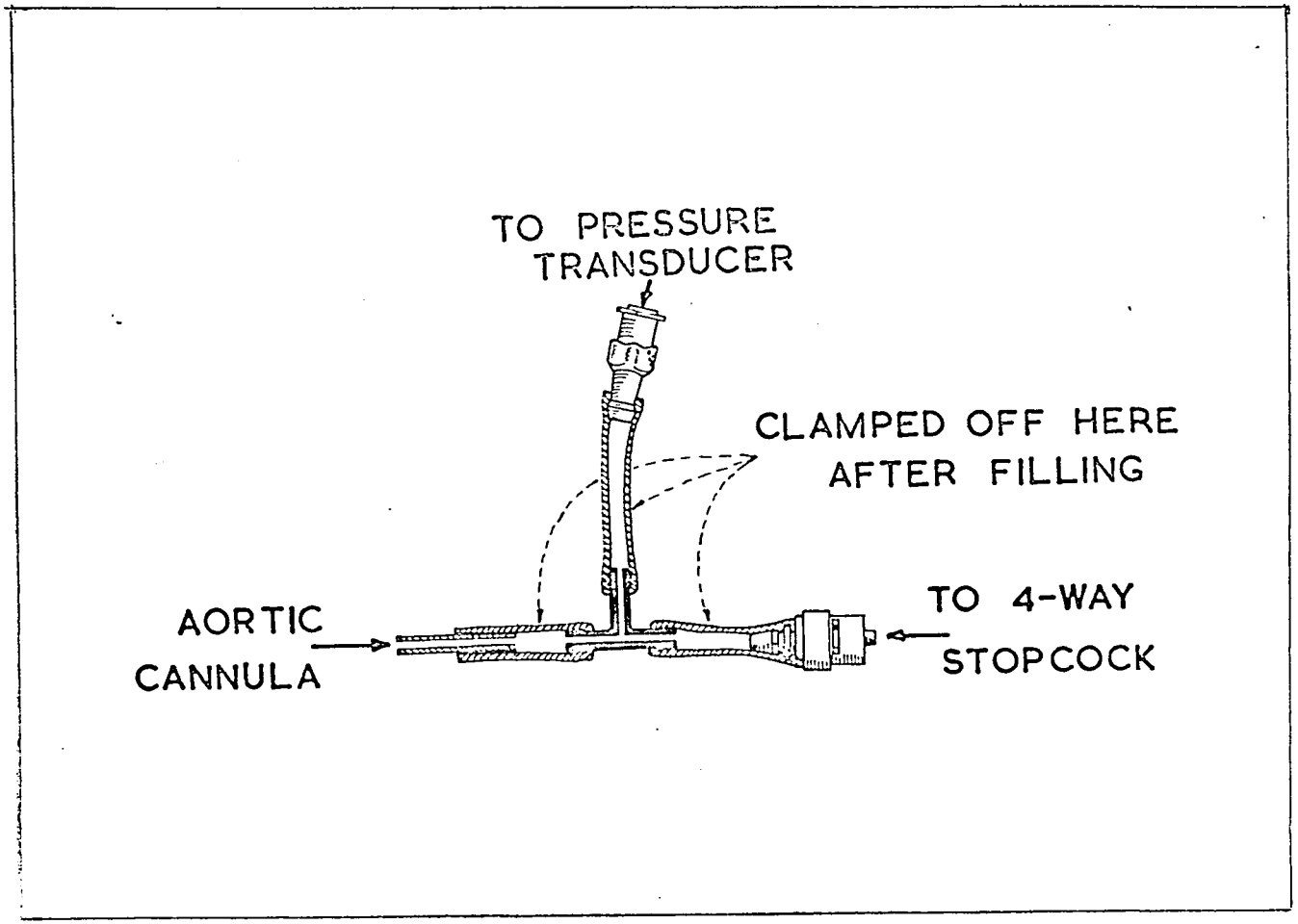

FIGURE 2. The Final Section of the Perfusion Line

one of the electrodes to record the electrical activity of the heart. After these preparations were completed, the heart and attached apparatus were transferred to the perfusion apparatus while still in the petri dish of cold saline.

III. PERFUSION AND RECORDING APPARATUS

Perfusion fluid. The perfusion fluid used in all control perfusions was of the same composition as that used by VAUGHAN WILLIAMS (1955). As described by Vaughan Williams, the solution contains, in milliosmoles, the following constituents: sodium 139.7, potassium 5.63, calcium 2.165, glucose 11.12, and bicarbonate together with chloride 149.66. This gives a total of 308 milliosmoles. Preferring to express these values in terms of mEq per liter, and expressing the separate concentrations for bicarbonate and chloride, the concentrations as used in these experiments were: sodium 139.7, potassium 5.63, calcium 2.165, glucose 11.12, bicarbonate 28, and chloride 121.66. In terms of the compounds used to yield the appropriate ionic concentrations, the concentrations used, in mEq per liter, were as follows: KCl 5.63, $CaCl_2$ 2.165, NaCl 111.7, $NaHCO_3$ 28, and glucose 11.12. Appropriate stock solutions were prepared so that the KCl and $CaCl_2$ could be added with a dilution of 1/100 and the NaCl and $NaHCO_3$ by a dilution of 4/100. Glucose was added as the dry reagent in the required amount for each experiment when the solution was prepared. The chemicals used were Fisher Certified Reagents in all cases. Solutions were constituted using water demineralized by a Barnstead "Bantam Demineralizer" with a Mixed Resin Cartridge, or by a

"Deeminizer" demineralizer.

In the experimental perfusions, the required changes might or might not necessitate changes in the above-described ionic constituents of the control perfusion fluid. When the objective of the experiment called for observation of the effects of changing both pCO_2 and pH of the perfusate, no change in the concentration of the chemicals added was necessary, since all changes were accomplished by changing the gas mixtures bubbling the perfusate. This will be described in a following section. In those experiments where pCO_2 was kept constant and it was required to change the pH, the concentration of bicarbonate was decreased in accordance with the relationship expressed in the Henderson-Hasselbalch equation

$$pH = pK + \log \frac{[HCO_3]}{[CO_2]}$$

Corrections for pK, CO_2 in solution, and salt error were incorporated into the basic relationship, and a modified form of the Henderson-Hasselbalch equation, as used by [illegible] (1955) was employed:

$$pH = pK + \log [HCO_3] + \log \frac{100}{x} + \log \frac{760}{(P-p)} \cdot \frac{22.4}{\alpha} - 0.5 \sqrt[3]{C}.$$

(In this equation P is the barometric pressure, p the vapor pressure, x the percentage of CO_2, α the solubility of CO_2 in volumes of gas absorbed by one volume of water when the pressure of the dry gas is 760 mm Hg, and C the molar concentration of cations.) When the bicarbonate concen-

tration was decreased, the difference between the required concentration of bicarbonate and the control bicarbonate concentration was replaced by sodium methyl sulfate (K & K Laboratories, Inc., Plainview, New York). To achieve a pH of 6.80 with the CO_2 remaining constant at 5%, it was necessary to use a bicarbonate concentration of 7.0 mEq per liter, so that 21 mEq of sodium methyl sulfate were used per liter. The total concentration of sodium methyl sulfate and sodium bicarbonate then equals the 28 mEq per liter of sodium bicarbonate in the control perfusate.

Sodium methyl sulfate was used to replace sodium bicarbonate for several reasons. First, the cation in both compounds is sodium; therefore, the change would not require a change in the ionic environment with respect to sodium. Second, the osmotic pressure of the solution would not change since, like the bicarbonate anion, methyl sulfate is a monovalent anion (BOISTEL and FATT, 1958). Third, the presence of the methyl sulfate anion does not cause calcium to precipitate (HUTTER and NOBLE, 1960). Fourth, the methyl sulfate anion does not penetrate the muscle membrane (HUTTER and NOBLE, 1960) and thereby behaves as does bicarbonate (see the discussion in Chapter I).

Sodium methyl sulfate could have a pharmacological action on the tissues of the heart. In control experiments used to check the possibility of such an effect, methyl sulfate replaced an equivalent amount of chloride ions

(21 mEq/liter). This represented a replacement of 17.8% of the chloride by another anion which is not without some danger of producing an effect due to chloride replacement alone. However, it has been reported (GARGELY, 1964) that methyl sulfate caused a potentiation of contraction when present at an optimal concentration of 100 mM. The concentration used in these experiments, 21 mM, is considerably smaller than the reported optimum and should give a minimal enhancement due to decreased chloride.

All solutions were made up from stock solutions on the same day as the experiments were performed. Stock solutions were always mixed vigorously before the pipeting. All solutes dissolved without difficulty. An eight liter batch of solution was routinely made for each experiment. The eight liter polyethylene aspirator bottle was then attached to the inlet of a Polyethylene Centrifugal Laboratory Pump (Cole Parmer). The impeller of this pump is polyethylene sealed to a stainless steel shaft within a polyethylene pump head. There should be no reaction with, or contamination of, the solution by this sort of pump. Saline was distributed to the perfusion tanks from the one eight liter batch in order to randomly distribute the saline and eliminate the effect of errors in concentration, which could have occurred, had each of the tanks of perfusate been constituted and filled separately.

The saline distribution system. The total perfusion apparatus was supported within a 2' x 3' framework of 1½" x 1½" angle steel (HEX-ANGLE) and mounted on casters. This type of free-standing support was found to facilitate a flexible, readily modifiable operation. The perfusion tanks and tubes were supported by a 1½' x 1½' superstructure rising 2½' above the main apparatus support and constructed of the same Hex-Angle steel supports. When the perfusion tanks were in position on this superstructure, the bottoms of the tanks were 195.0 cm above the top of the working surface and 167.6 cm above the level of the perfused heart.

The three tanks containing the perfusates were made by dividing a Plexiglass container, whose dimensions were 29.5 cm wide, 29.5 cm deep, and 30.5 cm high, into three compartments. The limiting factor in the design of the interior divisions into compartments, was the need to place the downward conducting tubes within a diameter described by the thermoregulating column. No attempt was made to keep the volume of all of the compartments equal since the maximum containable volume of each chamber was greater than the volume of fluid required for even the longest experiment. The tanks were designed to hold a larger volume than necessary in order to eliminate the effect of an appreciable fall in the hydrostatic pressure head during an experimental perfusion. Each of the compartments

was formed by 0.5 cm thick Plexiglass walls which were screwed together and then sealed with cement (TENSOL Cement Number 7). Each tank was checked for leakage to eliminate the possibility of intermixing of the perfusion fluids.

The three tanks were covered by a single cover to prevent contamination of the perfusate by airborne particulate matter. Three holes, of appropriate size, were bored through the cover to allow passage of three tubes for the introduction of the perfusate. Another group of three holes was bored to allow the escape of the bubbling gases. Restriction of the escaping gases, to one exit point of small size, was designed to retard the escape of gases, so as to provide an equilibrium environment above the perfusion fluid without increasing the pressure above the perfusate. It was felt that these measures would serve to assist in maintaining the steady state of the perfusate buffer system once it had been achieved. The amount of oxygen in solution also would be kept at equilibrium by retarding the escape of gases from above the solution. The stability of the HCO_3/CO_2 buffer system was ascertained by pH readings taken of the perfusate over long periods of time. This will be discussed and illustrated in Chapter Three.

The design and orientation of the various parts of the perfusion fluid conduction system may be seen by referring to Figure 3.

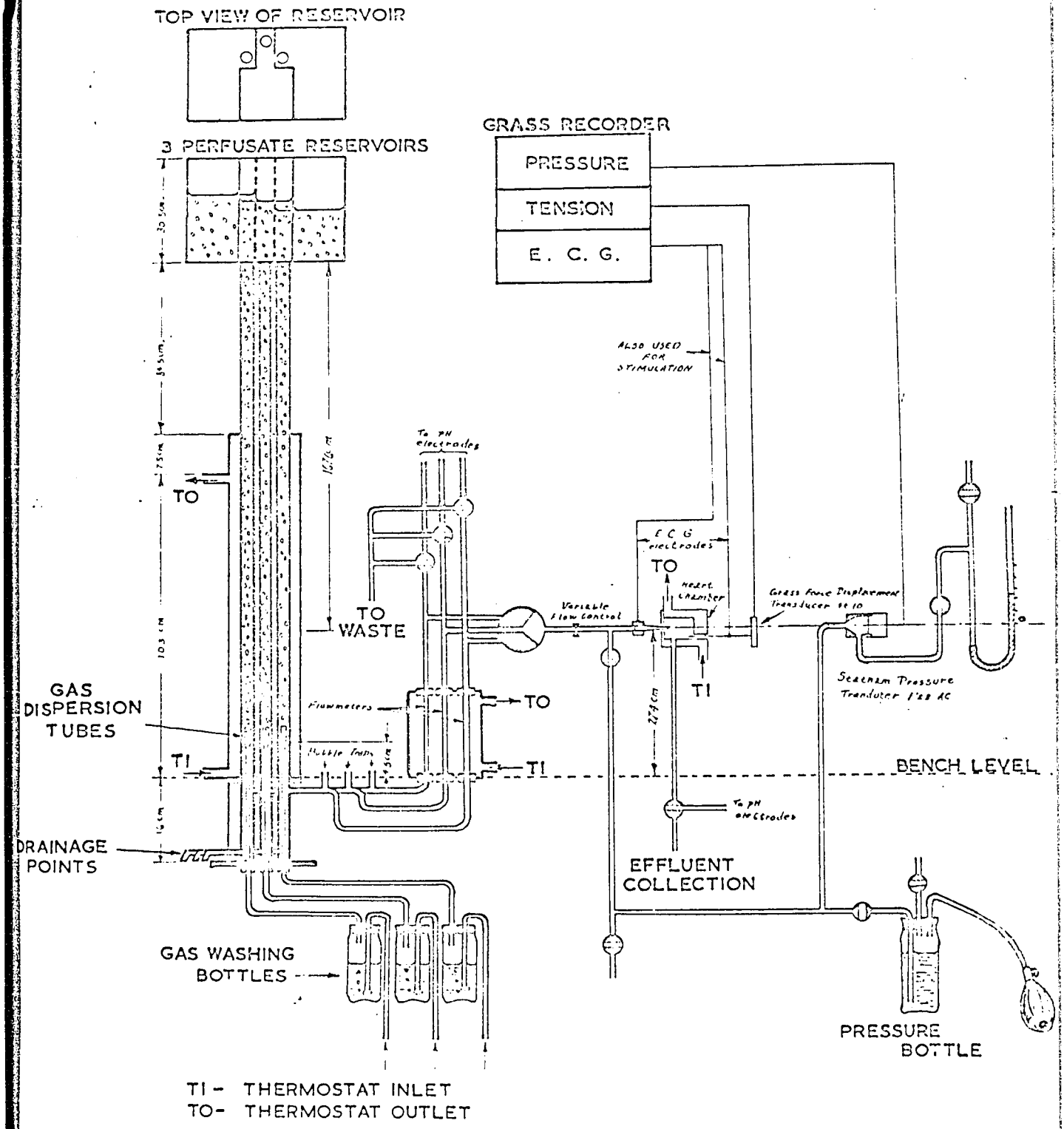

FIGURE 3. A Schematic Representation of the Total Perfusion and Recording Apparatus

The three tubes, designed to conduct the perfusate downward through a thermoregulating column, were Plexiglass cylinders with an internal diameter of 1.5 cm. This diameter was chosen to allow the insertion of gas dispersion tubes at the bottom of the columns. Each tube consisted of two sections; an upper section, 84.5 cm long, rigidly fixed to the perfusate reservoirs, and a lower section, 126.5 cm long, rigidly fixed inside the thermoregulating chamber. The two sections of each of the three tubes were joined by a flexible joint of 1.9 cm internal diameter Tygon Tubing, fitted over the contiguous ends of the tubes. The lower ends of the three tubes were sealed off by Neoprene stoppers, bored to allow the introduction of the gas dispersion tube stems. The stoppers were held in place by wire ties to the Plexiglass plate at the bottom of the column.

Efflux of the perfusate from the vertical columns was possible by several avenues. The exit of primary importance was that leading to the heart perfusion chamber. Auxiliary outlets were provided for draining the columns, adjusting perfusate reservoir levels, trapping bubbles, perfusion pressure determination, and perfusate pH determination. The primary outlets originated from the downflow columns at bench level by means of three Plexiglass tubes of 0.5 cm internal diameter and approximately 11 cm long. These outlets were placed approximately 4.5 cm below the base of the gas dispersion surfaces in order to reduce the possibility

of catching gas bubbles in the perfusate. The outlet tubes were connected to Pyrex or Polyethylene T-pieces in each line by Tygon tubing of the appropriate size. The horizontal limbs of the inverted T-pieces served as straight-through paths for the perfusate to pass on towards the heart. The vertical limbs of each of the T-pieces was attached to a 20 cm piece of Tygon tubing, which was clamped off near its end to serve as a bubble trap.

From the horizontal arms of the T-pieces serving as bubble traps, the perfusate passed through 5 cm of Tygon tubing to the bottom of rotameter type, glass flowmeters (Cole-Parmer) in each of the perfusate lines. The three glass flowmeters were thermoregulated within a Plexiglass box whose dimensions were 13 cm wide x 6 cm thick x 18 cm high. The upper end of each of the flowmeters was connected to a Polyethylene T-piece by 11 cm of 1/8" internal diameter Tygon tubing. The vertical arm of each of the T-pieces was connected to a three-way B-D MS05 stopcock (Becton-Dickenson) by 30 cm of 1/8" internal diameter Tygon tubing. These stopcocks could be closed, opened to a line leading to a waste bottle for flushing, or opened to a Tygon line leading to the pH electrode. The horizontal arm of each of the T-pieces was connected by a 1 cm length of 1/8" internal diameter Tygon tubing to a B-D MS14 four-way stopcock (Becton-Dickenson) which switched the flow of perfusate from any one column to the aortic cannula.

The object of placing the T-pieces immediately preceding the four-way stopcock, was to permit flushing out the dead space in each of the perfusate lines immediately before an experimental perfusion. Because of this location of the T-pieces, perfusate flushing or perfusate pH determination was possible without disturbing the flow of the perfusate in the line supplying the heart.

The final section of the perfusion conduction system was designed for easy removal and quick attachment, so that it could be attached to the aortic cannula, filled with cold saline, and reattached to the total perfusion apparatus with minimal difficulty. The attachment to the aortic cannula and the filling with saline has been described in the previous section on General Operative Procedures. Figure 8 shows this assembly.

To accomplish the design objectives discussed in the previous paragraph, the final section of the perfusate conduction system provided for quick and leakproof connection to the four-way stopcock, the aortic cannula, and to the pressure transducer. The attachment to the four-way stopcock was by a B-D 608/L, 1/6" tubing to male, Luer-Lok adapter. This was attached to one of the horizontal arms of a T-piece by 1.5 cm of 1/16" internal diameter Tygon tubing. The other horizontal arm of the T-piece was attached to a 1 cm length of the same Tygon tubing. Inside of this tubing was inserted the cannula of 1.67 mm internal diameter x 2.42 mm

external diameter Polyethylene (PE 240). The tubing lengths and diameters were kept minimal in order to minimize the dead space remaining after switching to another perfusate. The volume of liquid held by this assemblage of tubing was 0.4 ml. This represents a dead space which would be used up in approximately eight seconds at the mean flow rate of 3.0 ml/min. It was felt that this time was sufficiently small to be satisfactory.

The vertical arm of the T-piece, in the final section of perfusate tubing, was connected to an L/606 female Luer-Lok to hose end by a 3 cm length of 1/8" internal diameter Tygon tubing. This could be connected quickly, to a matching male Luer-Lok fitting attached to a line of tubing connected to Statham Model P23AC pressure transducer (Statham Instruments, Inc., Hato Rey, Puerto Rico).

The rate of flow of the perfusate was controlled by a clamp applied to the tubing just preceding the branch leading to the pressure transducer. A Castaloy Hosecock Clamp (Fisher Scientific) was used and kept stable by a Castaloy Versatile Clamp, Micro Size (Fisher Scientific). The threads of this hosecock clamp were machined well enough to allow fine adjustment of flow rate, by application of pressure to the outside of the tubing carrying the perfusate.

The remaining efflux pathway for the perfusion fluid was at the very bottom of the vertical downward conducting tubes. Plexiglass tubes of 0.5 cm internal diameter were

cemented to the vertical tubes. To the Plexiglass tubes were attached rubber tubes, closed off by pinch clamps to provide control of the flow of perfusate. This point of efflux permitted complete drainage of the perfusate in the tanks, columns, and in all of the small tubing after each experiment. This efflux also served the very important function of providing a means of equalizing the level in the perfusate tanks before each change to an experimental perfusate.

Although the perfusion system was drained after each experiment, evaporation of the remaining film of solution did leave a residue which became objectionable after a few experiments. Since this film consisted mainly of carbonates, it was found that a solution of weak hydrochloric acid (0.1 N) would dissolve it and completely clean the perfusion system. The cleaning was followed by five rinsings with demineralized water. This removed all traces of precipitate and acid. The removal of all traces of acid was verified by a check of the last rinse with pH electrodes. This procedure, for removal of the film of precipitate, was followed whenever the film became visible.

The heart chamber. The chamber to contain the heart during the experiments was designed to fulfill certain requirements which, in turn, permitted achievement of the

experimental objectives. The primary consideration was to keep the heart in an environment such that its cells would suffer minimum damage and would continue to function as normally as possible, despite the in vitro location. Of equal importance was the requirement that recording of all parameters must be possible with a minimum lag time. This meant that all dead spaces and points of inertia production must be kept minimal.

The required stable environment, external to the isolated heart, was found to be best delimited by a glass double-walled chamber with Plexiglass removable covers. Glass could be formed, in the hands of an expert glass blower, into a chamber with all of the required contours and openings. Mr. Lou Page of the National Research Council's Biology Workshop, constructed the chamber to the required specifications. The cover, to close off the open end of the chamber, was most efficiently made of Plexiglass since it had to resist dropping and an opening for the aortic cannula was required. To seal the Plexiglass-glass interface and other small openings, the best substance was found to be Vaseline. Vaseline was non-toxic to the heart tissues and furnished a minimum amount of inertia where it came in contact with the wire leading to the tension transducer. Therefore, using a glass chamber, a Plexiglass cover, and Vaseline seals, the heart could be isolated within its own new environment which could be controlled and which per-

mitted measurement of the desired parameters of heart function (Figure 4.)

The temperature of the heart's in vitro external environment could be controlled or kept constant by controlling the temperature of the water flowing in the wall of the double-walled chamber. The heart chamber was connected to the thermoregulating system which will be described in a following section. The same thermoregulating mechanism controlled the temperature of the perfusate, hence the temperature of both the internal and the external environment of the heart was controlled.

It was required that the cover over the open end of the heart chamber should be readily applicable without disturbing the heart on its cannula. As originally designed, the silver electrodes, for recording the electrical activity of the myocardium, passed through the cover by means of small holes drilled through it. To fill these needs and to eliminate the danger of breakage when accidentally dislodged, Plexiglass was used to construct the cover. A slot was cut out of the cover to allow it to be slipped easily over the cannula. The slot, cut out of the cover, was just larger than the outside diameter of the polyethylene cannula. After it was in position, the remainder of the slot, not occupied by the cannula, was filled with Vaseline to provide a seal from the outside environment. Vaseline was also used on the point of junction between the glass chamber and the

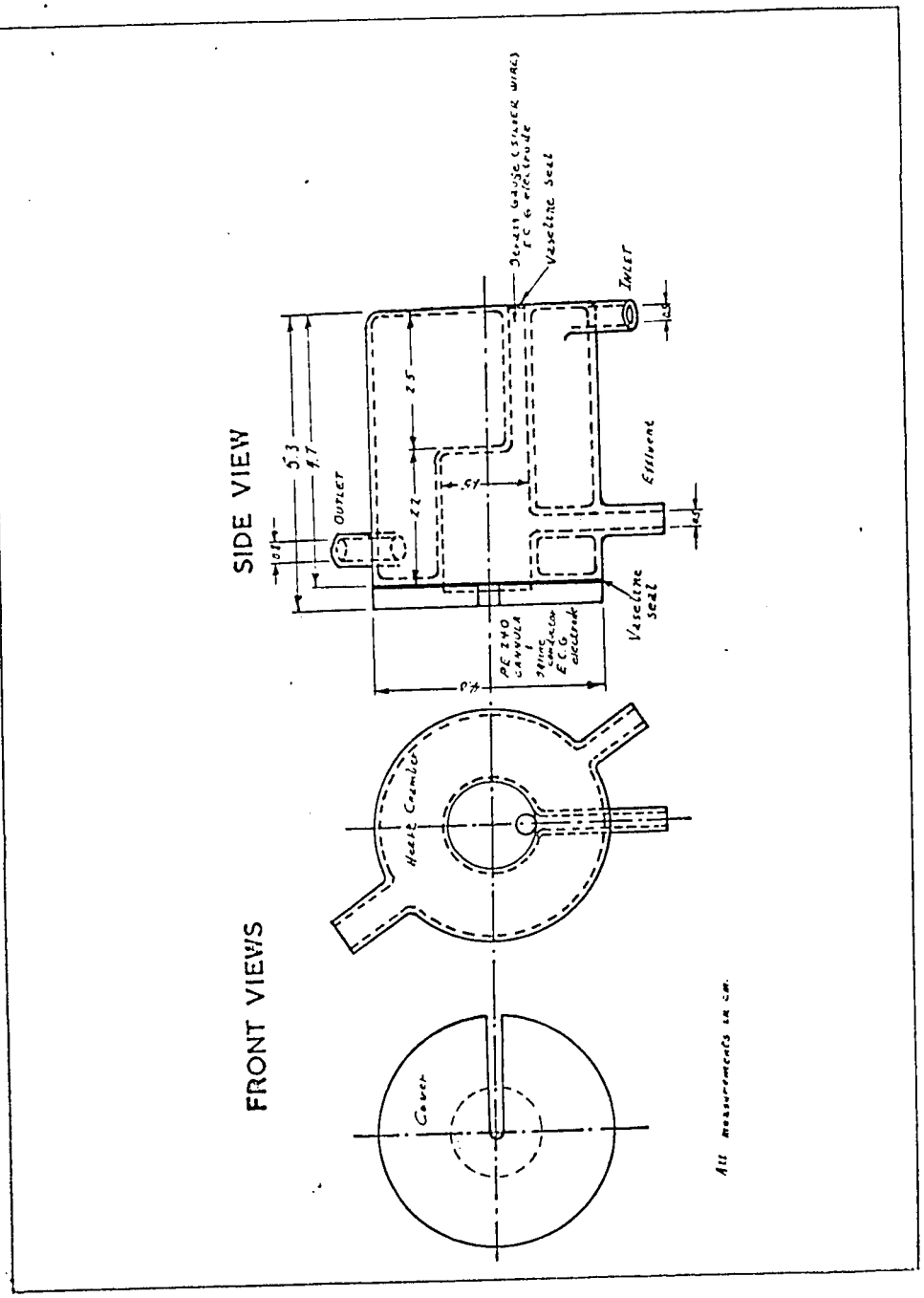

FIGURE 4. The Heart Chamber

Plexiglass cover. The covers used in the early experiments were bored in two places to permit insertion of two silver wires, used as electrodes for recording the electrical activity of the heart. Plexiglass O-rings were made to provide additional length to the chamber if required by larger hearts. These could be added beneath the cover using Vaseline seals on both surfaces. The cover was firmly held in place by a Castaloy-R Versatile, Micro-size Clamp to prevent breakage of the Vaseline seals during experiments.

The silver wire, affixed to the apex of the heart during preparation of the heart for perfusion, was easily guided through the small channel provided and as shown in Figure 4. This small channel could be sealed with Vaseline without affecting the tension recording system. In the later experiments, this silver wire served as one of the electrodes for recording cardiac electrical activity and for stimulating the heart when it was required to drive the heart at a constant rate. The heart chamber was clamped in position using a medium-sized, vinylized jaw, Castaloy-R Versatile Clamp attached to a flexible mount. This mounting arrangement permitted the adjustment of the chamber's position, so that the pull by the heart on the silver wire to the tension transducer could be transmitted without inertia, due to contact with the glass walls of the chamber.

The path of flow of the effluent from the perfused heart had to be controlled so that it could be channeled into the pH electrode or to sample collection. To accomplish this, a T-bored stopcock with a Teflon plug was used. Each of the arms was cut to a length of approximately 1 cm in order to reduce the dead space to a minimum. The cross bar of the T was placed in a vertical position to provide rapid, straight-through drainage when draining the chamber or collecting samples. The upper arm of the vertical portion of the T was attached to the heart chamber's effluent drain by a 1 cm piece of Tygon which provided a flexible, yet airtight, connection. The lower arm of the vertical path of the stopcock permitted sample collection or could be attached to a waste line to discard the effluent. The remaining horizontal arm of the T-bored stopcock was attached, by appropriate Tygon tubing, to the pH electrode. Since slight suction was required to draw the fluid into the pH micro-electrode, the presence of the added friction, imposed by the T-shaped pathway of the effluent to the pH electrode, posed no problem. Drainage of the chamber by the vertical pathway was by the force of gravity alone. Therefore, the Teflon plug was rebored to a slightly larger than normal diameter in order to allow rapid drainage. This capability of allowing enlargement of the bore to required size, as well as the inert nature of Teflon and its ease of operation, made a Teflon plug the most useful type available

for this three-way stopcock.

To measure pH reproducibly when a HCO_3/CO_2 buffer system is used, it is imperative that the system provides for minimum loss of CO_2. From the above description of the chamber, it can be seen that a gas-tight environment was provided around the heart and in the line leading to the pH electrode. It was desirable also to keep the dead space around the heart at a minimum, so as to provide a minimum sink into which CO_2 could be lost, and a minimum reservoir from which CO_2 could be gained. With this in mind, the heart chamber was designed so that it would just enclose a heart, without blocking the effluent drain. Also, all of the connections to the 3-way stopcock were kept as short as possible, and the diameter of the passageway for the silver wire was made as small as possible.

It was essential to keep a visual check on the functioning of the isolated heart and for this additional reason, the chamber made of glass was most desirable. A visual check of the heart revealed the nature of the heart's contractions which indicated the condition of the preparation. Complete and continuous drainage of the chamber was important in that a fresh sample for pH determination was always used, and this could be checked quickly with the glass chamber. The pull of the tension wire on the apex of the heart was checked frequently to insure that the pull was always direct, without causing the heart to wedge in one corner of the

chamber.

The thermoregulating system. The temperature of the thermostat was kept constant, at the preset temperature, by a Brownwill (Thermomix) Constant Temperature Circulator (Canlab). The circulator contained a built-in circulating pump which was used to thermoregulate the pH electrodes and the heart chamber. The circulator was immersed in a 28 cm x 28 cm tank filled to a height of 15 cm. From the bottom of this tank, an outflow to an external pump, a Polyethylene Centrifugal Laboratory Pump (Cole-Parmer), provided circulation to the 110.5 cm x 12.7 cm thermoregulating cylinder surrounding the downward conducting perfusate tubes and to the chamber surrounding the flow meters. The circulating water was returned from the top of the cylinder to the thermostat tank. See Figure 3. The water in this circuit was changed every 20 seconds, thereby providing a good thermoregulation. Since the thermoregulatory fluid surrounding the pH electrodes provided a shield for these electrodes, the thermostat bath was grounded by means of a copper wire immersed in the bath and attached to a water pipe.

Gas mixtures. The gases used to bubble the perfusates were mixed in two different ways. One group of gases were used routinely and could be readily obtained from a commercial supplier; another group consisted of special mixtures which were most efficiently mixed in the laboratory. The gases used routinely were the following: 95% O_2 with 5% CO_2, 80% O_2 with 20% CO_2, and 90% O_2 with 10% CO_2. These gas mixtures were Linde Gases and were obtained from Union Carbide Canada Limited in Ottawa. Those mixtures which provided stepwise changes in pCO_2 or pO_2, were mixed in the laboratory from tanks of the pure gases also obtained from Union Carbide.

The gases mixed in the laboratory were mixed in a spirometer (Warren E. Collins Inc., Boston, Mass.) with a capacity of 10 liters. The pure gases were introduced into the spirometer until the appropriate percentage of the total gas volume had been reached. After all of the required gases had been mixed, the dead space, which still contained the last gas introduced, was flushed out. The gas mixtures, mixed in the spirometer, were pumped through the gas dispersion system by either a Roll-Flex Laboratory Pump or by a Bantam Dyna-Vac Laboratory Pump (Cole-Parmer).

All gases used were saturated with the perfusing solution by passing through Pyrex Gas washing Bottles (Canlab). The flow of gas to the gas dispersion tubes was regulated by a needle valve in each line which, in the case

of the ready-mixed gases, was in addition to the two-stage regulators on the gas cylinders. All lines conducting the gas from the cylinders, or the spirometers, to the dispersion tubes were of Tygon.

The gas dispersion tubes were inserted into the bottom of the vertical perfusate columns, to provide a column of maximum length for solution of the gases and, at the same time, providing a minimum of solution as a dead space not recently gassed. The gas dispersion tubes used were Pyrex Brand, 4 inch, Tubular, Fritted Filter Tubes of fine porosity. These tubes provided multitudes of small gas bubbles for efficient solution of gases, without causing fluctuations of the perfusion pressure. To equalize the effect of the lifting action of the bubbling gases on the perfusion pressure, the flow of gases was adjusted by the needle valves so that the rate of bubble formation was equal in all columns. To check this method of cancelling pressure effects of bubbling for its effect on perfusion pressure changes, some experiments were done wherein the bubbling was stopped during the perfusion from a particular column. Results were the same in both cases. The pH of the perfusate remained constant for a period of time much in excess of any experimental perfusion period used. This is shown by the graph in Figure 5.

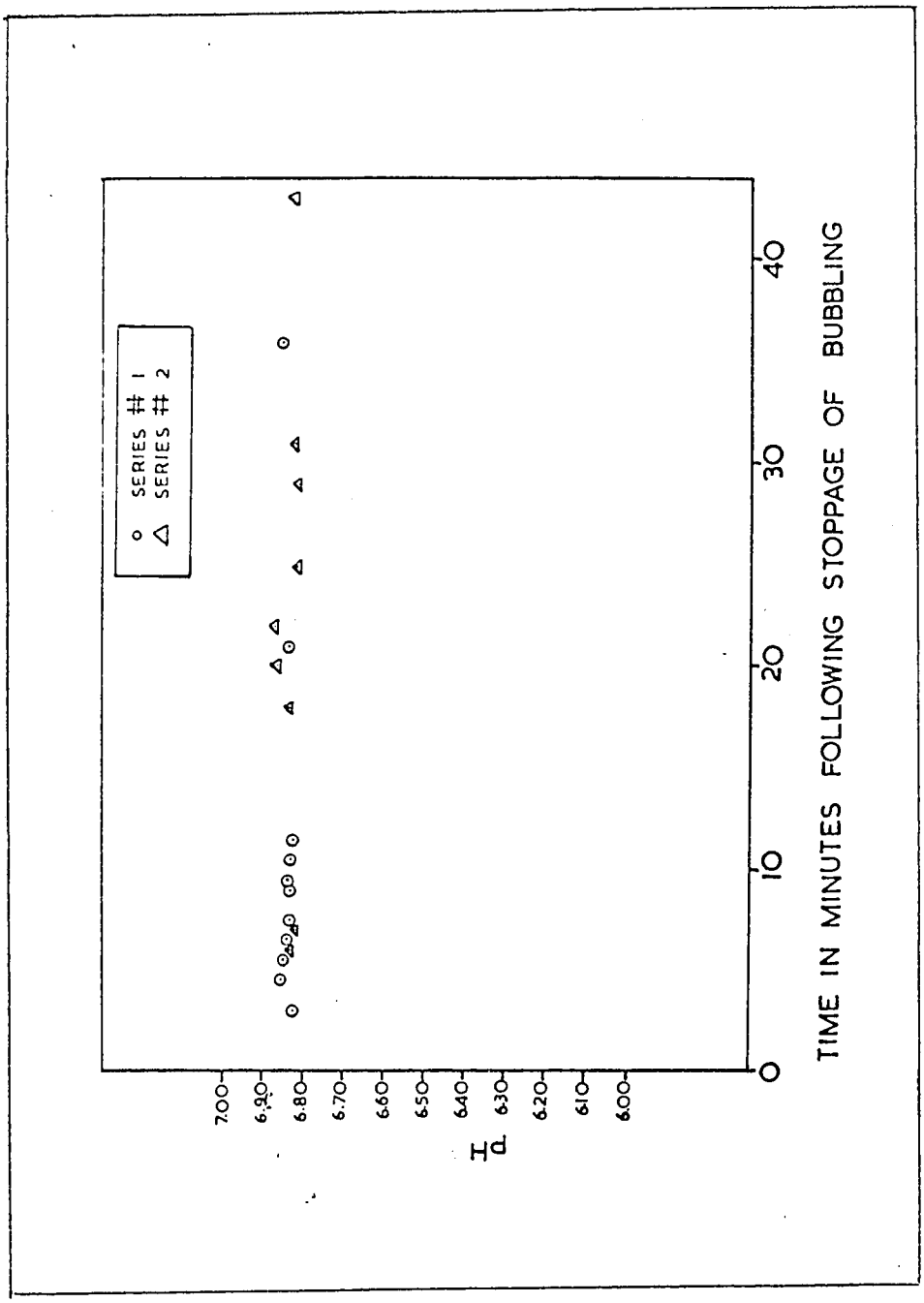

FIGURE 5. Perfusate pH Stability

Flow meters. The flow meters used to measure the flow rate of the perfusate as it was supplied to the isolated heart, were of the rotameter type with a spherical ball in a plain tapered tube. These flow meters were manufactured by Roger Gilmont Instruments, Incorporated, and distributed by Cole-Parmer. Since the fluid in the flow meters represented a considerable dead space, the flow meters were placed in the perfusion line before the switching stopcock by which the column of perfusate attached to the heart could be picked. This placement permitted flushing of this dead space before each perfusion. The flow meters were also thermoregulated by enclosing them in a Plexiglass chamber attached to the thermostat circuit.

The rate of flow of the perfusion fluids was read in relative values from 0 to 100. These could be related to actual flow rates by reference to calibration curves. The calibration curves were plotted from data acquired by using the perfusion fluid, at the perfusion temperature, to relate the absolute flow rate in ml/minute to the relative scale. These calibration curves are shown in Figures 6 and 7. Figure 6 shows the calibration curve for the three large capacity (0 ml to 35 ml) flow meters. Each point is the mean value of four determinations. Figure 7 shows the calibration curve for the small capacity (0 ml to 5 ml) flow meters. The small flow meters were found to give readings which were sufficiently similar to permit using

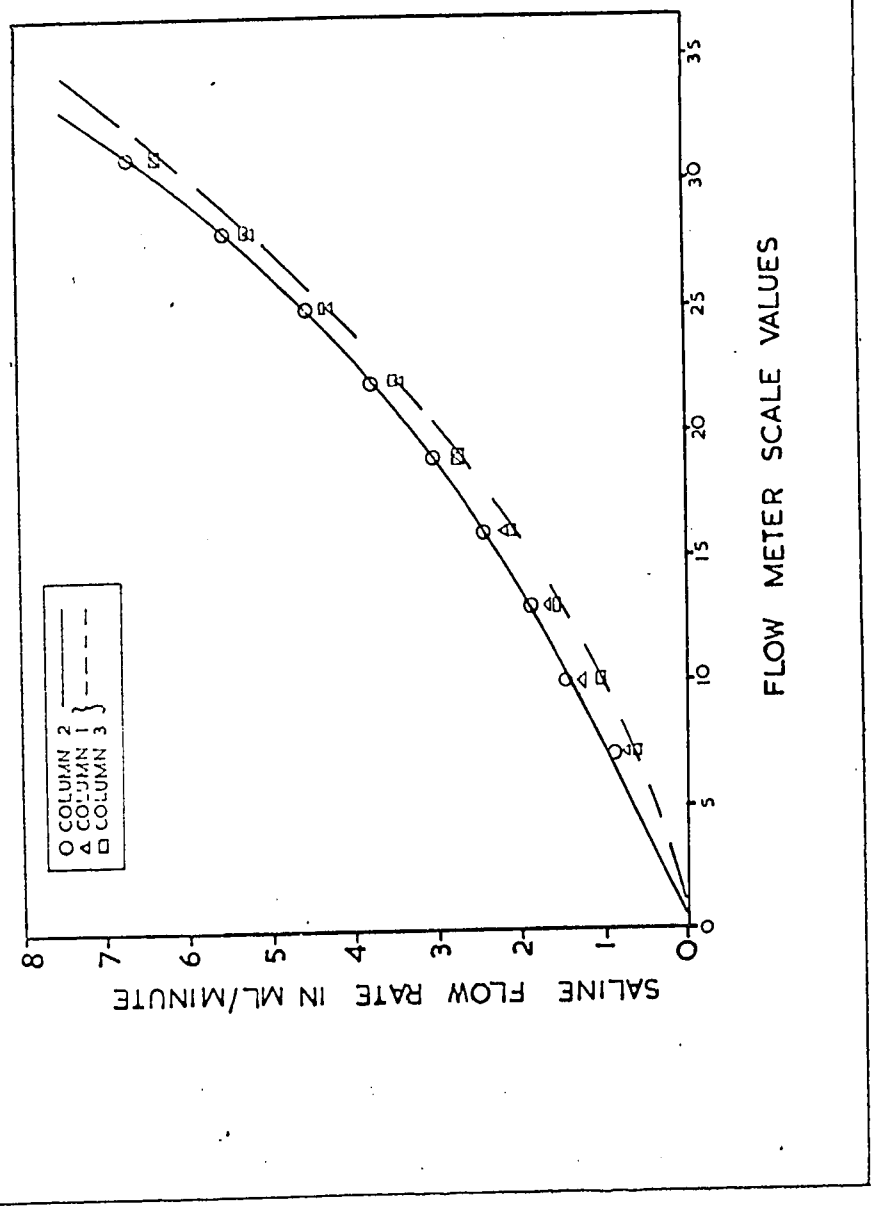

FIGURE 6. Large Flow Meter Calibration Curves

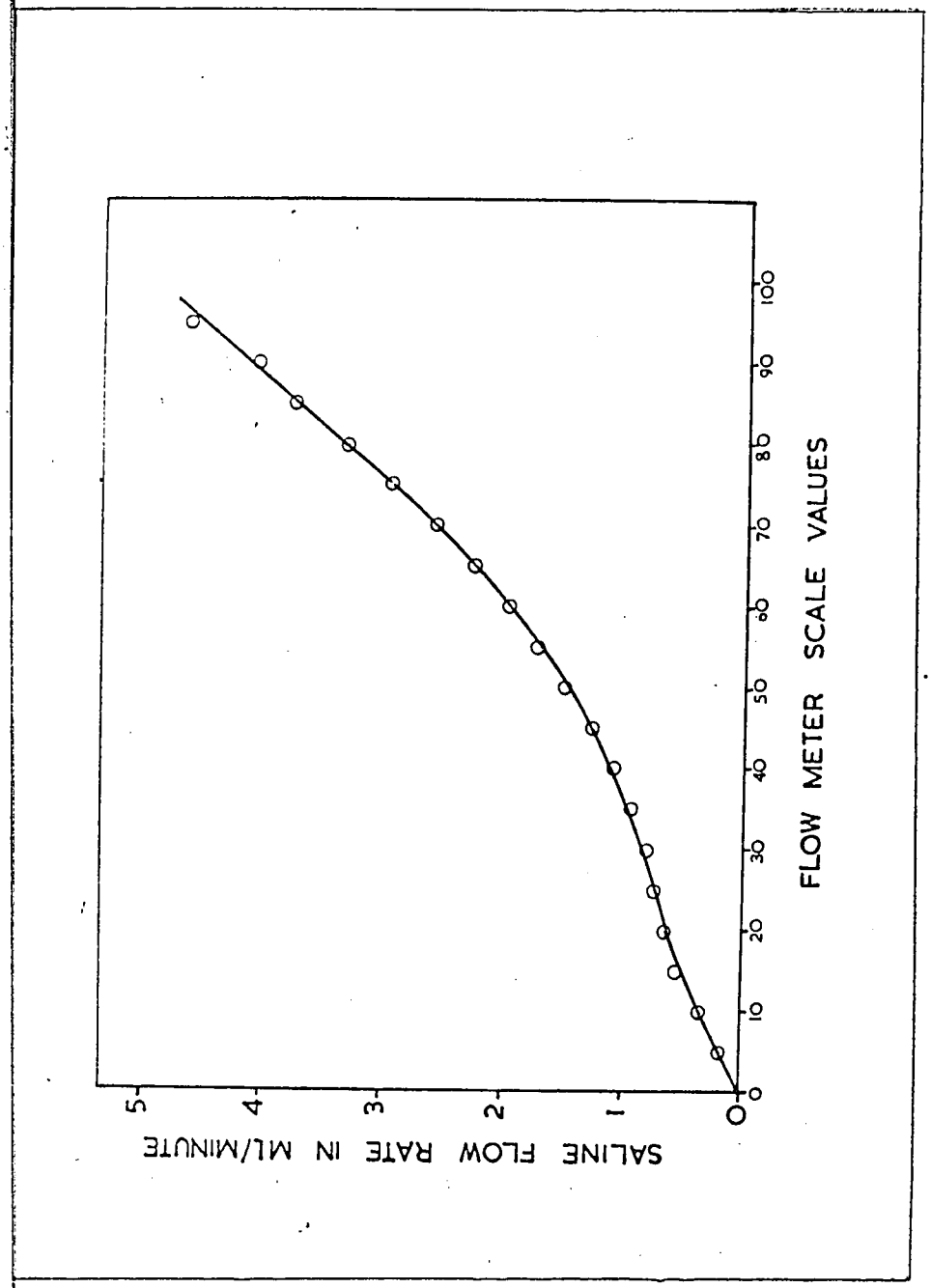

FIGURE 7. Small Flow Meter Calibration Curve

one calibration curve for the three flow meters. This curve is drawn from the mean value of two determinations for each of the three flow meters. The flow rate calibrations were made by collecting perfusate in a graduated cylinder for a measured length of time. The results were expressed in ml/min. These flow rates were then related to the values on the relative scale. All measurements for calibration were made when the flow had reached equilibrium. All calibrations and experimental readings were made at the center of the ball (rotameter) with the eye at the level of the rotameter to minimize the error from parallax.

Determination of pH. All pH determinations in these experiments were made using the Radiometer pH Meter 25 (Radiometer, Copenhagen, Denmark) with a Scale Expander, type PHA 925. This combination is referred to as pH Meter 25 SE. When used with the scale expander, the 0-14 scale of the pH meter is expanded to a 0-1.4 scale, the zero of which can be set at any value between 0 and 14 pH units. With the scale expander, the reproducibility of the pH Meter 25 SE is $\pm$0.002 pH units.

The electrode chain used with the pH Meter 25 SE was the Micro Electrode Unit (Radiometer) consisting of a glass electrode G297 and a calomel reference electrode K497. This Micro Electrode unit is shown in Figure 6. The two

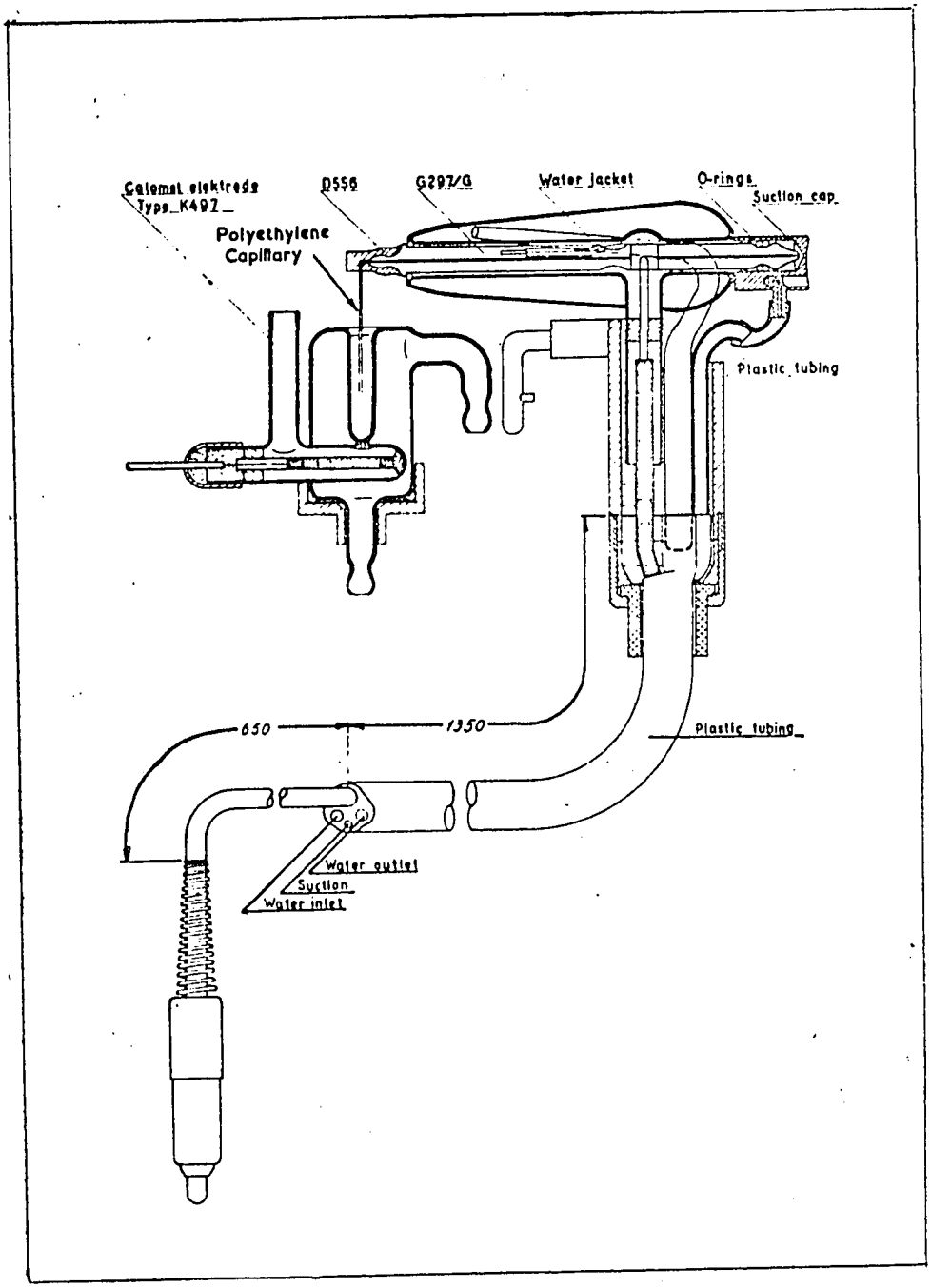

FIGURE 8. Microelectrode for pH Determination

electrodes and the liquid junction in this unit were thermoregulated by connection with the thermostat previously described. The sample volume required by this electrode was 20 microliters so that it was possible to take repeated samples from even the smallest volume of perfusate or effluent. In the sampling position, the polyethylene capillary of the glass electrode (see Figure 8) was lifted out of the calomel electrode by lifting and rotating the complete glass electrode assembly. In this position, the polyethylene capillary could be quickly attached to polyethylene tubing (PE 190) of 1.19 mm internal diameter, by simply inserting the capillary tubing into the PE 190 tubing. Since the capillary is tapered, a tight seal was made between the two pieces of tubing. This linkage completed the airtight connection between the heart chamber or the perfusate outflows (see Figure 3) and the pH electrodes. With this airtight chain of tubing and chambers, the pH of the perfusate or the effluent of the isolated heart could be measured with the assurance that CO_2 was neither gained nor lost. The solutions were drawn into the glass electrode by a slight negative pressure from a suction apparatus.

Standardization of the pH meter was done before each experiment, midway during the experiment, and at the end of the experiment, using Beckman 3501 Buffer Solution (Beckman Instruments, Inc., Fullerton, California). Since buffer pH values were given for 10 C increments of tempera-

ture change, a calibration curve was drawn to quickly give the buffer values in pH units at the temperatures most likely to be used in the experiments (Figure 9). At the usual experimental temperature of 32 C, the pH of this buffer was 6.984.

Effluent collection. Effluent collection for the determination of sodium, potassium, and bicarbonate concentrations was accomplished by placing Polyethylene Chemical Containers (Bel-Art, Pequannock, N.J.) under the three-way stopcock attached to the bottom of the heart chamber, and collecting the required amount of effluent. The containers used had a capacity of 15 ml and had leakproof closures of polyethylene. The samples collected were placed in the refrigerator immediately upon collection. When it was not being collected, the effluent drained into a waste container.

The physiological recorders. Two recorders were used during the complete series of experiments. The Physiograph "Four" Recorder (E & M Instrument Company, Inc., Houston, Texas) was used for experiments one to twenty-six. For experiments twenty-seven to fifty-seven, the Grass Model 7 Polygraph (Grass Instrument Company, Quincy, Mass.) was used. The use of these two different machines and sets of transducers provided a check against the possibility

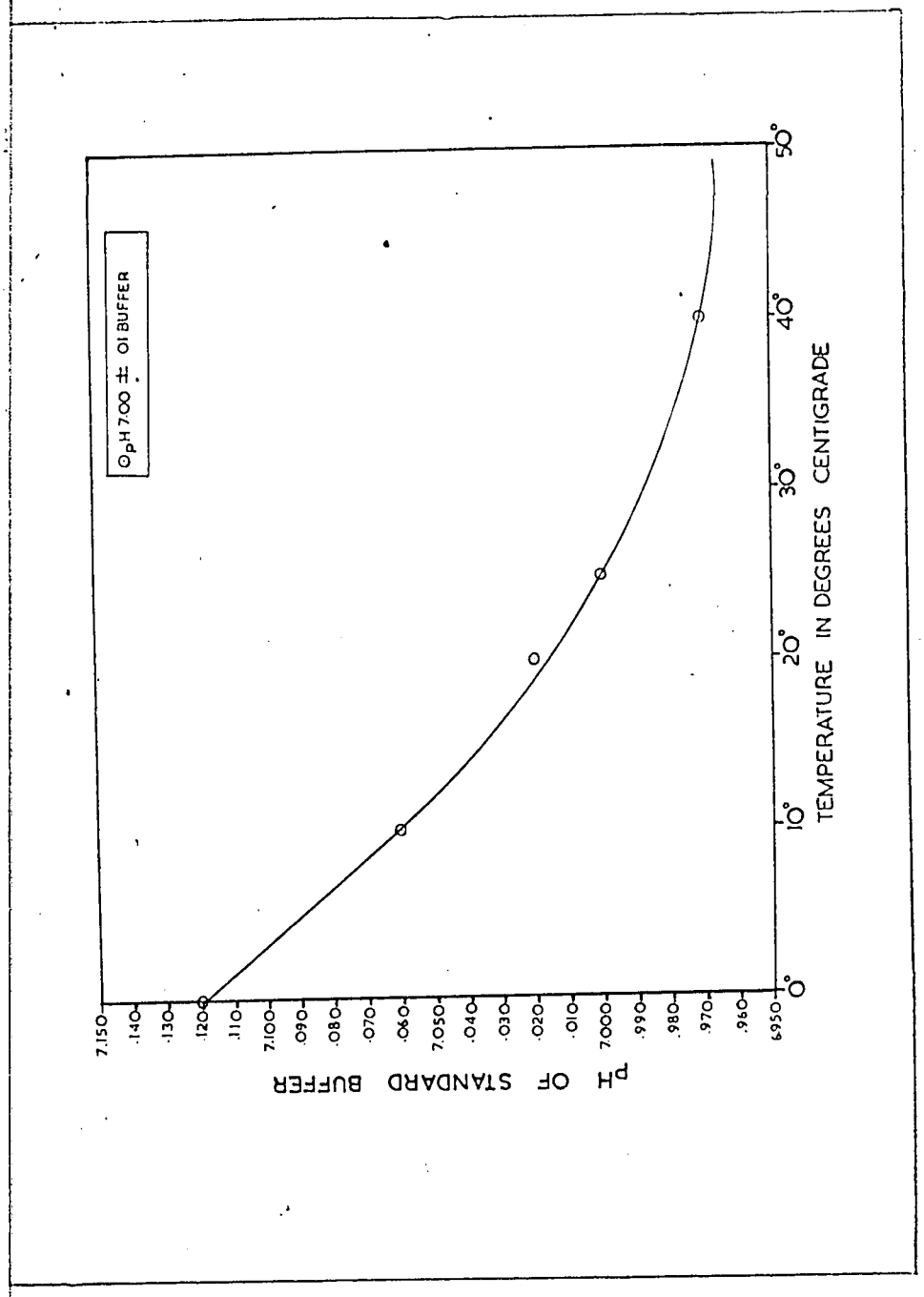

FIGURE 9. Buffer Temperature Calibration Curve

that a modification of the results observed might have been due to the characteristics of the recorder and associated transducers.

Three channels were available in the Physiograph and each consisted of a D.C. Amplifier, with channel number three having an added Hi-Gain Preamplifier for recording the electrical activity of the heart. Channel one was used to record tension, channel two to record perfusion pressure, channel three to record the heart's electrical activity, and a fourth channel provided time and event markings.

The paper control in the Physiograph was a continuously variable type. The paper control unit in the Grass Recorder was the Instant Shift Chart Drive System, Type 925 (Standard), with speeds of 100, 50, 25, 10, 5, 2.5, 1, 0.25 mm per second. The speeds chosen were those adequate to separate the events which were of interest.

Tension recording. The tension produced by the heart was recorded by the E & M Myograph, Type B, when using the Physiograph. This transducer is of the photoelectric type, and has a maximum range of 0 to 30 grams. The transducer and amplifier were calibrated before each experiment by hanging 5, 10, 15, 20 and 25 gram weights on the transducer's hook, and adjusting the amplifier's sensitivity control to produce the desired pen deflection.

When recording tension with the Grass Polygraph, the Grass Force-Displacement Transducer, Model FT10 was used. This transducer used four bonded strain gauges to form a bridge connection to measure the strain produced on a cantilever beam when a force was applied to it by the preparation. The maximum working range of this instrument was 0 to 50 grams when used without a spring. This range was adequate for these experiments, hence this transducer was always used without springs. Calibration was accomplished by hanging weights on the lug for preparation attachment. The weights used were as described above for the other transducer. The balance voltages on the preamplifier, required for making any one of several 10 gram steps of tension increase the new baseline zero value, were checked and recorded before each experiment. Using any one of these values during the experiment permitted the expansion of the tension recording in a given range with a resultant measurement of greater accuracy.

Perfusion pressure recording. Perfusion pressure was measured by an E & M Pressure Transducer, Bourdon Type, when recording with the Physiograph. This is a photoelectric transducer with a range of 0 to 300 mm Hg pressure. The volume displacement of this transducer is approximately 15 cubic mm per 100 mm of mercury pressure. This transducer

and the amplifier were calibrated in 10 mm Hg pressure steps from 0 to 200 mm Hg pressure at the beginning of each experiment.

When using the Grass recorder, a Statham Pressure Transducer, Model P23Ac (Statham Transducers, Inc., Auto Key, Puerto Rico) was used. The volume displacement of this transducer was 0.8 cubic mm per 100 mm Hg pressure. The transducer and amplifier were calibrated before each experiment as described above for the Physiograph recorder and its transducer. The balance voltages on the preamplifier were checked and recorded before each experiment so that any one of the 10 mm Hg pressure steps could be made the zero base line. This permitted expansion of the pressure scale within the range at which each heart was functioning, and provided more exact measurement of pressure changes.

Recording myocardial electrical activity. The third channel of both recorders was used to record the electrical activity of the heart. True electrocardiograms were recorded while the rats were under anesthesia by inserting pin electrodes in the four extremities. This permitted monitoring heart rate variations during the operation, during heparin injection and during saline infusion. After the heart was slowed by the cold saline infusion, the pin electrodes were removed and the ECG preamplifier was coupled

to the electrode system for recording the potential changes from the surface of the heart. The term electrogram will be used to refer more properly to the records of the heart's electrical activity as recorded by electrodes in contact with the surface of the heart (BELL, DAVIDSON and SCARBOROUGH, 1961; GOLDMAN, 1956).

The electrograms from the isolated heart were taken using two different types of electrodes. In the early experiments (1-26), the electrodes consisted of two 20 gauge silver wires passed through the heart chamber's Plexiglass cover with one touching an atrium and the other touching a ventricle. To make placement of the heart in the chamber as simple as possible, the electrodes were changed in Experiments 27 to 57. In these experiments the saline column perfusing the heart served as one electrode, and the silver wire through the apex of the heart served as the other electrode. Attachment to these electrodes could be made quickly, without disturbing the heart, by connecting the leads from the preamplifier to the surface of B-D Luer-Lok connection in the perfusate line and to the tension transducer. The electrograms and the electrocardiograms were used to analyze heart rate only, and no attempt was made to correlate ionic changes with changes in cardiac electrical activity.

Stimulator. When the experimental procedure called for driving the heart at a constant rate, the stimulator used was a Grass Stimulator S4G. The electrodes used for stimulation were those used to record the electrogram. The voltage used in all cases was that voltage which was just greater than threshold.

Integrated use of the equipment. After the heart had been isolated from the body of the rat, as described in a previous section, the heart was brought to the perfusion apparatus. The final section of the perfusion tubing, which was already attached to the heart, could be quickly attached to the main section of the perfusion apparatus by Luer-Lok fittings. To insure that no bubbles were present and that saline at the proper temperature and pH was presented immediately to the heart, the perfusate was allowed to flow through at a normal rate for a minimum of five minutes prior to attachment of the heart to the perfusion system. The perfusate in the tanks was bubbled and thermoregulated for a minimum of an hour before each experiment. The control perfusion used to begin all experiments, was with the perfusate saturated with 95% O_2 and 5% CO_2. Upon attachment of the cannulated heart to the perfusion apparatus, clamps on the tubing were released so that the pressure transducer line was next opened. This permitted drainage of any bubbles

present. This line was then attached to the pressure transducer while saline flowed in order to prevent the accumulation of bubbles. The clamp on the heart side of the T-piece (see Figure 2) was then released so that a bubble-free stream of perfusate now passed through the heart's coronary system. If the heart did not start beating spontaneously at this time, the preparation was discarded.

The recorder had been allowed to operate since the rat was placed on the operating board, in order to keep track of elapsed time as well as to record heart rate changes. After attachment of the heart to the perfusion apparatus the recorder amplifier for pressure was turned on. Perfusion flow rate was adjusted to the desired level, and the base line of the pressure amplifier was set to a range calculated to best show the anticipated pressure changes.

When the attachment of the pressure recording apparatus was complete, the silver wire attached to the apex of the heart was threaded through the passageway in the heart chamber (see Figure 4) and attached to the tension transducer. The heart chamber then was slipped in place over the heart and the cover, with its Vaseline seal, was applied over the open end of the chamber. The tension wire opening also was sealed with Vaseline. The tension transducer was adjusted to apply the approximate tension required on the heart. Tension adjustment was by means of a microscope mechanical stage modified to be firmly attached in position by its base

and to rigidly hold the transducer. After the approximate tension had been applied, the tension channel amplifier was switched on and the tension adjusted to the desired value.

With the silver wire in place and the perfusate column complete to the heart, the electrodes could be attached to record the electrogram of the isolated heart. One electrode was attached to the Luer-Lok connector in the perfusion pressure line and the other on the tension transducer. With the circuit thus completed, the ECG amplifier was turned on to produce a recording of the electrogram used for rate analysis.

Initially the heart was perfused for a minimum of fifteen minutes with the control perfusate to allow equilibrium to be achieved. Usually at the end of the fifteen minute period, no further changes were evident in perfusion pressure, tension, or rate, but if changes were occurring, additional time was allowed for equilibrium to be reached. Flow rate values, in relative units, were read during the equilibrium period and recorded on the polygraph record paper above an event mark made at the exact time of the determination.

The change to an experimental perfusate was preceded, in each case, by a flushing of the perfusate line lasting at least one minute in order to present fresh solution to the heart at the instant of change. Also, immediately before each change, the level of the saline in the perfusion tank

was adjusted to insure that the same hydrostatic pressure was presented to the heart as that under the control perfusion.

Switching from the control perfusate to the experimental perfusate, and the subsequent determinations and recordings, required the participation of two people. The division of duties was made so that one operator watched the recorder, switched to new baselines or amplifier sensitivities, read and recorded flow rates, recorded effluent pH values, recorded the samples taken, and noted and recorded the elapsed time on a particular experimental mixture. The other operator, who directed the overall management of the experiments, assured that the desired experimental conditions were maintained, switched to the various columns of perfusate, took effluent samples, sampled for effluent pH, read out the effluent pH values, and flushed through the perfusates to adjust tank level or to remove fluid in the dead space.

The two variables which were recorded manually during the experiment, were flow rate and perfusate pH. By using two remote event markers, the point at which these two variables were read, and the occurrence of other events could be indicated exactly on the record. For example, when the four-way stopcock was turned to switch from one column of perfusate to another, an event mark was made and labelled. The records from the experiments, completely

labelled in this manner, minimized the possibility of error due to misplaced experimental values.

At the end of each experiment, the perfusate pH values were read again for comparison with the perfusate pH values from the beginning of the experiment. The heart was removed from the cannula and all tissue was dissected away leaving only the two ventricles. The ventricles are more definitively outlined, hence it was felt that the water content of myocardial tissue could be more exactly sampled by using only the ventricles. The ventricles were cut open and blotted dry on filter paper and then weighed on a Mettler Gram-atic Balance (E. Mettler; Zurich, Switzerland). This balance weighs accurately to 0.1 milligram. The ventricles were then placed in an oven at 120 C for a minimum of twenty-four hours. At the end of this time, the ventricles were weighed again and their water content was calculated.

After completion of the experiments, the polygraph record was completely analyzed and the results tabulated. The values extracted from the record and tabulated included: Time from the beginning of perfusion, elapsed time from the initiation of a particular experimental perfusion, elapsed time from the beginning of recovery on the control perfusate, tension at diastole, tension at systole, relative flow rate values, absolute flow rate values, perfusion pressure at diastole, perfusion pressure at systole, heart rate, effluent pH, and remarks about any changes in experimental or control

conditions.

Heart rate was calculated from the record by counting the number of beats in fifteen seconds, as recorded by the electrogram, and converting to the number of beats per minute. Flow rate readings were made at approximately ten second intervals during periods of change, and at approximately thirty second intervals during periods of equilibrium. The relative flow rate readings were converted to absolute values, in ml/min, by reference to the calibration curves previously described. The other variables were measured from the continuous record at the same intervals as the flow rate measurements.

The resistance of the coronary circulation at systole and at diastole was calculated immediately from the experimental values for flow rate and pressure. The resistance to flow was calculated using the relationship $R = \frac{\Delta P}{F}$ (BURTON, 1965). When ΔP, the pressure on the arterial side, minus the pressure on the venous side, is expressed in dynes/cm^2 (mm Hg pressure x 1333 dynes/cm^2) and F, the flow rate, is expressed in cm^3/sec, R is the resistance in dynes-sec/cm^5. The flow in cm^3/sec is closely equivalent to ml/min, the expression of flow rate used in the experiments, divided by 60. The conversion factor, 1333 dynes/cm^2 is calculated as follows (FUCH and FULTON, 1960):

$$1 \text{ mm Hg pressure} = \frac{13.6 \text{ grams}}{\text{cm}^3} \times \frac{980 \text{ dynes}}{\text{gram}} \times 0.1 \text{ cm}$$

$$= \frac{13.6}{\text{cm}^2} \times 980 \text{ dynes} \times 0.1$$

$$= 1332 \text{ dynes/cm}^2.$$

IV. SODIUM, POTASSIUM AND BICARBONATE DETERMINATIONS

Sodium and potassium. The determination of sodium and potassium concentrations in the perfusate and in the effluent was done by flame photometry. A P.H. Coleman Flame Photometer, Model 21, coupled to a P.H. Coleman Galvo-Meter, Model 22 (Coleman Instruments, Inc., Maywood, Ill.), was used for all analyses. This is a filter instrument and the appropriate filters were used for sodium and potassium.

All volumetric glassware used to measure or dilute the solutions for analysis was Kimax, Class A, Glassware (Kimble Glass Co., Toledo, Ohio). This glassware is of borosilicate glass which has a low alkali content and which has no calcium present. All volumetric glassware used was of precision grade meeting the accuracy requirements of the United States Bureau of Standards.

Before each determination, calibration curves for sodium and for potassium were drawn from the appropriate standards. Sodium chloride, of the grade previously described, provided the varying sodium concentrations for the sodium standards. To cancel out the effect of the interaction of cations, it seemed analytically sound to provide a background in all the standards equivalent to that which would be present in the experimental samples. Therefore, preparation of the standards was equivalent to a preparation

of the perfusate saline, but with sodium, or potassium, concentrations limited to the values required by the standards.

Each cation has an optimal concentration for its determination by flame photometry. The optimum concentration range for sodium, specified by the manufacturer, is 0.05-5.0 mEq/liter and the optimum concentration range specified for potassium, is 0.02-18.0 mEq/liter. The total sodium present in the perfusate was 139.7 mEq/liter, and the total potassium present was 5.63 mEq/liter. The effluent samples were diluted 1:50 to bring the concentration of the cations down to the level for optimal flame photometric function. This was based on the assumption that the heart would alter the composition of the perfusate only minutely. Based on this assumption, the effluent concentrations still would be of the same order of magnitude as the perfusate concentrations. The estimated concentration of sodium after dilution would be approximately 2.8 mEq/liter and that of potassium would be approximately 0.11 mEq/liter.

The standards were made to locate these approximate final concentrations in the middle range of the emission scale. With the sodium standards, 2.0 mEq/liter was made equal to 0% emission and 3.0 mEq/liter equal to 100% emission. Standards were also prepared for each 0.2 mEq/liter step between 2.0 and 3.0 mEq/liter.

The standards for potassium determinations were made so that 0.06 mEq/liter gave 0% emission and 0.14 mEq/liter gave 100% emission. Intermediate standards were made up at 0.02 mEq/liter steps between the 0% and 100% values. Two typical standard curves are shown in Figure 10.

In all standard solutions and in all dilutions of effluent samples, Acationox (Scientific Products; Evanston, Illinois) was added to give a final concentration of 0.02 g/ 100 ml. The inclusion of Acationox served to prevent fouling of the aspirator capillary. Demineralized water, from the source described previously, was used to dilute standards and samples in all cases.

The samples of perfusate and effluent were stored in the refrigerator immediately upon collection. The storage temperature was approximately 5 C. The solutions were warmed to room temperature immediately before pipeting. Two 2.0 ml samples were pipeted, using 2.0 ml pipets (calibrated to deliver), into two 100 ml volumetric flasks. Acationox was added as previously described. Demineralized water was added to volume. These dilutions provided samples for the sodium and potassium determinations. Refrigerated storage of all solutions was carried out whenever analysis was interrupted for extended periods of time.

For the determination of the concentrations in the standards and in the experimental solutions, certain precautions were routinely used to insure the optimum performance

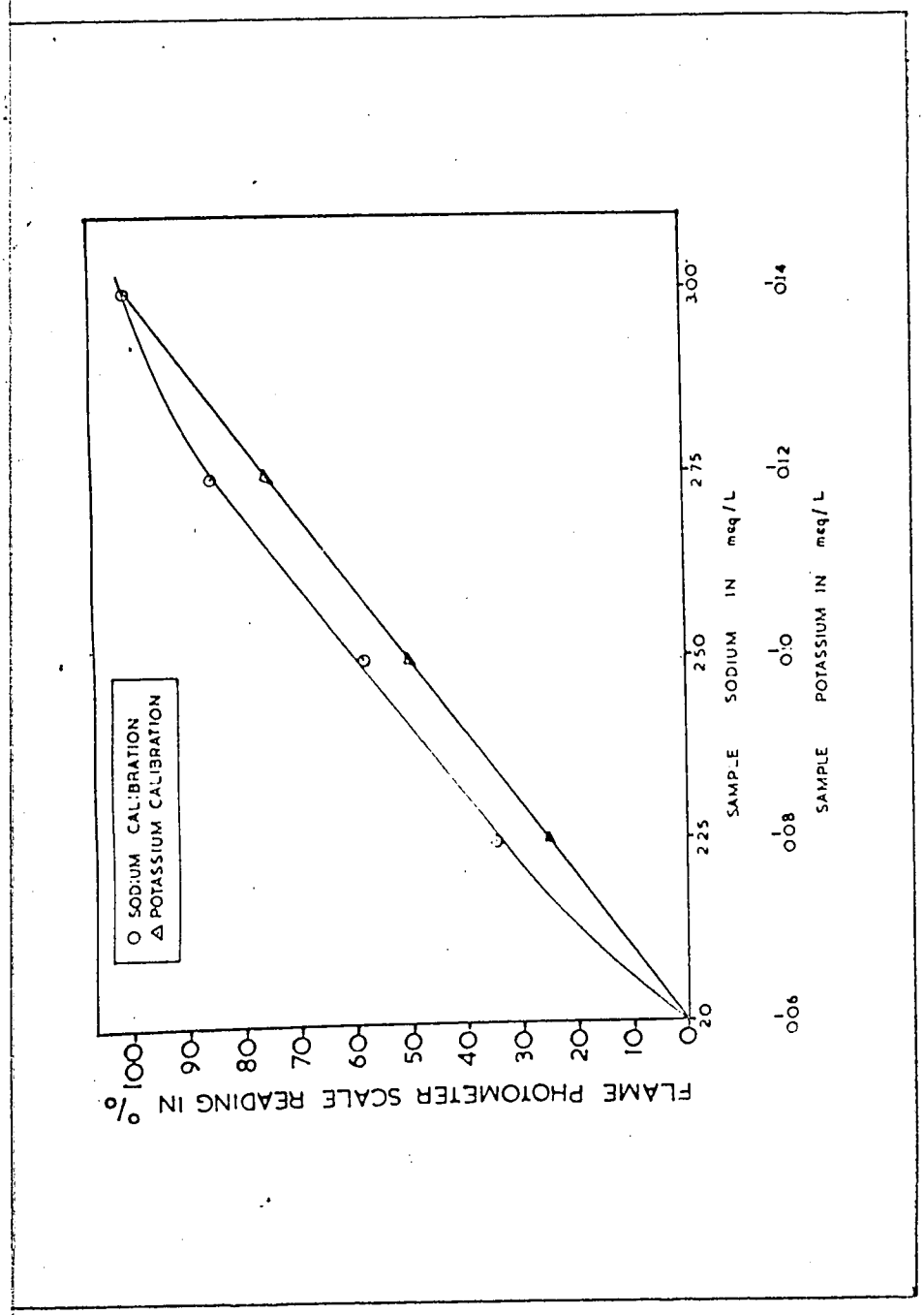

FIGURE 10. Sodium and Potassium Calibration Curves for Flame Photometry

of the instrument. Fresh silica gel was placed in the electronics compartment of the flame photometer, and the electronic circuitry was allowed to remain "on" overnight prior to the determinations. These precautions displaced most of the moisture from the electronic components and enhanced the stability of the instrument.

The aspirator assembly was stored in demineralized water between determinations. Prior to each experiment, the capillary was blown dry with compressed air. The screen surrounding the flame was stored in demineralized water also and cleaned before use. These measures ensured that no accumulation of particles of residue from previous experiments remained to contaminate current determinations.

The 10 ml glass beakers, used to present the samples to the aspirator assembly, were made of borosilicate glass in order to prevent alkali contamination. These beakers had been washed in a non-ionic washing solution, had been rinsed five times with demineralized water, and had been oven dried before use. The solution to be analyzed was always used to give a final rinse before analytical use of the beakers.

To minimize the effect of decreased flow rate of the solutions through the flame as the level in the beakers dropped, the level in the beakers was kept within the same limits for all determinations. A minimum of four determinations was made for each of the two dilutions of the

effluent samples.

Bicarbonate. The sample bicarbonate concentrations were determined volumetrically. The scheme of the determinations was to add a known excess of HCl, allow the reaction with HCO_3^- to occur while assisting the evolution of CO_2, and titrate the excess HCl with NaOH. The reaction, which is fundamental to this scheme of determination, is:

$$H^+ + HCO_3^- \longrightarrow H_2CO_3 \longrightarrow CO_2\uparrow + H_2O.$$

The quality of volumetric glassware used in all of these determinations was as described in the previous section for sodium and potassium.

Sodium carbonate was used as the primary standard for the HCl and the NaOH. Sodium carbonate (Fisher Certified Reagent) was heated to 270 C to remove water completely and to convert bicarbonate to carbonate (FISHER and HAENISCH, 1948). This sodium carbonate was weighed on a Mettler Gram-atic Balance which could be read accurately to 0.1 mg. The standard sodium carbonate was titrated to pH 6.0 by the HCl using Gramercy Universal Indicator (Fisher Scientific Co.; Fairlawn, N.J.). The titration of this salt of a weak acid, Na_2CO_3, by the strong acid, HCl, forms a weak acid at the stoichiometric point, hence an indicator with the end point on the acid side of neutrality was most satisfactory (FISHER and HAENISCH, 1948). The standardized

HCl was then used to standardize the NaOH by titration to pH 7.0 using the same indicator.

The apparatus used to react several samples concurrently consisted of a rack of twenty 10 mm diameter Micro-Filter Tubes (C.H. Johns Glass Co., Ltd.; Toronto, Canada). These tubes will contain slightly more than 10 ml; a convenient volume for these titrations. At the base of these tubes is a sintered glass disk which serves to distribute a gas as a mass of fine bubbles. These fine bubbles served to mix the solution thoroughly and to remove the CO_2 from the site of the reaction, thereby shifting the equilibrium point of the reaction far to the right. The gas used for this purpose was CO_2-free N_2. The gas line was attached to the tapered gas connection below the sintered glass filter.

Titration of the excess HCl by NaOH was done by means of a Piston Motor Buret, Model E298 (Metrohm AG Herisau, Switzerland). Using the 5 ml barrel, each division on the scale represented a 0.005 ml volume change. Within each of these divisions, it was possible to read to the nearest 0.001 ml of volume change. The tubing used to deliver the NaOH to the sample in the Micro Filter tubes, was small bore polyethylene which provided flexibility and small drop delivery. See Figure 11 for a schematic representation of this equipment.

The addition of the Universal Indicator caused undesirable frothing of the solution during bubbling with the

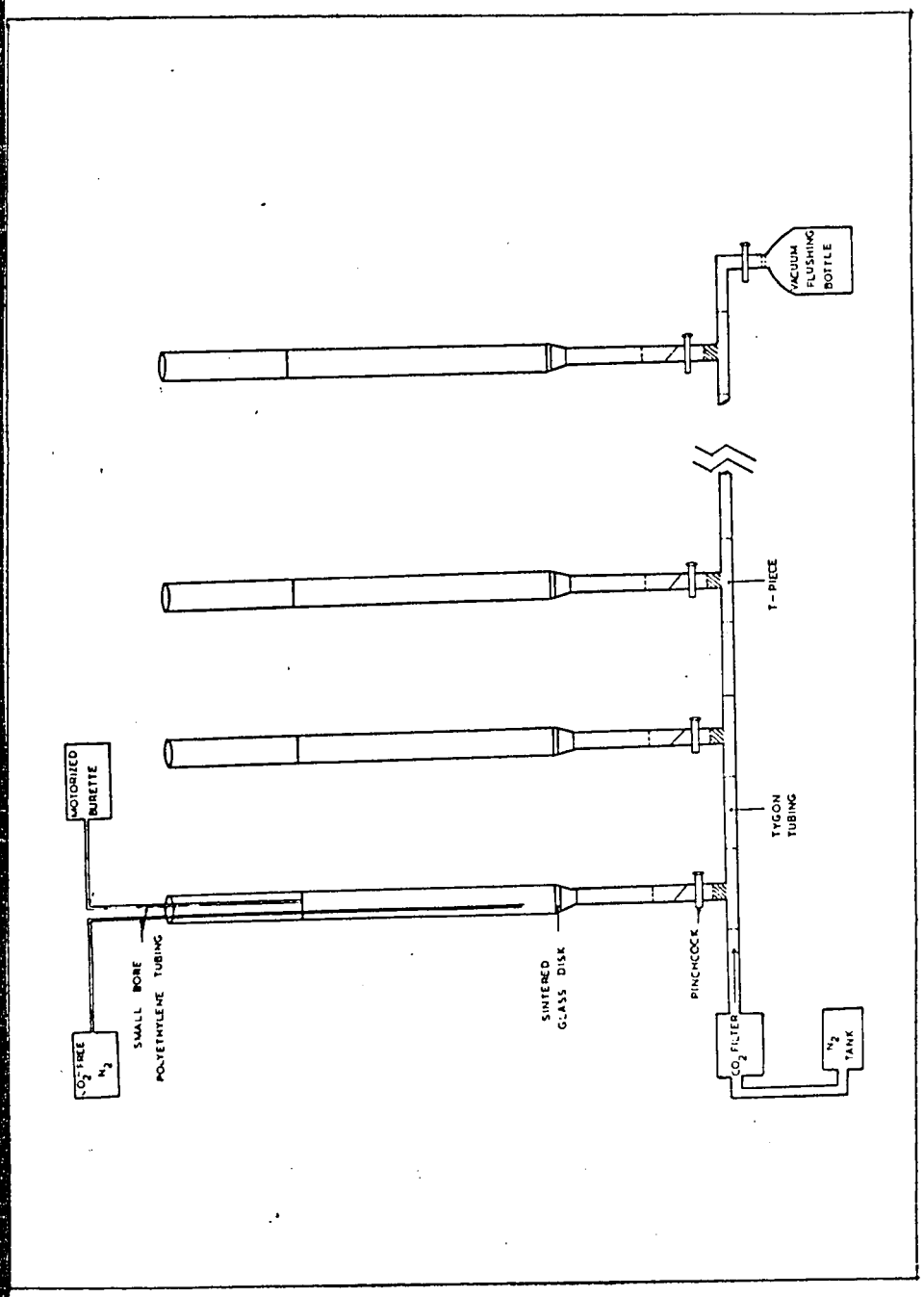

FIGURE 11. The Bicarbonate Determination Apparatus

sintered glass disk. To overcome this difficulty, 3/4 of the NaOH calculated to be required was added to the sample and this was bubbled vigorously for two minutes without the indicator. The approach to the end point was then done with the indicator in the solution, the gas flow through the sintered glass disk closed off, and bubbling with a very small bore polyethylene tube (see Figure 11). This tube provided bubbles adequate to agitate the solution and drive off any CO_2, but large enough so that frothing did not occur.

The time required for the HCl to react with the standard sample of $NaHCO_3$ was determined. It was found that bubbling for five minutes drove off all of the CO_2 and allowed completion of the reaction.

The effluent samples previously described supplied the samples for the bicarbonate determinations also. It should be recalled that these samples were kept refrigerated in airtight containers. Two samples were taken from each of the effluent samples collected and diluted 1:50 with demineralized, CO_2-free, water. Two ml of the diluted samples were pipeted into the bubbling tubes. Into the two ml samples was pipeted 0.5 ml of the previously standardized, approximately 0.005 N HCl. The acidified sample was allowed to bubble by means of the CO_2 free N_2 for at least five minutes. Using the motor buret, approximately 3/4 of the calculated NaOH required was added and bubbled for two minutes. The bubbling was then stopped and the indicator

added (0.5 ml). Bubbling was continued using the polyethylene tubing while titration to pH 6.5 was completed. A minimum of two determinations was done on each of the two dilutions from each effluent sample.

The calculation of the number of mEq/liter of bicarbonate present was done by subtracting the number of mEq of NaOH required to titrate the excess HCl from the total number of mEq of HCl added.

V. STATISTICAL ANALYSIS OF THE RESULTS

To test the changes in the several variables for significance, the means of the changes were analyzed for significance by assuming that these were paired experiments. Two observations on the same individual may always be paired (SNEDECOR, 1962). The first member of each pair of values was the control quantity obtained during perfusion with the saline bubbled with 95% O_2 and 5% CO_2. The second member of the pair was the experimental result obtained during perfusion with a perfusate other than the control. The test for significance on the grouped pairs was done by evaluation according to the "Student" t-Test. If the probability of a larger value was 0.05, or less, the experimental change was considered significant.

Comparison of the experimental results against the control values was done at selected time intervals after the change to the experimental perfusate. From experience with the preparation, it could be seen that analysis of the results at 1/2, 2, and 3 minutes would encompass all changes in the variables. For some variables, it was decided to analyze the changes at a time of 1 minute to test whether this value fell on the curve plotted by the use of the other three time intervals. Since the 1 minute value always fell properly on the curve plotted using the other three values, it was concluded that the original

assumption was valid.

Experimental changes were placed in homogeneous groups determined by the type of experimental perfusate used. For each of the selected times, the mean change, the standard error, the standard deviation, and the t-value, was calculated by machine. The method for machine calculation was as described by SNEDECOR (1962).

The test used for correlation was the Quadrant Sum Test for Association (OLMSTEAD and TUKEY, 1947). To test the possible association of two continuous variables, a scatter diagram of the points, located by the value of the two variables, is drawn. Two lines are drawn to form the x and the y axes representing, independently, the median point of the y and the x values respectively. This divides the scatter diagram into four quadrants. The upper right and the lower left quadrants are labelled as positive, while the other two are labelled as negative. Moving a straight edge successively from each side of the paper toward the origin, those points present in one of a pair of quadrants, before the appearance of a point in the other quadrant, are tabulated with the appropriate sign. The sum of these points is taken algebraically and analyzed for significance according to the values given in Table 1.

TABLE 1. Working Significance Levels for Magnitudes of Quadrant Sums*

Significance level (Conservative)	Magnitude of quadrant sum**
10%	9
5%	11
2%	13
1%	14-15
0.5%	15-17
0.2%	17-19
0.1%	18-21

*Adapted from original table, OLMSTEAD and TUKEY (1947).

**The smaller magnitude applies for large sample size, the larger magnitude for small sample size. Magnitudes equal to or greater than twice the sample size less six should not be used.

CHAPTER III

RESULTS

I. SOME OBSERVATIONS ARISING FROM THE GENERAL OPERATIVE PROCEDURES

Observations such as heart rate change with the cold saline infusion were made at the beginning of experiments, regardless of the protocol followed during the experiments. In this section those results will be presented.

Chest temperature change with cold saline infusion. In four experiments, the changes in chest temperature accompanying the infusion of cold saline were recorded. In all cases, the chest temperature refers to the temperature measured on the exposed chest musculature after removal of the skin. Figure 12 shows the change in chest temperature observed in experimental rat number 35. It can be seen that chest temperature fell gradually with progress of the cold infusion and then reached an equilibrium level. The equilibrium level was limited by the temperature of the infusate.

Experiment 35 was used to show this change in temperature because it provided the most complete series of readings. The other determinations, while less complete,

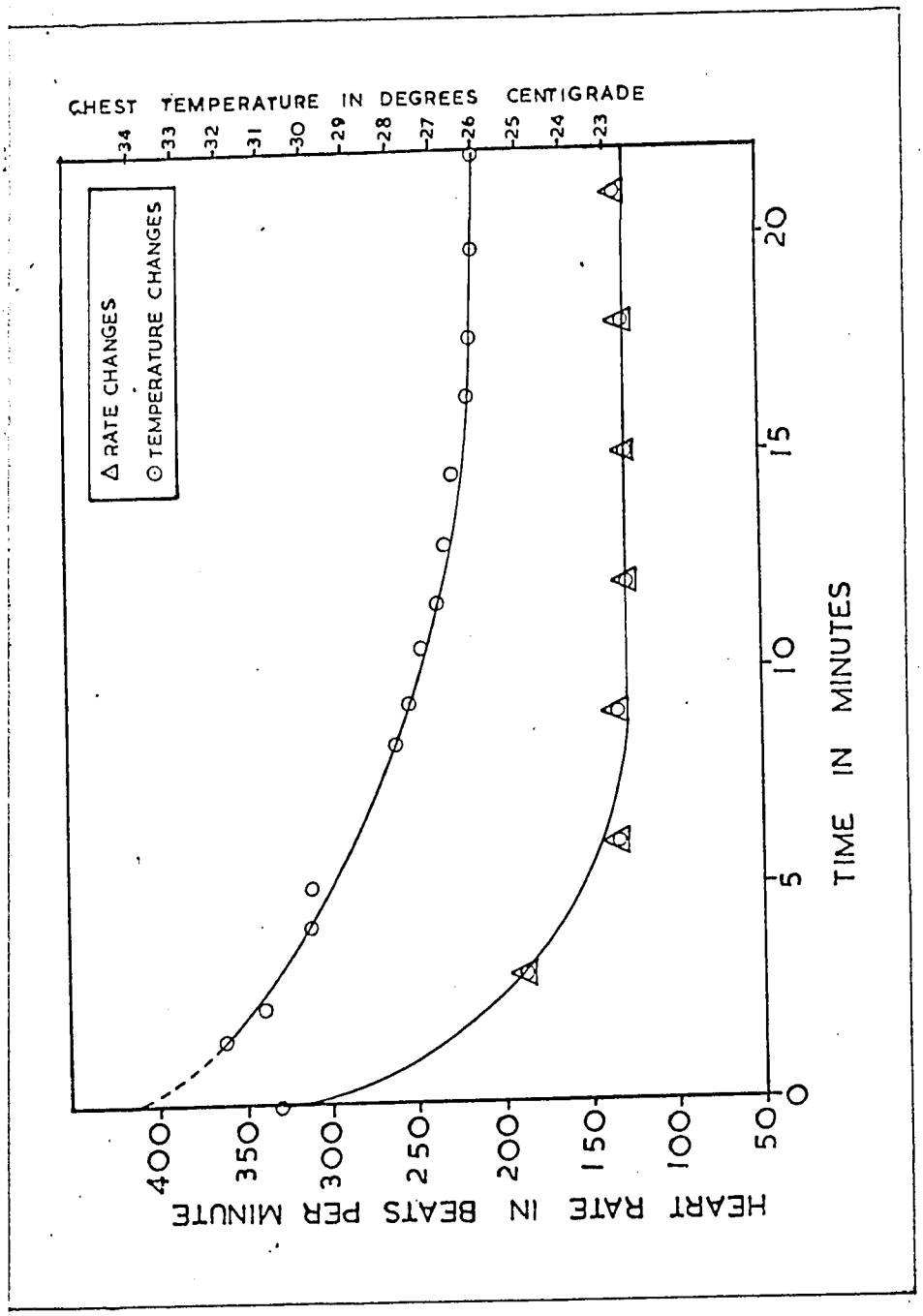

FIGURE 12. Heart Rate and Chest Temperature Changes with Cold Saline Infusion

provide a curve of the same shape as shown in Figure 12. The temperature extrapolation to zero time is an estimate since no readings were taken at zero time, due to the technical demands of initiating infusion of the cold saline.

Change in heart rate with cold saline infusion. Figure 12 also shows the change in heart rate with cold saline infusion as seen in Experiment 35. It can be seen that the rate of contraction falls rapidly with onset of the saline infusion and then plateaus out to an equilibrium rate. The slope of the curves varied from experiment to experiment, but the effectiveness of the cold saline infusion, as manifested by the rapid slowing of heart rate, is evident in each experiment. Fibrillation was not observed during cold infusion in any of the experiments.

The criterion used to end the cold saline infusion was the attainment of a rate equilibrium. When it could be seen by an inspection of the polygraph recording of the ECG that the rate was no longer decreasing, then the cold infusion was terminated.

Water content of the experimental hearts. To check the amount of water gained by the experimental hearts, hence the degree of edema, the percent of the wet heart

weight to dry weight (tissue weight) was calculated and analyzed statistically. A comparison was made between the experimental rats and a group of control rats.[1] The mean percentage of the control rats' (dry heart weight)/(wet heart weight) was 20.40 ± 1.33% (SD). The mean percentage of (dry heart weight)/(wet heart weight) for the experimental rat hearts was 16.51 ± 4.37% (SD). There were thirty-nine hearts in the experimental group and seventeen in the control group. Since the dry tissue weight of the experimental rat hearts was less than that of the control hearts by 3.89%, the experimental rat hearts contained an average of 3.89% more water than the control group. An analysis of significance of the difference of the means of the two groups, according to the method for two groups of different sizes (SNEDECOR, 1962), was carried out. By this test, the significance of the difference of the two means was much less than 0.001, therefore the greater water content of the experimental hearts was significant and likely due to the experimental procedures.

[1]The control data was obtained from Dr. M. Beznak's laboratory. The hearts were from animals within the same weight range as the experimental rats. The ventricles were removed and weighed without undergoing any experimental procedures, except their removal.

Number and duration of experimental changes. Each rat heart successfully used for a series of experimental changes was numbered sequentially. Therefore, experiment numbers refer to the number assigned to the rat heart used. Each experimental change performed on the numbered rat heart was lettered consecutively. Any experimental change which suffered a departure from proper control conditions was eliminated and the results were not used in the statistical analysis of the variables. Such departures from proper control conditions included changes in control tension applied to the apex of the ventricles due to accidental movement of the transducer, movement of the heart chamber during an experimental change, sticking of the flow meter ball following a change in perfusate, entrance of air bubbles into the perfusate stream, or control perfusion periods of inadequate length for proper recovery from the previous experimental perfusion. The number and duration of the successful experiments with proper control conditions is shown in Table 2.

TABLE 2. Number and Duration of Experimental Changes

Nature* of change	Number of changes	Mean duration in minutes	Range in minutes
80% O_2-20% CO_2 pH 6.80	78	6.50	2.00-29.50
95% O_2-5% CO_2 pH 6.80	27	4.10	2.00- 7.00
Methyl sulfate control	6	3.33	3.00- 5.00
90% O_2-10% CO_2 pH 7.10	4	8.33	7.15-10.00
Other changes	75	6.90	0.50-20.25

*All changes were from 95% O_2-5% CO_2, pH 7.40

Within the group, "Other changes", are included those changes to gas mixtures in which O_2 or CO_2 was changed in a stepwise fashion.

The effect of the duration of the experiments on the observed responses. Since the isolated heart will ultimately fail, the possible modification of response, due to reaching a point of failure, must be considered. One heart was allowed to continue under the usual perfusion conditions to check this possibility. Function, as determined by heart rate and perfusion pressure, was almost identical with

the initial level after 12 hours.

Various times have been reported as maximal for isolated heart perfusion without failure. Taking 90 minutes as an average opinion, the responses to experimental perfusion with 80% O_2 - 20% CO_2 bubbled saline were grouped according to whether they occurred between 0 and 90 minutes or whether they occurred after 90 minutes of perfusion. The percentage change in heart rate and the percentage change in contraction tension from control values were then calculated for the two groups. These results are shown in Table 3.

From the results in Table 3, it can be seen that there is no essential difference between the responses in the 0-90 minute time interval and the responses in the 90+ minutes group.

Another control on the effect of perfusion duration was carried out for each heart. An experimental perfusion with 80% O_2 - 20% CO_2 was conducted near the beginning and at the end of each experiment. The responses were always qualitatively equivalent.

TABLE 3. The Effect of Time on Contraction Tension and Rate Responses to Perfusion with 80% O_2-20% CO_2 Bubbled Saline, pH 6.80

Time Range of Experimental Occurrences in minutes	Number of Experiments	Time in minutes following initiation of perfusion 1/2	2	3
		Contraction tension decrease ± S.D.		
0 - 90	14	53.09 ± 21.06%	61.92 ± 23.12%	58.24 ± 21.43%
90+	7	37.60 ± 10.00%	60.51 ± 19.29%	68.59 ± 24.23%
		Rate decrease ± S.D.		
0 - 90	18	9.88 ± 5.97%	22.97 ± 7.90%	19.12 ± 11.39%
90+	7	3.51 ± 3.40%	12.00 ± 3.95%	13.02 ± 4.72%

II. THE EFFECT OF VARIOUS EXPERIMENTAL PERFUSATES ON THE TENSION DEVELOPED BY THE MYOCARDIUM

General. The tension, as measured by a transducer attached to the apex of the heart, is not the same contractile tension as that applied to the mass of blood during the heart's pumping action in situ. However, when the apex of the heart is attached to a transducer, the transducer then measures the resultant of the forces applied by all the myocardial cells regardless of their orientation. Since the cells cannot change their orientation and since the connecting wire does not move during the experiment, the only change in tension measured by the transducer must be the result of changing myocardial cell contractility.

The terms systolic tension and diastolic tension will be used for the sake of brevity to refer to the tension measured at systole and to the tension measured at diastole, respectively, in these isolated heart preparations. It is understood that this usage of systolic and diastolic tension is different from that used as applied to the heart's function as an organ within the circulatory system.

The terms twitch tension or contraction tension will be used in reference to the difference between the tension at systole and the tension at diastole. This then represents the amount of tension generated during contraction over and above the resting tension.

The typical response to a perfusate bubbled with 80% O_2 and 20% CO_2. When changing from a control perfusate bubbled with 95% O_2 and 5% CO_2 to an experimental perfusate of the same ionic composition but which was saturated with 80% O_2 and 20% CO_2, the responses observed were as shown in Figure 13. This represents a typical response to the change in perfusate and was taken from the record of Experiment 37 b. The complete figure is made up of eight second sections of the original record at time intervals which show all phases of the changes. Zero time is the point of change of perfusate. Minus one minute shows the control conditions at one minute prior to the change in perfusates. The one-half, one, two and three minute sections are portions of the record at those respective times after the change of perfusates. Since there was a change in the sensitivity of the pressure amplifier between the one and the two minute time intervals, the two and three minute records are slightly separated from the remainder of the records and a new scale is placed at the right border of the record.

The response of the heart's contractility to the change in perfusate can be seen to be quick and well defined. The systolic tension decreases sharply between the time of change and the one-half minute time interval. Diastolic tension, however, does not change with the change of perfusate. The twitch, or contraction tension, therefore, decreases from the control value. In summary, it can be

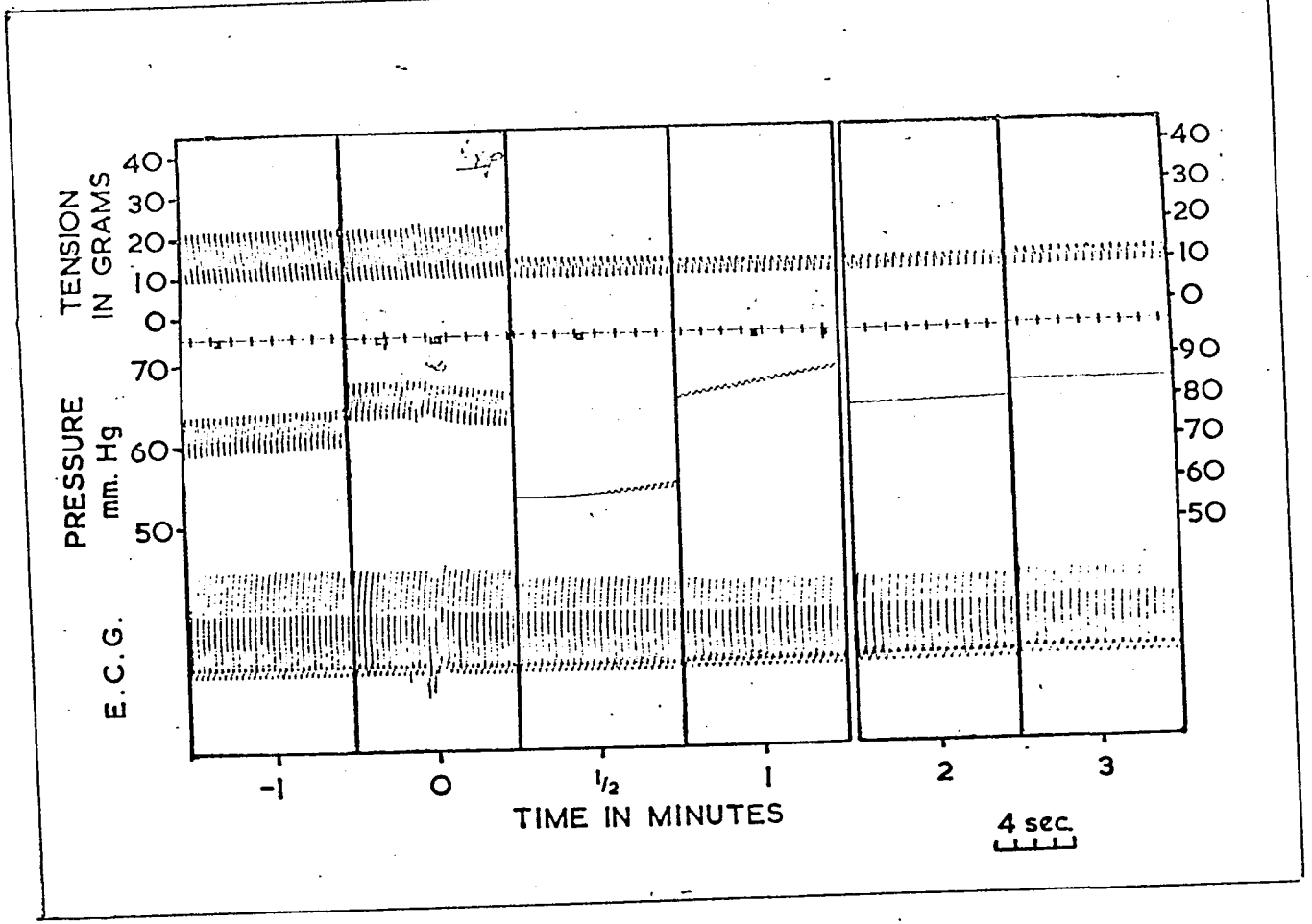

FIGURE 13. The Recorded Effect of 80% O_2-20% CO_2 on Heart Function

These are sections of the recording taken from Experiment 37 b. Time 0 is the point of change from control to experimental perfusate, and the other times are the elapsed times from 0 time. The right scale applies to the two right sections of record.

said that the perfusate bubbled with 80% O_2 and 20% CO_2 had a negative inotropic effect on the isolated rat myocardium, at the times studied.

Since it will be necessary to consider the effect of the change of perfusates on tension as possibly resulting from changes which the experimental perfusate caused in other variables, it is desirable, at this time, to define the effect of the perfusate change on coronary resistance and on rate of myocardial contraction.

The heart rate changes can be seen, in a qualitative fashion, by inspection of the electrogram which is here labelled ECG for the sake of brevity. By examination of the interval between the peaks of electrical activity, it can be seen that the frequency of depolarization decreases. That this is coupled with a decreased frequency of contraction also can be seen by noting the coupled decrease in rate of the tension twitches as displayed by the tension transducer. The change in frequency will be shown in a quantitative fashion in a subsequent figure.

The change in coronary resistance is indicated in this record as raw and qualitative data by the change in the perfusion pressure. As described previously, the coronary resistance may be expressed by a relationship analogous to Ohm's Law for electrical circuits, wherein resistance varies directly with the pressure difference between the arterial and the venous side of a circulatory circuit and, inversely,

with the volume flow of fluid through that circuit. Since the effluent runs off freely on the venous side so that the venous pressure is effectively zero, then the change in pressure on the arterial side varies directly with a change in coronary resistance. This is true if flow rate varies in an inverse fashion as compared to the pressure variations; and from experience, it was seen that flow rate did vary inversely with pressure. Therefore, it can be seen, in an approximate fashion by examining the perfusion pressure recording, that with a change in perfusates, the coronary resistance first decreases and then increases above the level observed under control conditions.

The portion of the record labelled as minus one minute is included to show the stability of the variables during control perfusion. It can be seen that for tension the control values were very stable. This sort of stability also applies to heart rate and will be shown in a quantitative fashion in subsequent figures. The pressure recording does not seem to have this stability; however, the control recording represented a recovery period from a previous experimental perfusion and the perfusion pressure was still recovering. At the time of the experiment, it was ascertained that the perfusion pressure had returned to the control level before beginning the experimental change shown in the record.

Figure 14 shows a graph of the complete results from Experiment 37 b for the changes in systolic and diastolic tension occurring with the change to the perfusate bubbled with 80% O_2 and 20% CO_2. Zero time again represents the change from control to experimental perfusate. This graph shows the stability of the baselines by the values plotted at -1 and -1/2 minutes. The period of no change after initiation of the experimental perfusion lasts for approximately ten seconds. This agrees well with the 8 seconds calculated in Chapter II to overcome the physical dead space. Ten seconds will be used to approximate the dead space with the assumption that this most probably includes a physiological dead space, or latent period.

As predicted from the selected samples from the polygraph record (Figure 13), the tension at diastole does not change with the change to the experimental perfusate. The systolic tension change is quick and marked, however, and it has a 1/2-time of approximately 12 seconds. Since these variables reach equilibrium assymtotically, it is useful to calculate the 1/2-time, i.e., the time required to reach a value equal to 1/2 the equilibrium value. Subtracting the dead space time from the gross 1/2-time gives an approximate net 1/2-time of 2 seconds. The 1/2-times from the variables studied will be used in later discussions as an estimate of their possible inter-relationship.

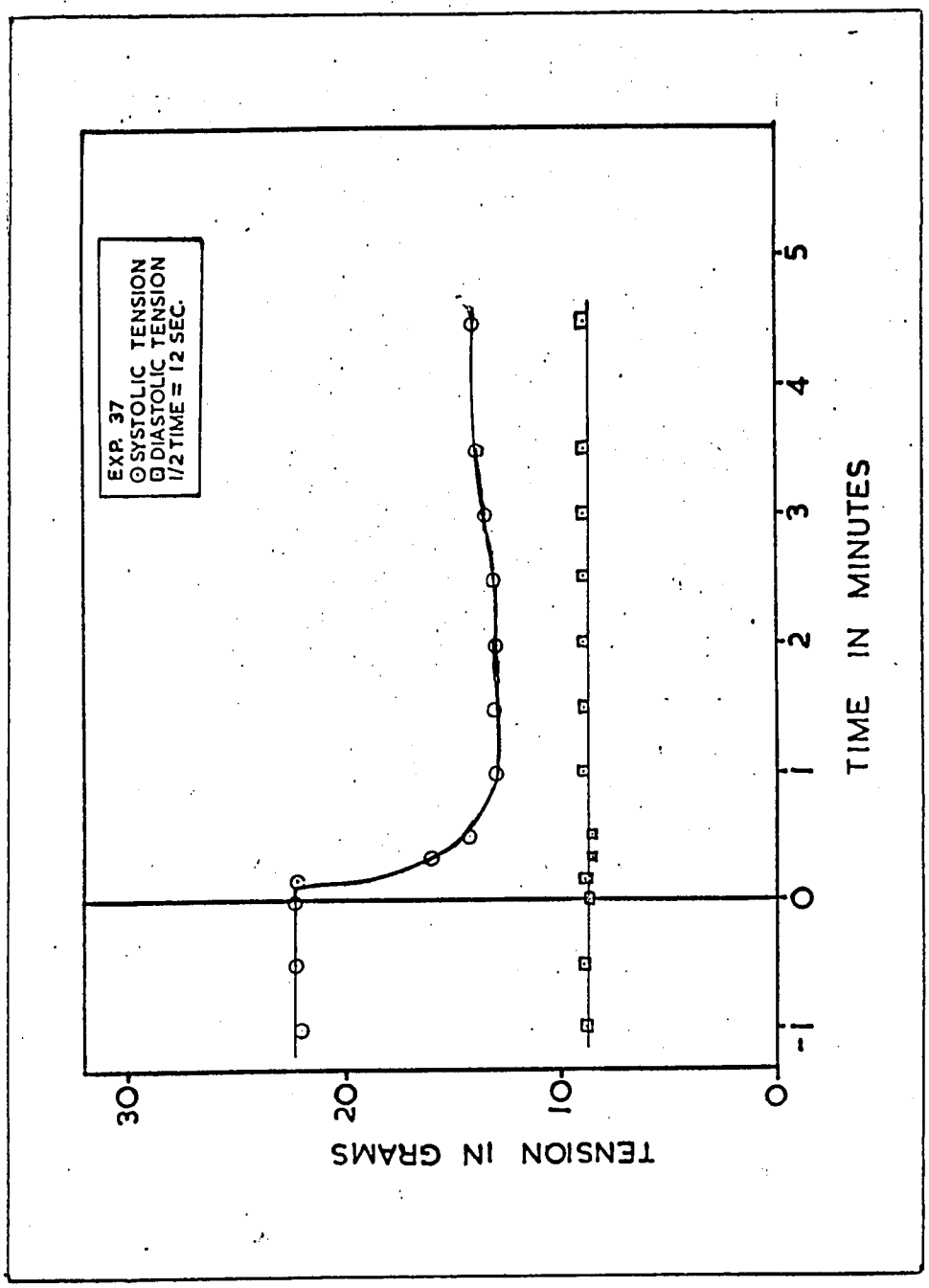

FIGURE 14. A Typical Tension Response to 80% O_2-20% CO_2

The systolic tension appears to recover in the last two minutes of perfusion. Since the fractional portions of grams of tension were estimated from the space between two lines on the polygraph record, these changes of less than a gram in magnitude must be interpreted with caution.

Calculation of the percentage of the control twitch tension reached by the twitch tension at equilibrium during the experimental perfusion, shows that twitch tension decreases to 33% of the control value.

Figure 15 graphs the complete time course of coronary resistance change in Experiment 37 b. These results will be discussed in more detail in the section on coronary resistance changes. However, it should be noted, at this time, that the gross 1/2-time of the apparent vasodilation--decreased coronary resistance--is 12 seconds. The net 1/2-time obtained by subtracting the dead space time would be 8 seconds. The gross 1/2-time for the vasoconstriction--increase in coronary resistance--is 75 seconds, and the net 1/2-time would be 65 seconds.

Figure 15 also shows that the postulated method of quick estimation of coronary resistance from examination of the perfusion pressure recording, as discussed previously, is valid. It should be noted again that the coronary system was still recovering from the previous experimental change, as was indicated by inspection of the pressure changes at -1 minute in Figure 13.

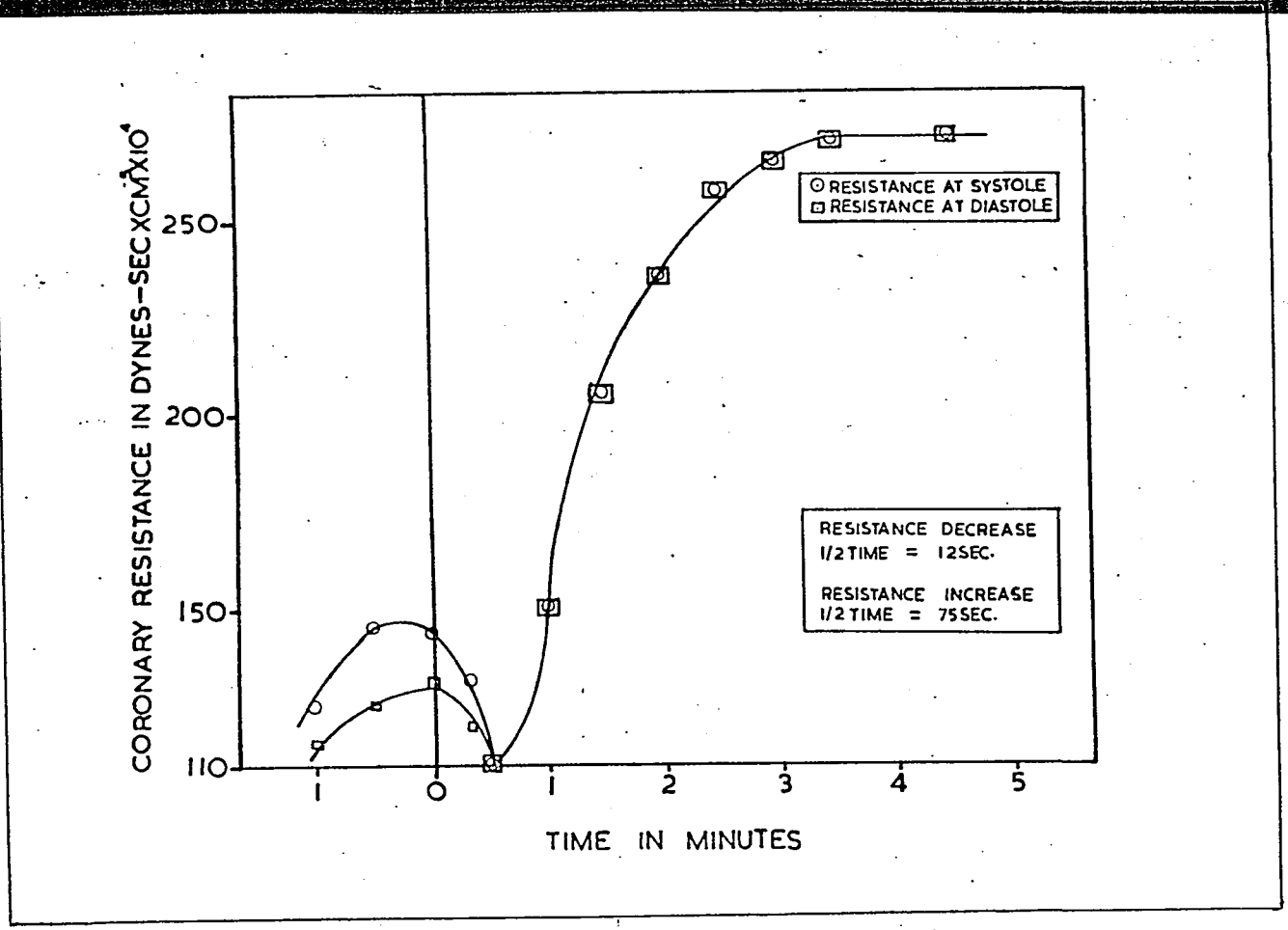

FIGURE 15. A Typical Coronary Resistance Response to 80% O_2-20% CO_2

This shows the response as observed in Experiment 37 b.

Figure 16 shows the complete data from Experiment 37 b on the effect of the experimental perfusate on contraction frequency. Contraction frequency changes will also be discussed fully in a later section. It is to be noted, at this time, that the dead space time is again indicated by a 10-second period of no change following the initiation of experimental perfusion. The gross 1/2-time of the rate change is approximately 50 seconds, and the net 1/2-time is 40 seconds.

By examining Figures 14, 15 and 16, it can be seen that if the changes occurring at 1/2, 2 and 3 minutes were analyzed, then a fairly complete picture of the effects of an experimental perfusate would result. This was the basis for selecting the above time intervals as the points of statistical analysis of the changes. The validity of this selection of times was further emphasized by the finding that the values later analyzed for the one minute time fell on the curve previously plotted through the 1/2, 2, and 3 minute times.

In Figure 17 are graphed the mean changes in systolic and diastolic tensions, $\pm$ the standard error, as derived from all of the experiments with this experimental perfusate. Zero time again represents the point of change from the control perfusates bubbled with 95% O_2 and 5% CO_2 to the experimental perfusates bubbled with 80% O_2 and 20% CO_2. The amount of change from the control tension was calculated

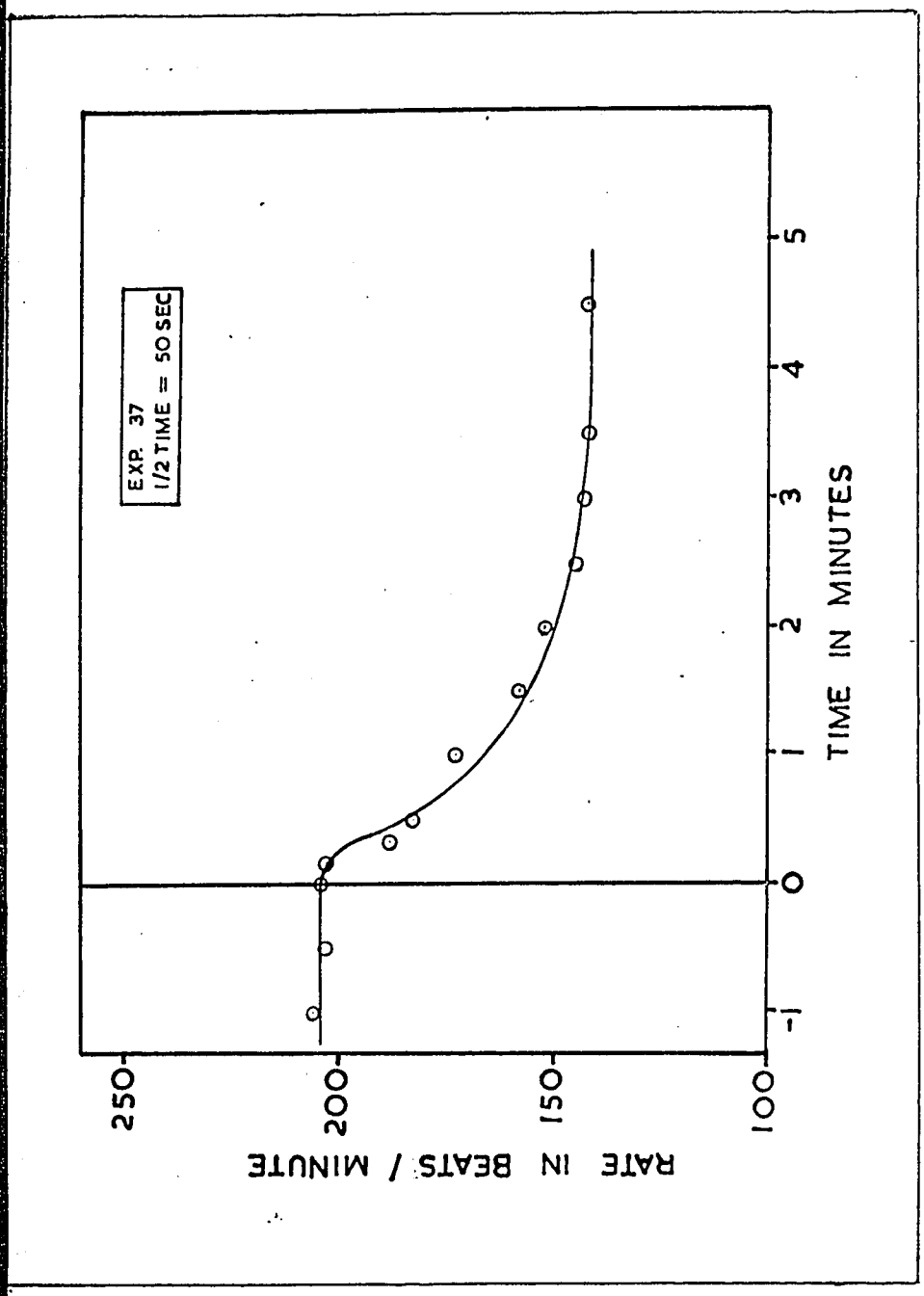

FIGURE 16. A Typical Heart Rate Response to 80% O_2-20% CO_2. This is the response as observed in Experiment 37 b.

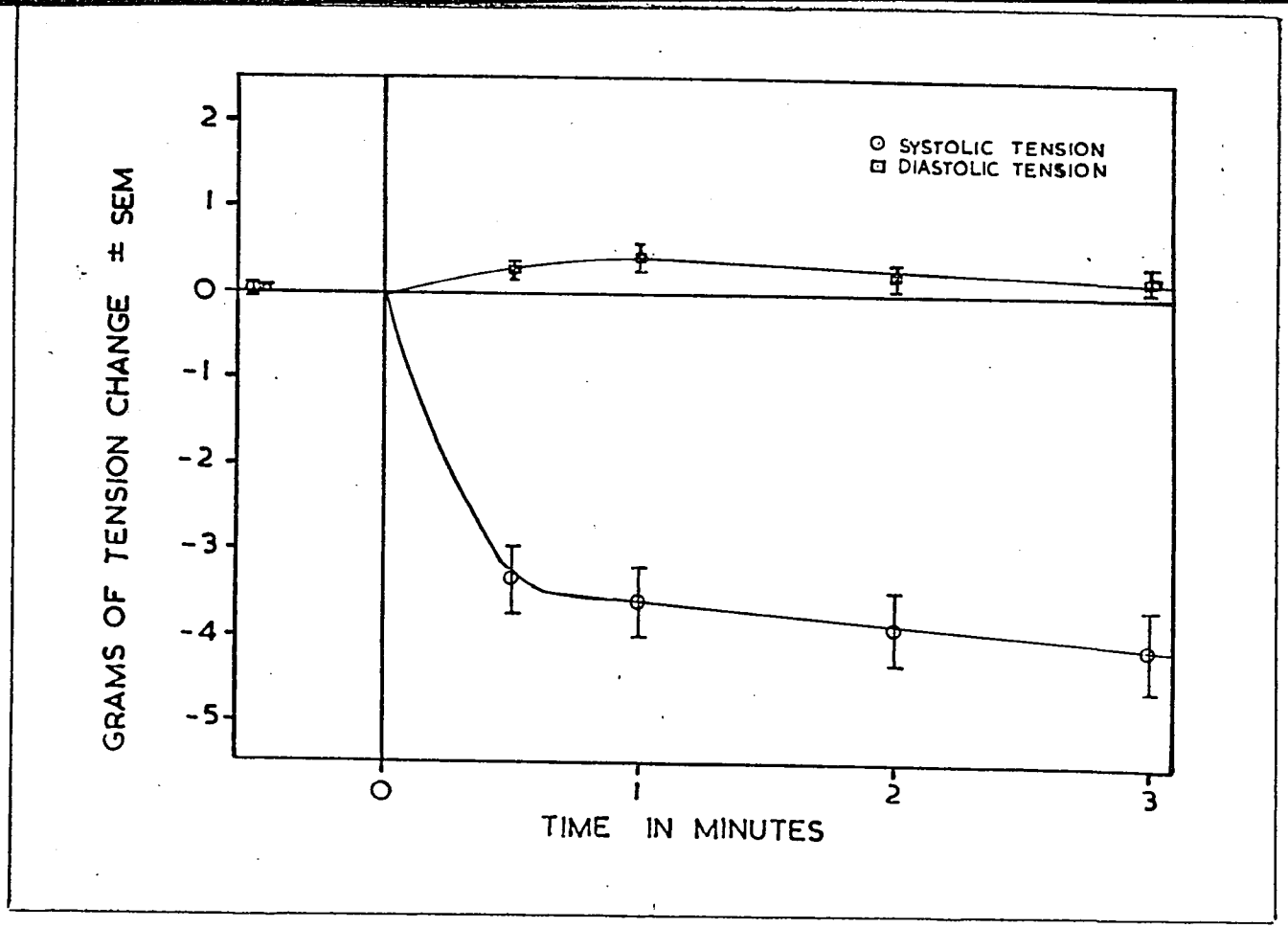

FIGURE 17. A Statistical Analysis of the Effect of 80% O_2-20% CO_2 on Tension

The control diastolic tension is 8.86 grams (mean) and the control systolic tension is 18.02 grams (mean). The diastolic tension change at 1 minute is significant. All systolic experimental changes are significant.

for each of the times in every experiment, and the mean, standard deviation, and the standard error calculated for this grouped data. The control tension used in each case was the value for systolic and diastolic tension immediately prior to the change of perfusates.

To test the control values for stability, changes in tension from the zero time control value were calculated at 1/2 minute before zero time. The means of these variations, and their standard error, are plotted at time equal to -1/2 minute on the graph of Figure 17.

Table 4 summarizes the above data for the diastolic and systolic tension changes; and adds other information of value such as mean control tension, standard deviation, percentage change in tension from control values, and p, the probality of such deviations from the means resulting from chance occurrences.

From Figure 17 and Table 4, it can be seen that systolic tension decreases quickly and significantly with the onset of perfusion with 80% O_2 and 20% CO_2 saline. These results agree well with the results from Experiment 37 b, the typical experiment previously used to illustrate this effect. The behavior of diastolic tension from the population of hearts is slightly different, however, from that of diastolic tension in Experiment 37 b. Whereas diastolic tension did not appear to change in Experiment 37 b, diastolic tension appears to increase significantly

TABLE 4. Summary of Diastolic and Systolic Tension Changes Upon Perfusion with 80% O_2 - 20% CO_2 Bubbled Saline

Number of Hearts	Time of Determination	Mean Change in Grams	± Standard Deviation	± Standard Error	p	Percent Change
			DIASTOLIC TENSION			
56	-1/2	+ 0.02	0.25	0.04	0.50	+ 0.23
56	0	8.86			----	Control
56	1/2	+ 0.26	0.87	0.12	<0.05	+ 2.94
56	1	+ 0.44	1.17	0.16	<0.01	+ 4.94
56	2	+ 0.22	1.16	0.15	>0.10	+ 2.48
52	3	+ 0.20	1.19	0.17	>0.20	+ 2.26
			SYSTOLIC TENSION			
56	-1/2	-0.03	0.44	0.06	> 0.50	-0.1
56	0	18.02			----	Control
56	1/2	-3.33	2.52	0.38	< 0.001	-18.50
56	1	-3.61	3.03	0.40	<0.001	-20.01
56	2	-3.90	3.26	0.44	<0.001	-21.64
52	3	-4.11	3.44	0.48	<0.001	-22.82

at the 1/2 and 1 minute times when the whole group of hearts is considered. However, at the 2 and 3 minute times, no significant change in diastolic tension is also indicated in the grouped hearts.

Since both systolic tension and diastolic tension vary, albeit little in the case of diastolic tension, a more compact determination of the effect of the experimental perfusate should result if twitch, or contraction, tension is analyzed. Contraction tension would then combine the changes of two variables into changes in a single variable. Figure 18 shows a graph of the change in twitch tension in Experiment 37 b, and the same change analyzed statistically for the whole experimental rat heart population. It can be seen that the curves both show the quick drop in twitch tension, although they are of different magnitudes. The curve from the population of hearts shows no indication of recovery at 3 minutes, whereas the heart in Experiment 37 b indicates slight recovery in twitch tension beginning at 3 minutes. Again this must be interpreted with caution due to the nature of the recording.

According to the "law of the heart" (STARLING, 1915), the energy of contraction is proportional to the amount of stretch put on the muscle fibers. When dealing with isolated hearts, this mechanism has been found to operate as predicted by Starling and by many other investigators. The possible adherence of these isolated heart preparations

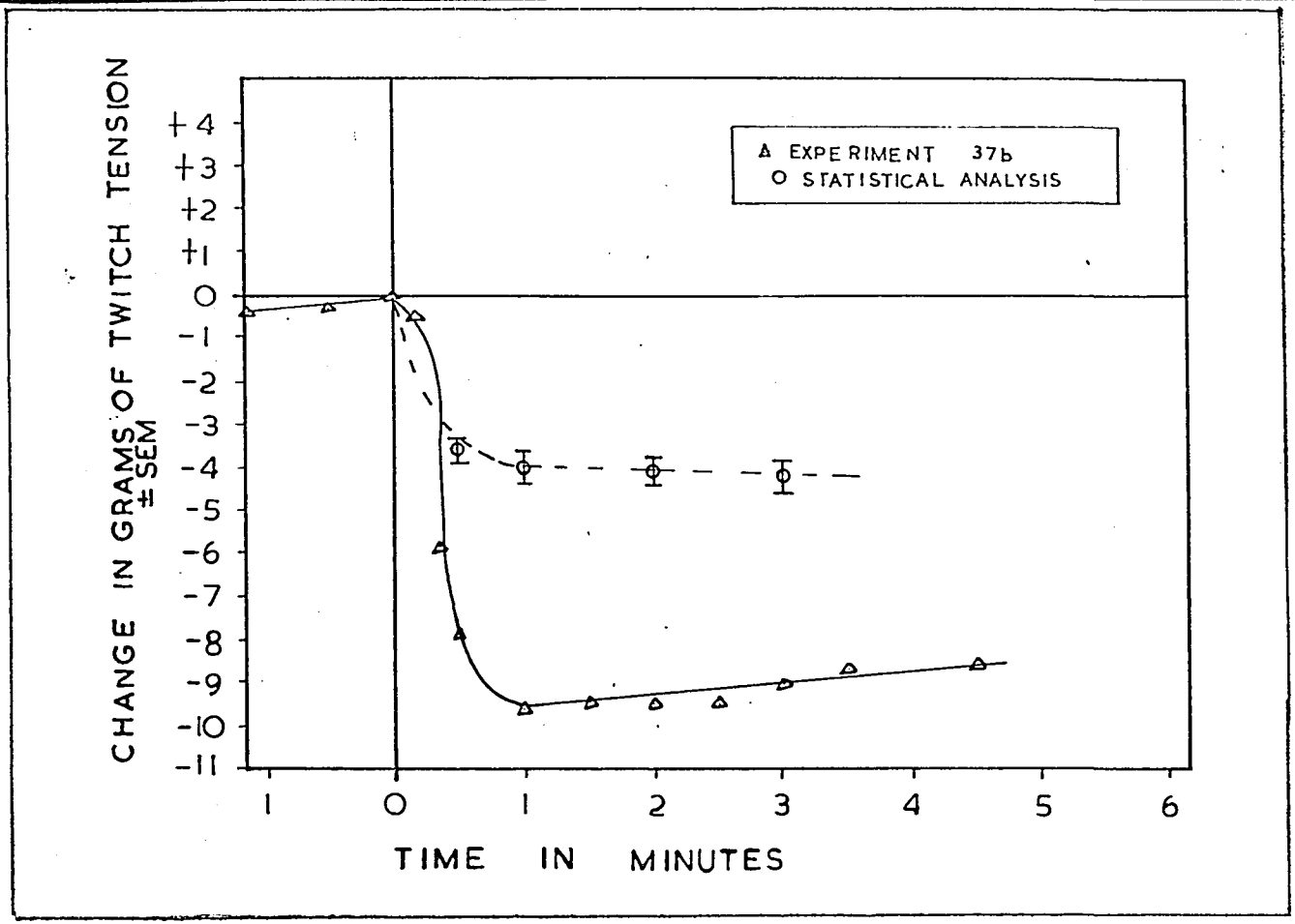

FIGURE 18. A Comparison of the Statistical and Typical Twitch Tension Responses to 80% O_2-20% CO_2

The control twitch tension for Experiment 37 b is 13.5 grams and the mean control twitch tension from the statistical data is 9.16 grams. All of the responses statistically analyzed were significant.

to Starling's "law of the heart" was checked by sixty stepwise changes in stretch applied to various hearts.

Figure 19 shows the quadrant sum test for correlation between the grams of stretch and the contraction, or twitch, tension. Considering all magnitudes of stretch applied, from 0 to 107 grams, the quadrant sum is +11. This gives a probability value of 0.05, according to Table 1. There is a significant correlation then between the amount of stretch and the resulting contraction tension. Starling observed, however, that this law of the heart was valid up to a maximum amount of stretch. The maximum stretch for this group of isolated heart preparations can be readily determined by visual inspection of Figure 19. At 80 grams of stretch, the positive correlation very abruptly changes to a negative correlation. If the quadrant sum is determined for the range of stretch from 0 to 80 grams (coordinates for this determination are the broken lines), then the value of this quadrant sum is +30, or a probability of less than 0.001. If the ten remaining points in the range of 80 to 107 grams of stretch are analyzed by the quadrant sum test, then, even with such a small number of points, the quadrant sum is -8. This indicates a trend towards a negative correlation which is very close to significance (-11) even with only ten points.

Following the demonstration of conformity of these isolated hearts to the principle that the amount of con-

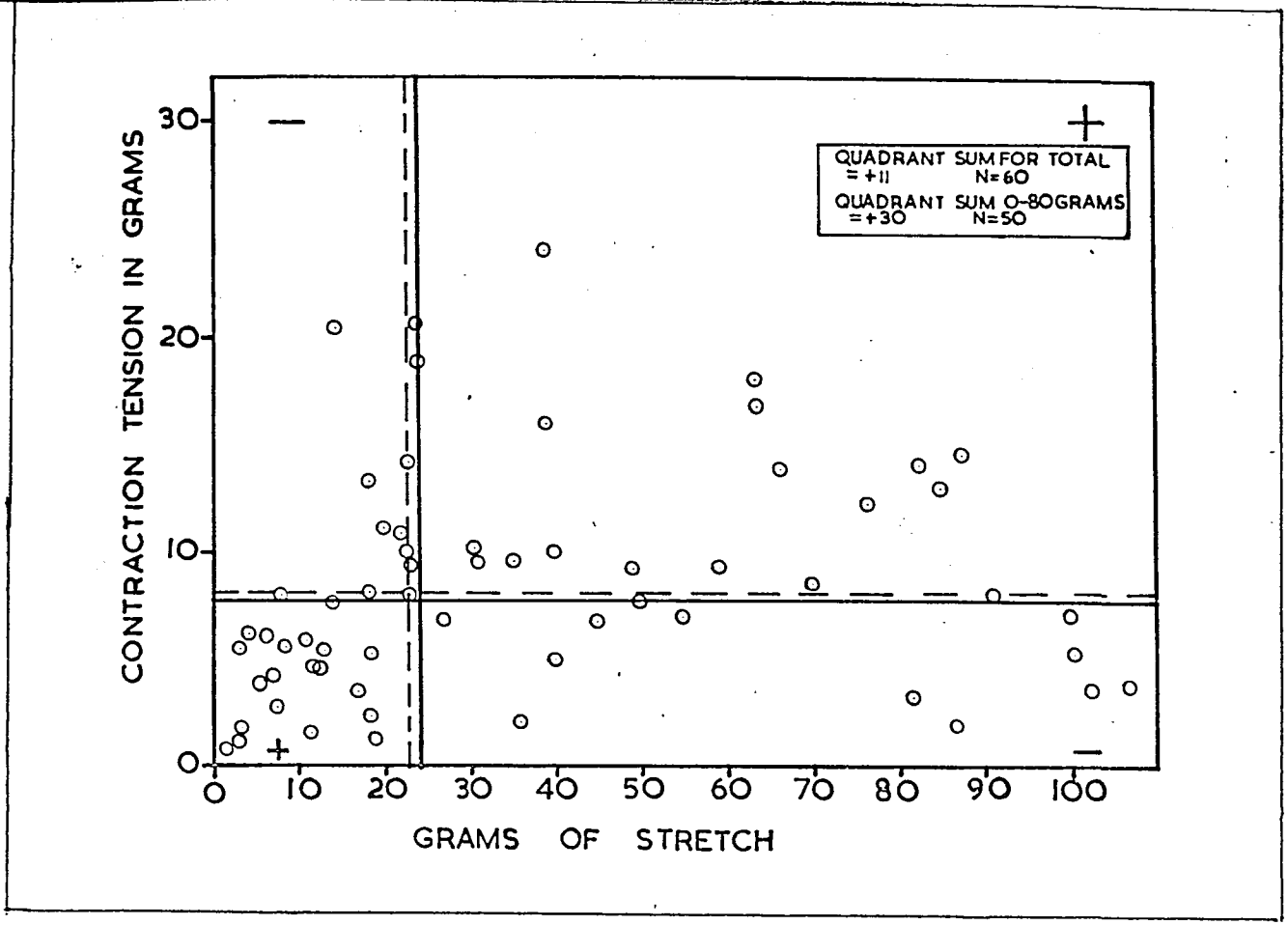

FIGURE 19. Quadrant Sum Test for Correlation Between Contraction Tension and Initial Stretch

The solid lines are the coordinates for the analysis of the total group. The broken lines are the coordinates for the analysis of the 0-80 grams of stretch group.

traction tension developed was dependent on the amount of initial stretch applied, it was reasoned that perhaps the amount of contraction tension decrease, resulting from perfusion with 80% O_2 and 20% CO_2 bubbled saline, was also proportional to the amount of stretch applied. Since it had already been demonstrated that the amount of contraction tension depended on the amount of initial stretch applied under control perfusion, it seemed logical to bring the correlation check one step further and to look for correlation between the decrease in grams of contraction tension during experimental perfusion and the grams of contraction tension during control perfusion. The tension values used were those prepared for the statistical analysis at times of 1/2 and 2 minutes following initiation of the experimental perfusion. The 1/2 minute value was chosen to see if the response correlated immediately, and the 2 minute value was used to check correlation of the tension upon achievement of equilibrium following the change.

Figure 20 shows the quadrant sum test for correlation at 1/2 minute and Figure 21 shows the correlation at 2 minutes. Both figures show a very significant positive correlation between the control contraction tension and the grams of decrease in contraction during experimental perfusion. Therefore, the variable, decrease in grams of contraction, is dependent on the value of the other variable, grams of contraction tension during control perfusion.

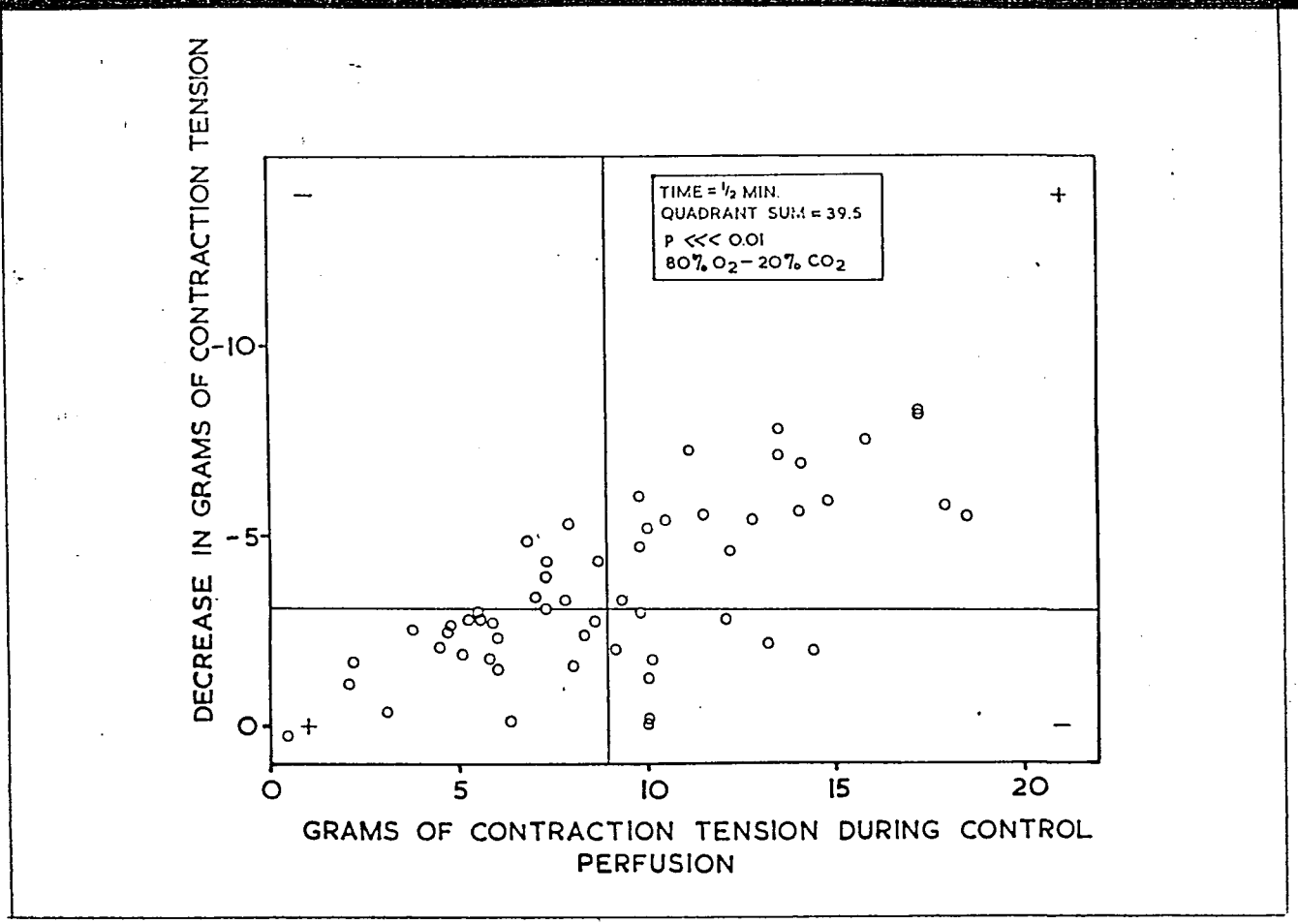

FIGURE 20. Quadrant Sum Test for Correlation Between Contraction Tension Decrease and Control Contraction Tension

This is an analysis of the data at 1/2 minute following initiation of experimental perfusion with 80% O_2-20% CO_2.

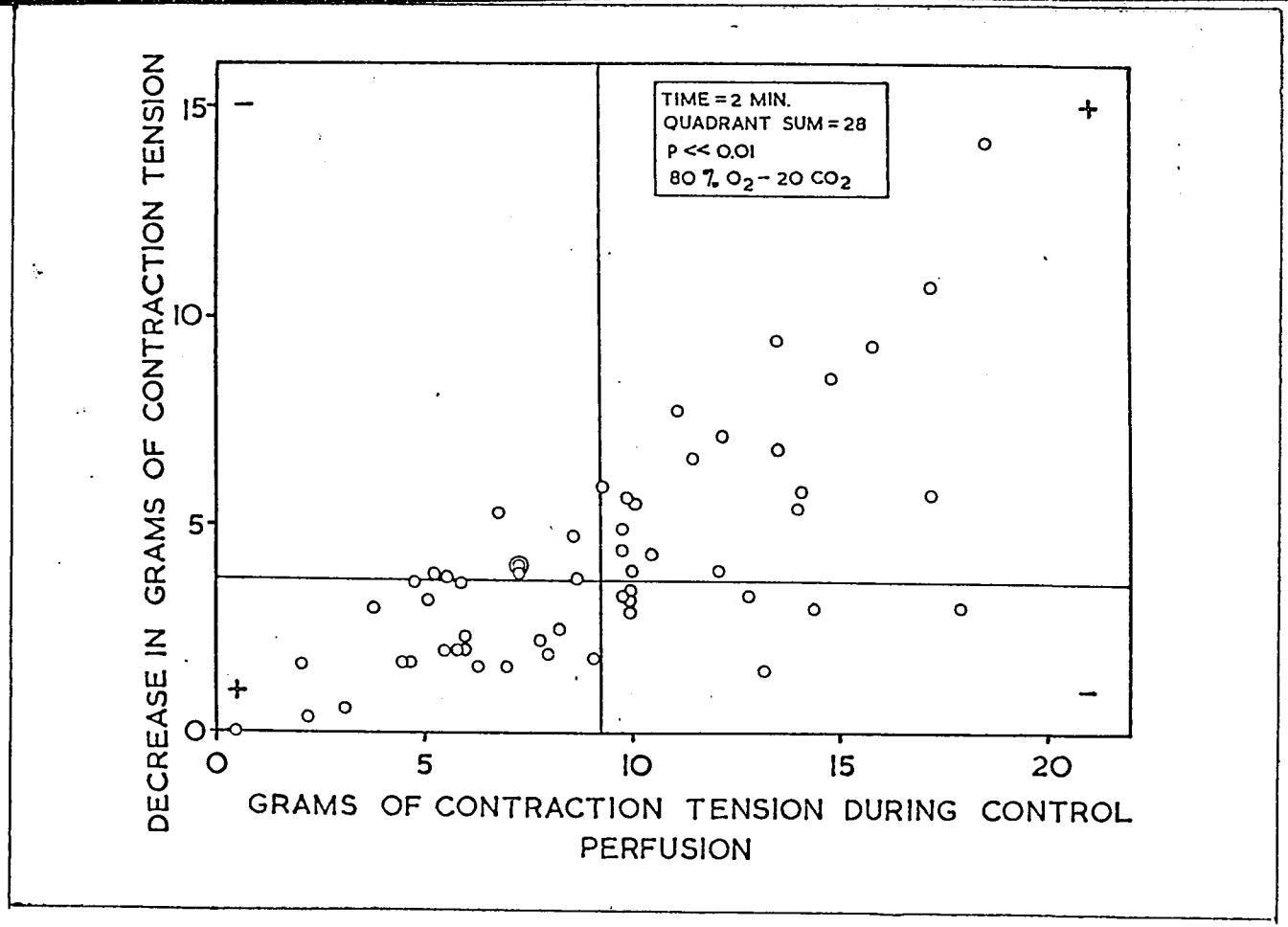

FIGURE 21. Quadrant Sum Test for Correlation Between Contraction Tension Decrease and Control Contraction Tension

This is an analysis of the data at 2 minutes following initiation of experimental perfusion with 80% O_2-20% CO_2.

Incorporating these two variables into one new variable, the percentage decrease of the experimental contraction tension from the control contraction tension, should give a more appropriate definition of tension change for purposes of comparison. This can be stated as the following mathematical relation:

$$\frac{\% \text{ decrease in tension}}{100} = \frac{\text{grams of contraction tension decrease.}}{\text{grams of control contraction tension}}$$

Figure 22 shows the graph of the percentage decrease in contraction tension, and its standard error, for the various times after onset of experimental perfusion. Also plotted are the results from the typical Experiment, 37 b. Although this protrayal of the data presents a more efficient picture of what happens to tension upon initiation of experimental perfusion, neither the significance of the data nor the display of the pattern of change is altered by this additional calculation. This can be verified by comparing Figures 16 and 22.

However, it is pertinent to wonder if Experiment 37 b is atypical since the mean values for contraction tension decrease of the population are much smaller than those for Experiment 37 b. The standard deviations for the population of twitch tension changes, at 2 and 3 minutes following initiation of perfusions, are ±2.70 and ±2.86 grams respectively. Thus, two standard deviations, 95% of the population, do include Experiment 37 b. That it shows

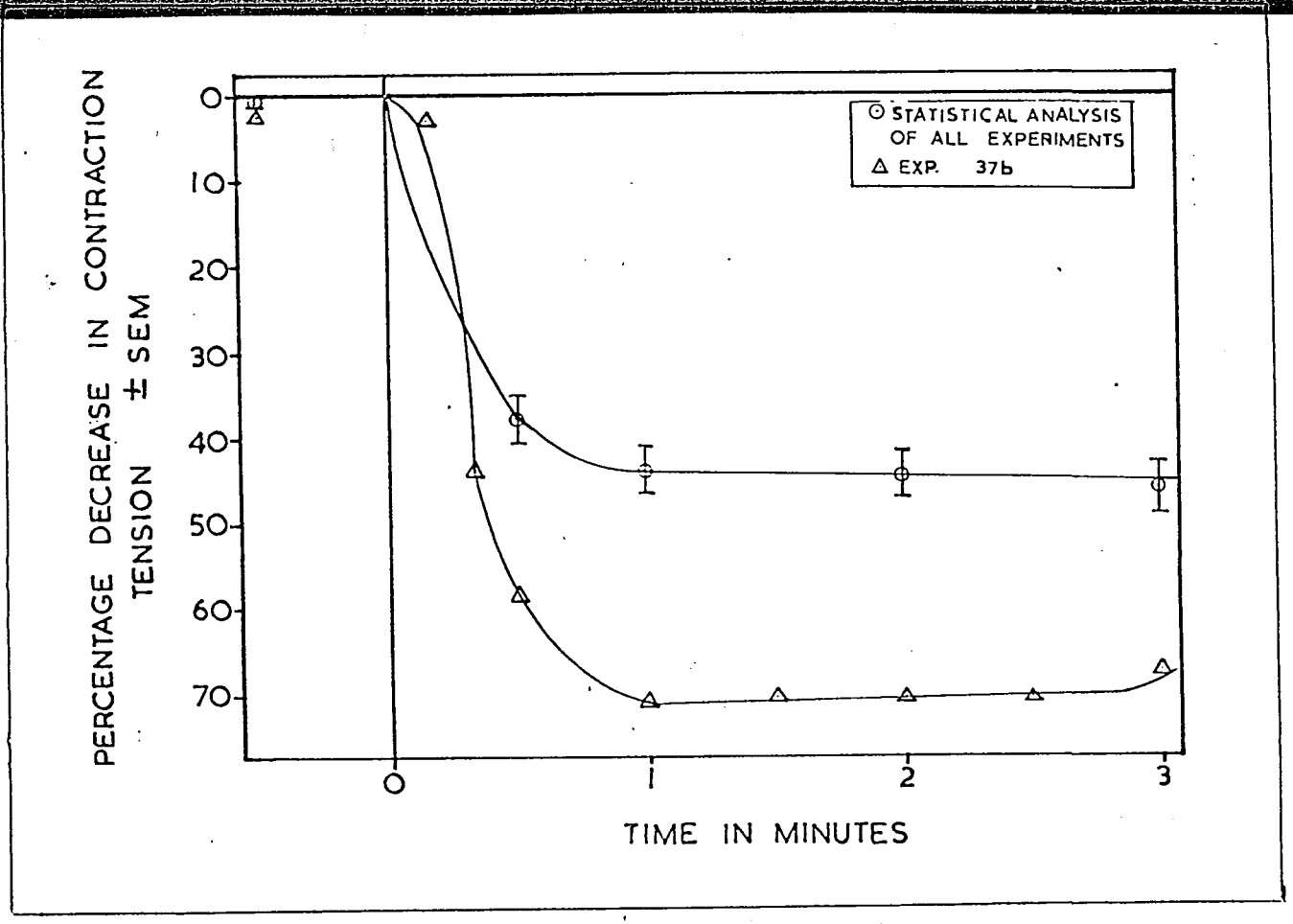

FIGURE 22. A Comparison of the Statistical and Typical Percentage Twitch Tension Responses to 80% O_2-20% CO_2

Control twitch (contraction) tension is 13.50 grams for Experiment 37 b and the mean control contraction tension from the statistical analysis is 9.16 grams.

one of the larger decreases in twitch tension is verified by inspection of the original data.

That the calculation of percent change of experimental twitch tension from control twitch tension does make the data more suitable for comparison, is evidenced by the location of the results from Experiment 37 b in Figure 22 with respect to the population means. The standard deviation for the population of percent decrease in twitch tension is 19.56% at 2 minutes and 21.30% at 3 minutes. This now places the results of Experiment 37 b at approximately one standard deviation away from the population means.

From the above demonstrated correlations of decrease in twitch tension to the amount of stretch or diastolic tension, the maintenance of a constant diastolic tension should minimize experimental variations. Therefore, in the comparisons of the relative effects of pH versus CO_2 on tension, the control diastolic tension was kept as close to a constant value, 10 grams, as possible. This value was also approximately the mean from the population, and gave good results without damaging the preparation.

Recovery from the effects of perfusion with 80% O_2 and 20% CO_2 bubbled perfusate. The next logical question is whether the heart recovers from the effects described

in the previous section. Figure 23 is a composite record, made, as previously described for the experimental perfusion, from the polygraph record of the recovery from experimental perfusion in Experiment 37 b. As seen previously in the description of the change to the experimental perfusate, the diastolic tension does not change during the recovery process either. The apparent change between the 1/2 minute segment of record and the 1 minute segment is due to a difference in width of the polygraph paper. There is also a similar, but smaller, difference between the 2 and 3 minute segments. Systolic tension increases sharply and definitely upon return to the control perfusate.

Perfusion pressure decreases after a very slight increase following initiation of control perfusion. From this response it can be estimated that coronary resistance increases slightly and then decreases markedly following the initiation of recovery.

The rate of contraction increases following the introduction of the control perfusate. This can be seen from the change in density of the electrograms per unit of record as well as the density of contraction twitches per unit of record.

When the complete data for tension recovery in Experiment 37 b is plotted, in Figure 24, it is shown that the recovery of tension from experimental perfusion is complete. The gross 1/2-time is 22 seconds, and the net 1/2-time is

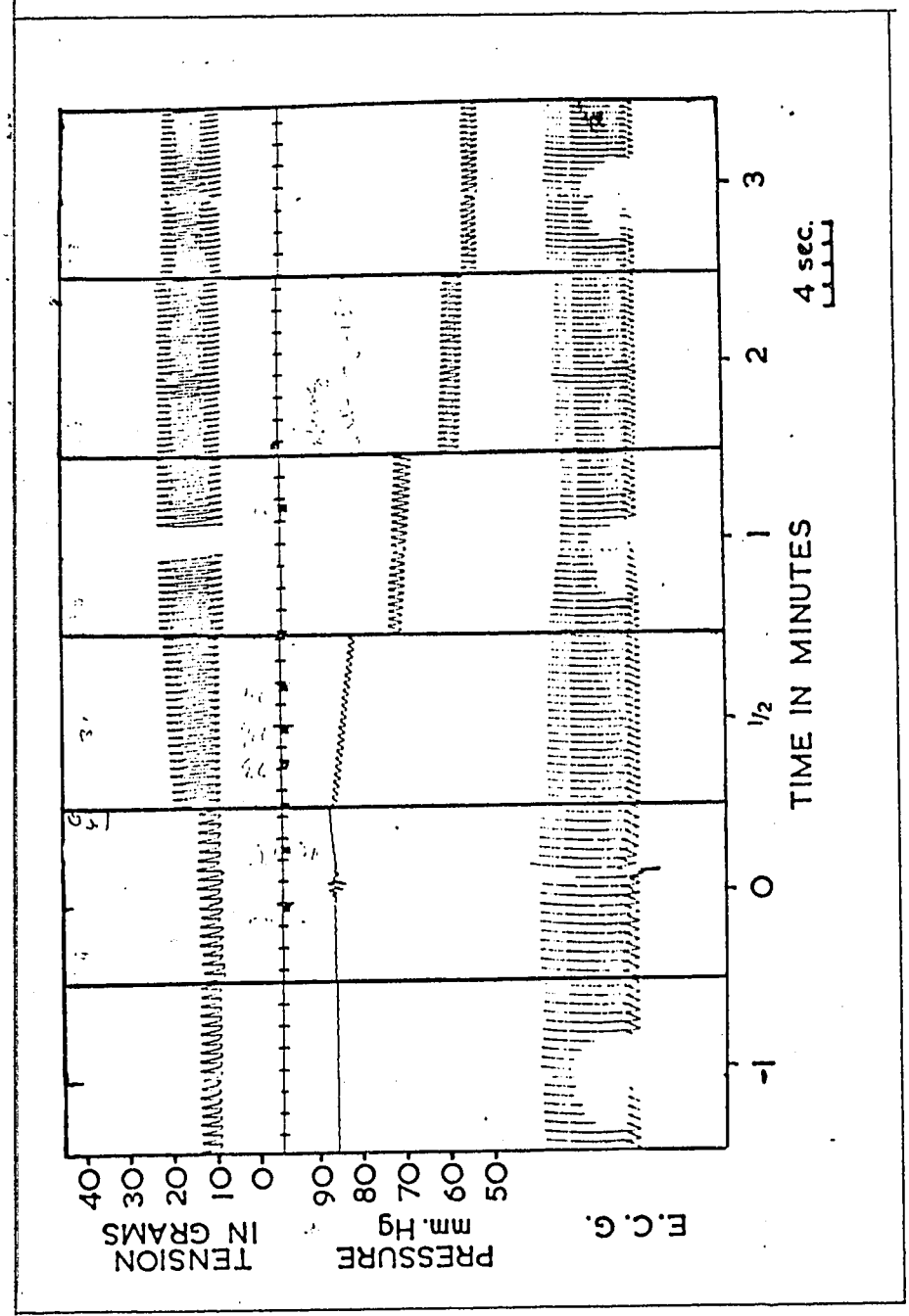

FIGURE 23. The Recorded Recovery of Heart Function from the Effects of 80% O_2-20% CO_2.

Time 0 is the time at which control perfusion was reinstated. Experiment 37 b.

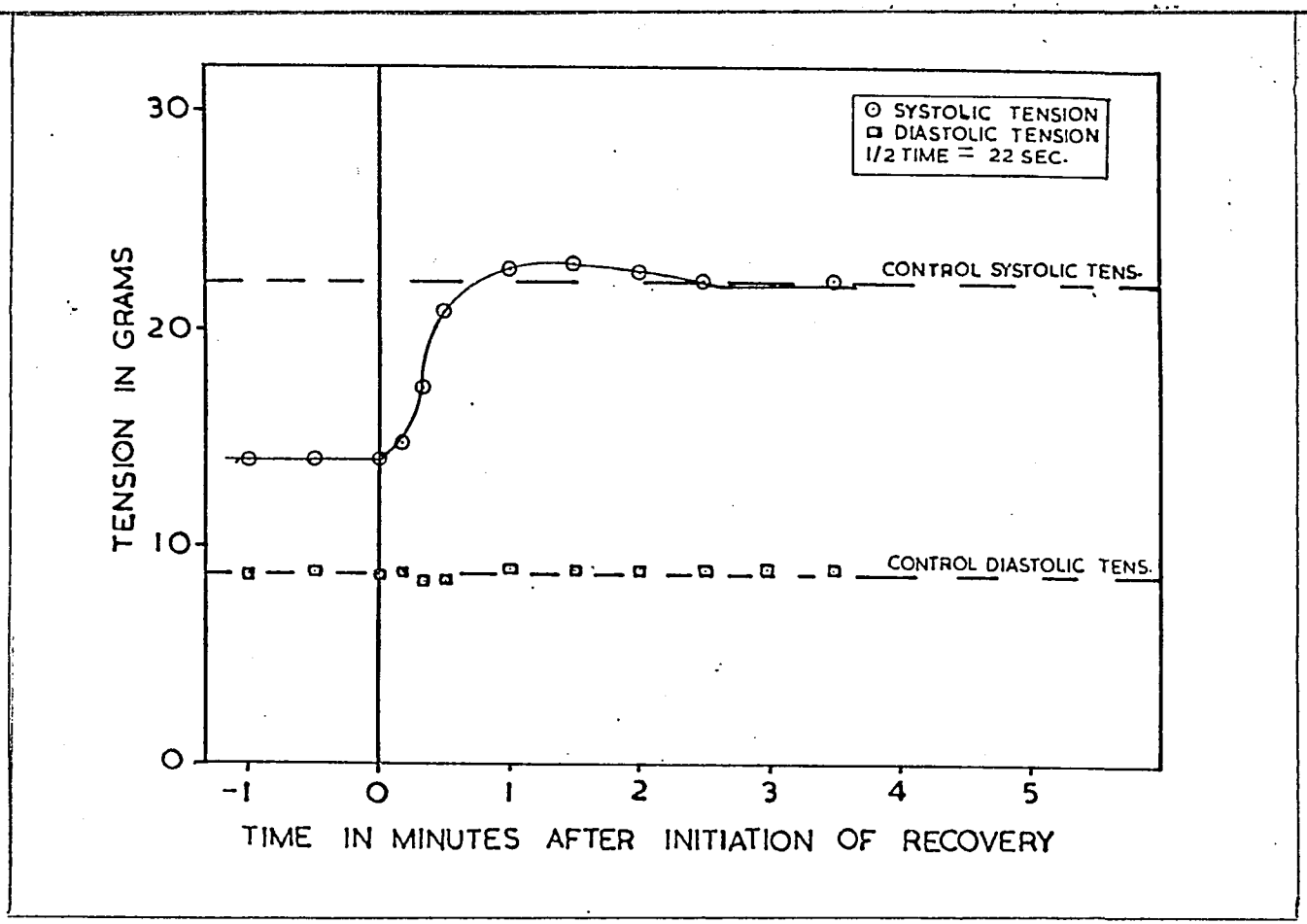

FIGURE 24. Recovery of Diastolic and Systolic Tension from the Effects of 80% O_2-20% CO_2 as Seen in a Typical Experiment

The control tensions are from the control perfusion immediately prior to the experimental perfusion which terminated at 0 time.

12 seconds. That the time required to clear the dead space is again 10 seconds is shown by the time interval with no reaction after the perfusate change in Figures 24 and 26.

Figure 25 shows the return of coronary resistance to control values after return to control perfusate. The return to control levels is accompanied by separation of resistance into distinct portions identifiable as the resistance at diastole and the resistance at systole. The gross 1/2-time is 20 seconds and the net 1/2-time is 10 seconds.

Figure 26 graphs the recovery of rate to its previous control value. The gross 1/2-time is 44.5 seconds and the net 1/2-time is 34.5 seconds.

Figure 27 graphs the results of a statistical analysis of all the experimental hearts as they recover from the effects of experimental perfusion with 80% O_2 and 20% CO_2 bubbled saline by being perfused with 95% O_2 and 5% CO_2 bubbled saline. Control diastolic and systolic tensions indicated on the graphs are the tensions at diastole and systole during the control perfusion period preceding the experimental perfusion. Specifying these control tensions indicates the degree to which the preparations recovered to pre-experimental conditions. The return to control levels is slower than the departure from control levels when the experimental perfusion was initiated. This is

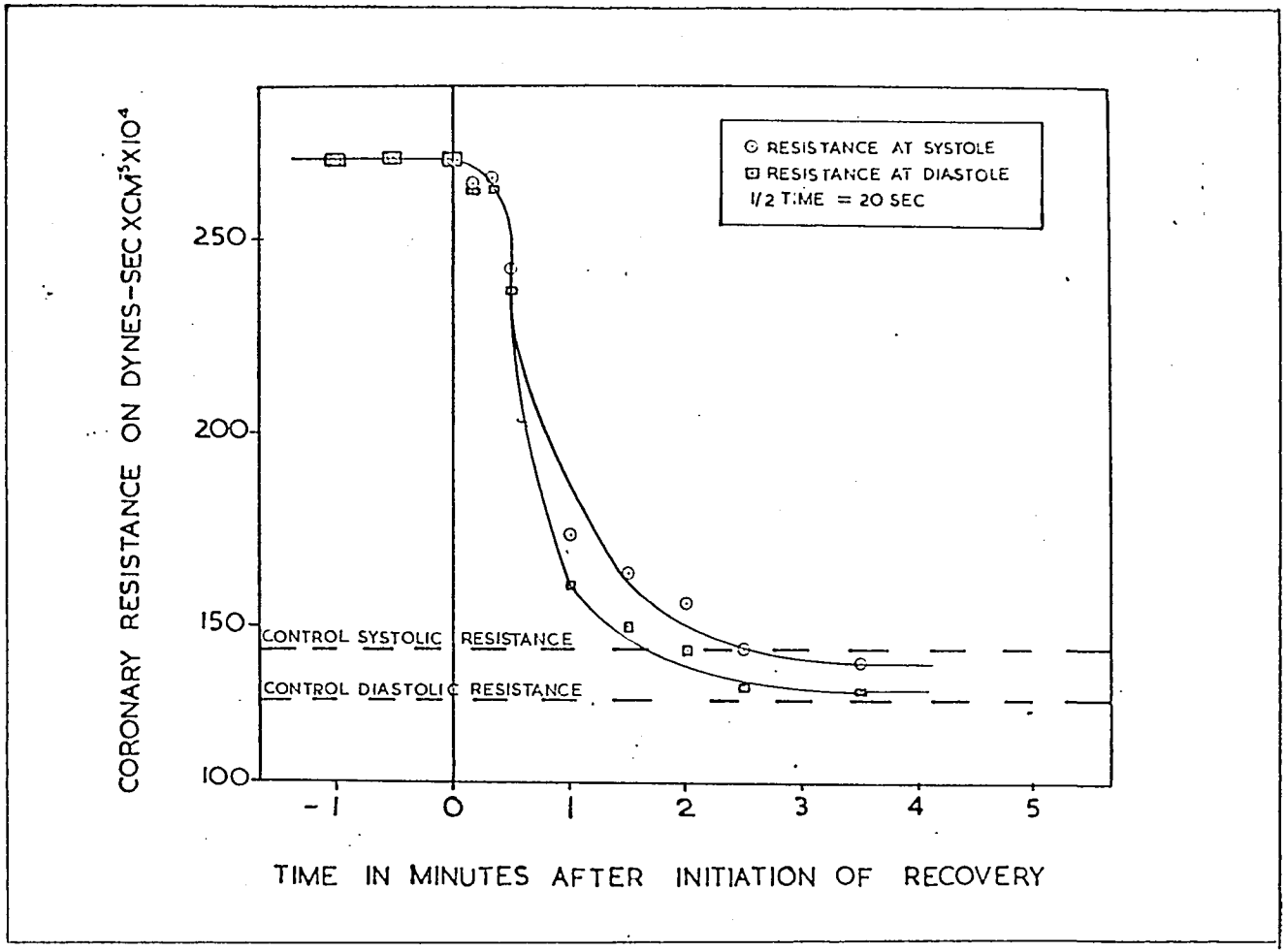

FIGURE 25. Recovery of Coronary Resistance from the Effects of 80% O_2-20% CO_2 as Seen in a Typical Experiment

Control resistances are those observed during the control perfusion immediately preceding the experimental perfusion which terminated at 0 time. Experiment 37 b.

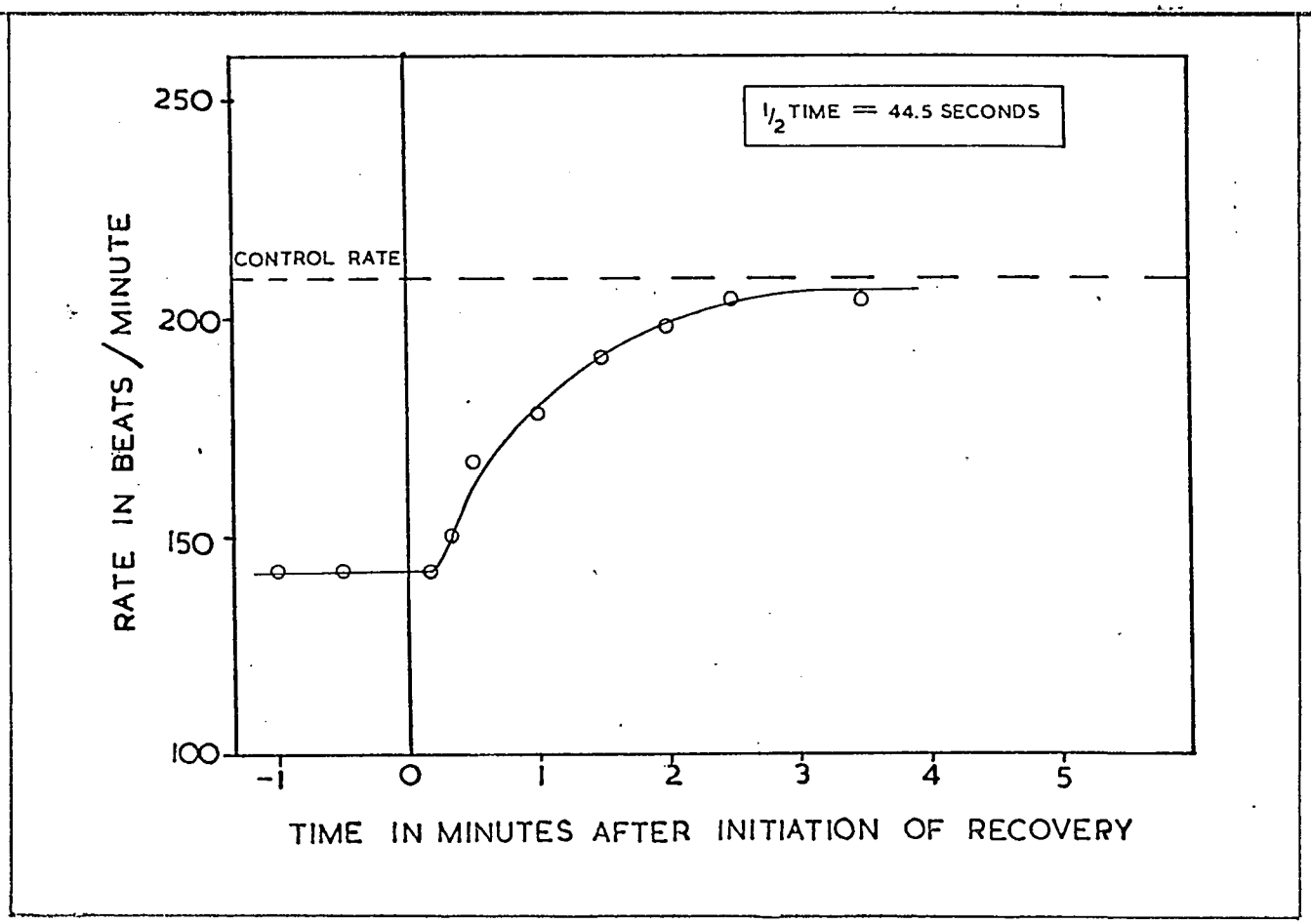

FIGURE 26. Heart Rate Recovery from the Effects of 80% O_2-20% CO_2 as Seen in a Typical Experiment

Control rate is that observed during the control period immediately preceding the experimental period which was terminated at 0 time. Experiment 37 b.

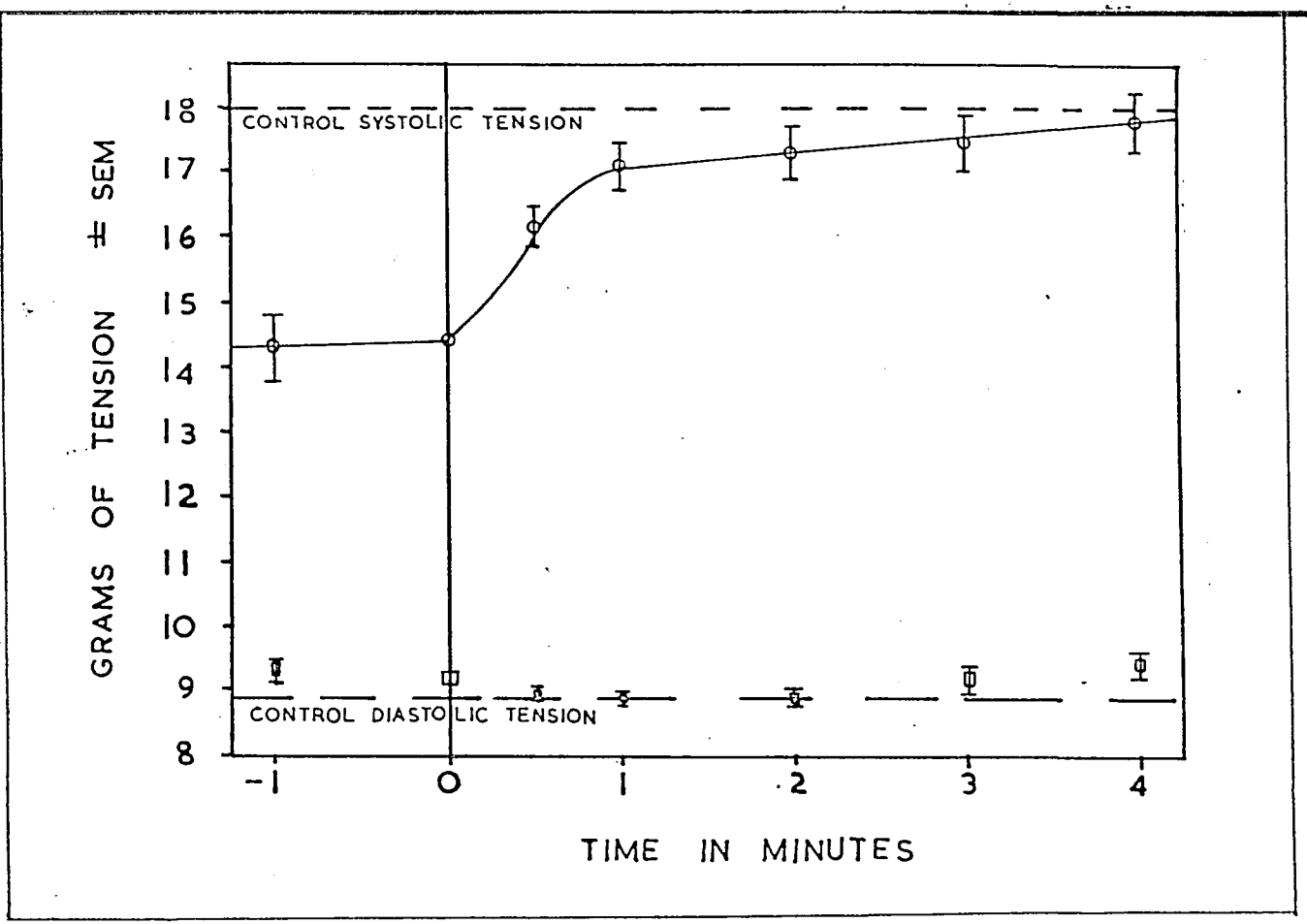

FIGURE 27. Statistical Analysis of the Recovery of Systolic and Diastolic Tension

Control tensions are the mean values of the tensions during the control perfusion immediately preceding each of the tension values used.

also shown when the 1/2-times for onset and for recovery of systolic tension in Experiment 37 b are compared, 2 seconds and 12 seconds respectively.

It should be noted also that following both the onset and the recovery from experimental perfusion, the initial change in tension occurs very rapidly whereas the final portion of the change is completed more slowly.

Diastolic tension shows very little change. The changes in diastolic tension at the 1/2 and the 1 minute times are significantly different from the pre-recovery values. The changes at the other times are not significant.

General questions relative to the performance of the preparation. Since this isolated heart preparation included some steps in the preparation which certainly are not a part of the usual physiology of the circulatory system, it may be validly asked whether or not these steps may not have been, in some way, directly responsible for the responses described. The first such possibility lies in the portion of the preparation consisting of the pre-operative steps taken to prepare the heart. This included the anesthetic, the heparin, and the cold saline infusion. The effect of removing all three of these steps was analyzed by stunning a rat with a blow on the head (Experiment 34). The heart was then removed as rapidly as possible while ice

cold saline was poured in the chest cavity. After cannulation of the aorta, the heart was then placed in the perfusion apparatus without further treatment. Figure 28 shows a comparison of the negative inotropic effect of the experimental perfusate (80% O_2-20% CO_2 bubbled saline) of all of the hearts as compared with the effect in the stunned rat's heart. It is readily seen that there is no significant difference in the negative inotropic response in the two types of preparations. An analysis of the percent decrease of contraction gives equivalent results.

The determination done with the stunned animal indicates that if the combined effects of anesthesia, heparin, and cold saline infusion are removed, there is no change in the tension response. Further evidence that the removal of these factors as a group was not masking the deleterious effect of one, had it been removed singly, was obtained from experiments wherein the cold infusion was not successful but in which the heparin had been injected. These showed the characteristic negative inotropic effect also. The evidence for the lack of effect of heparin is only circumstantial and indirect. When the heparin was injected, the ECG was observed carefully for changes in rate or pattern. None were observed in any of the experiments. This indicates that the heart at least superficially continues to function normally during the infusion of heparin. Therefore, having observed the lack of effect

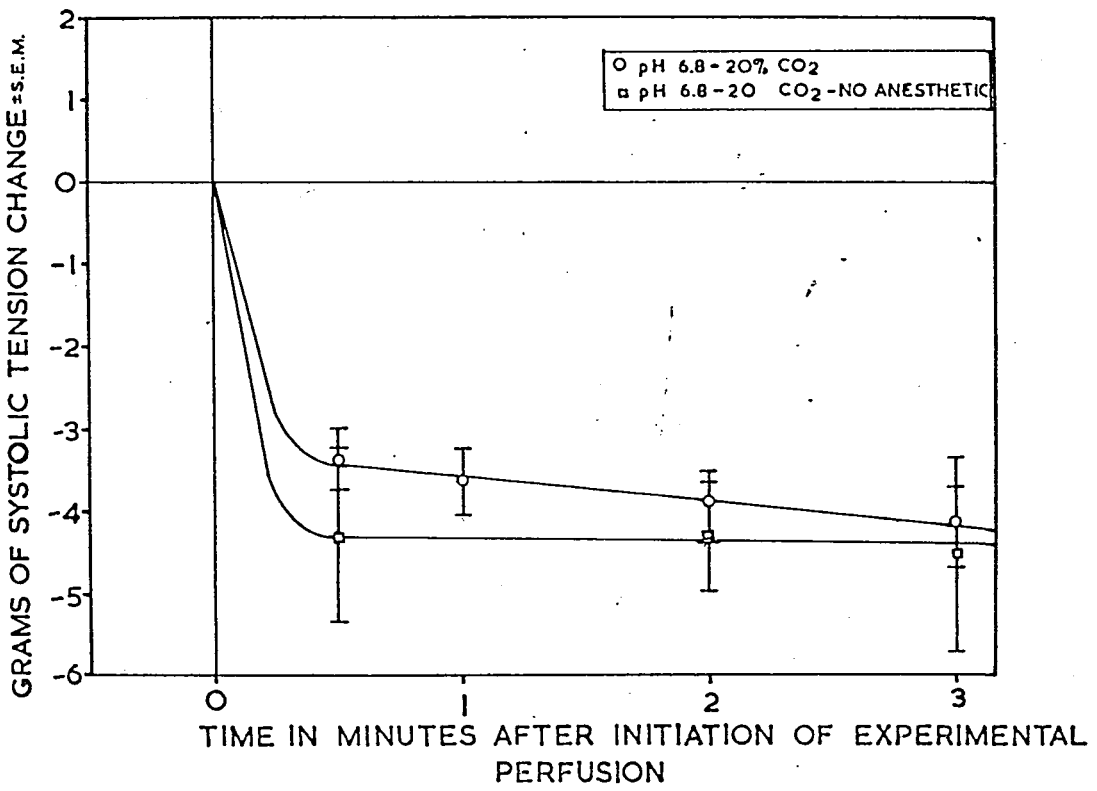

FIGURE 28. The Effect of Anesthesia, Heparin, and Cold Saline Infusion on the Negative Inotropic Response to 80% O_2-20% CO_2

The data for the non-anesthetized animal is from Experiment 34. This is compared with the data from the statistical analysis of tension response by the animals undergoing complete pre-perfusion treatment. Mean control systolic tensions were 37.45 grams and 18.02 grams for the non-anesthetized and anesthetized animals respectively.

of heparin and the cold saline infusion separately, and the lack of effect of the three procedures when conducted as a unit, it is concluded that this portion of the protocol did not cause the negative inotropic effect of the experimental perfusate.

The other non-physiological condition which might have affected the response to the experimental perfusate was the perfusion at 32 C rather than at 37 C, the more physiological temperature. To analyze the effect of temperature, Experiment 35 was done with the temperature initially at 37 C and then the temperature was lowered to 32 C. Figure 29 compares two changes to the experimental perfusate at 37 C with one change at 32 C. The equilibrium level of twitch tension decrease is approximately the same for the three experimental changes. The tension change at 32 C is slower than that for the two changes at 37 C; however, it should be noted that the 32 C was Experiment 35 f, one of the last changes for Experiment 35. The later changes in an experimental sequence are characteristically slower than the earlier ones in all experiments. It is theorized that this was probably due to the increasing edema with progress of the experiment. Therefore, based on the ability of a heart to reach an equivalent equilibrium level at both temperatures, it is concluded that the temperature routinely used for the control and experimental perfusions (32 C) was not the cause of the

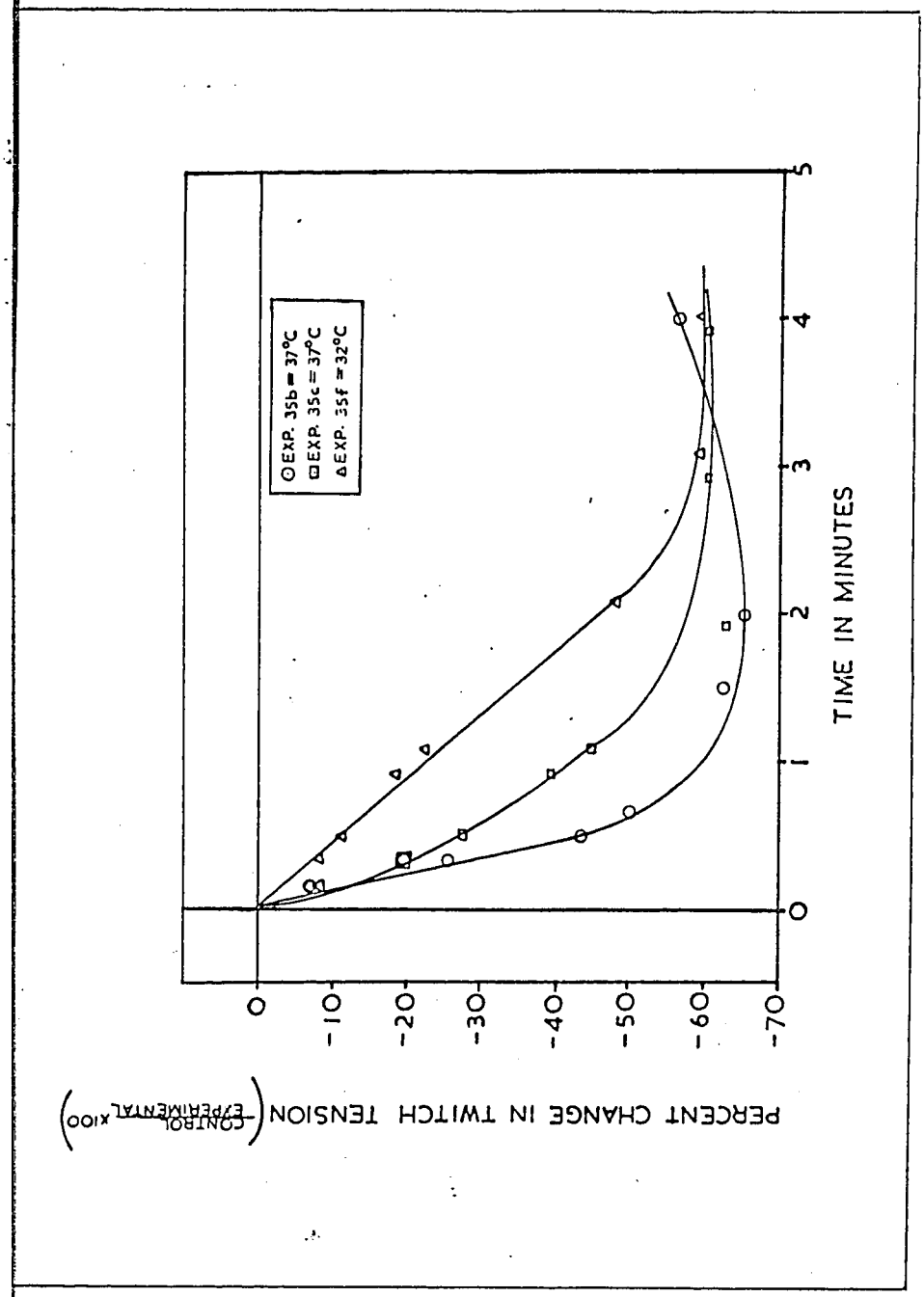

FIGURE 29. The Effect of Perfusate Temperature on the Twitch Tension Response to 80% O_2-20% CO_2

Grams of control twitch tension: 35 b, 4.9 g; 35 c, 3.8 g; 35 f, 9.7 g.

negative inotropic response in these isolated hearts.

The final general question about factors in the experimental procedures, which may have altered or caused the typical responses described in the previous sections, is the question of baseline stability. VAUGHAN WILLIAMS (1955) found that in his isolated rabbit atria the tension baselines were not stable under control conditions. This was not a problem with the preparations in these experiments. The stability has been indicated by the very small and non-significant standard errors of the means at the 1/2 minute times before the experimental changes in the typical response graphs already presented. Figure 30 shows the stability of the recordings of the variables during a control perfusion. While this record only shows approximately one minute of record, this sort of stability could be seen for long periods of time. To further insure that control values were well defined for all experiments, each experimental perfusion was preceded by a control perfusion period of sufficient length to achieve a new equilibrium.

The effect of decreased pH versus increased pCO_2 on the characteristic negative inotropic effect observed with 80% O_2-20% CO_2. To undertake answering the key question of the relative effects of decreased pH and increased pCO_2, the pH of the perfusate was changed by decreasing the

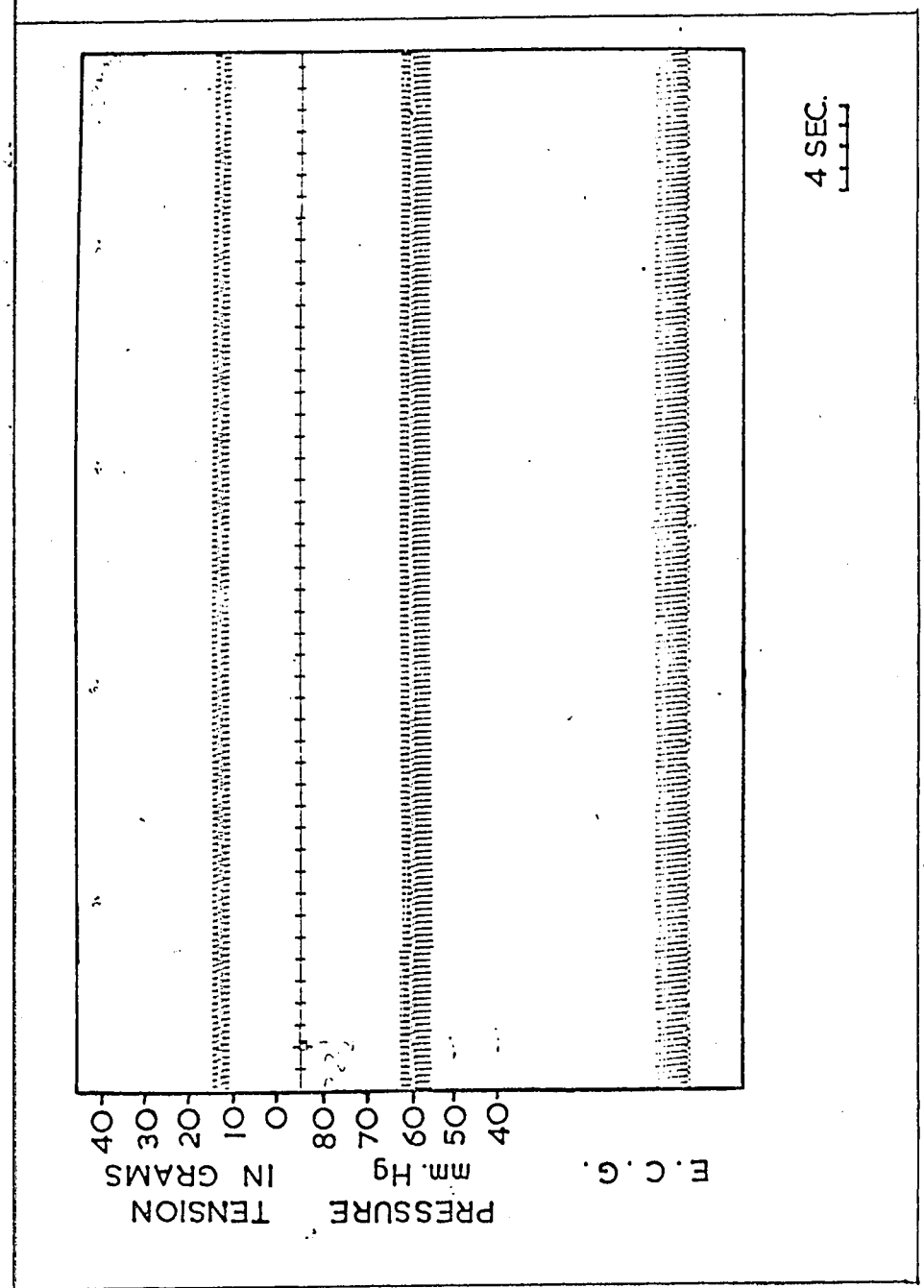

FIGURE 30. An Example of Baseline Stability

bicarbonate concentration while keeping the pCO_2 at the same level as for the control perfusate (5%). The method whereby this was accomplished was discussed in Chapter II.

Figure 31 shows sections of the polygraph recording from Experiment 57. These records compare the effect of the two types of perfusates. Although other factors not yet considered may possibly have an effect, it can be tentatively called a comparison of the effect of low pH and high pCO_2 on the isolated heart. The left panel of the Figure shows the typical response to high CO_2 (Experiment 57 d) which has already been characterized in a previous section. The right panel is the response to perfusion with a saline having a reduced pH without an increased pCO_2. The time 0 is the instant of change from the control perfusate to the experimental perfusate, and the other times are the minutes after initiation of experimental perfusion. From the record, it can be seen that the response to high CO_2 is as previously described for tension, pressure and rate. The initiation of perfusion with saline of low pH without the high CO_2 can be seen to cause no large change in tension, possibly a small change in perfusion pressure, and no obvious change in contraction rate.

When all of the values for tension from Experiment 57 c are graphed, the results shown in Figure 32 are obtained. This shows that there is no change in tension

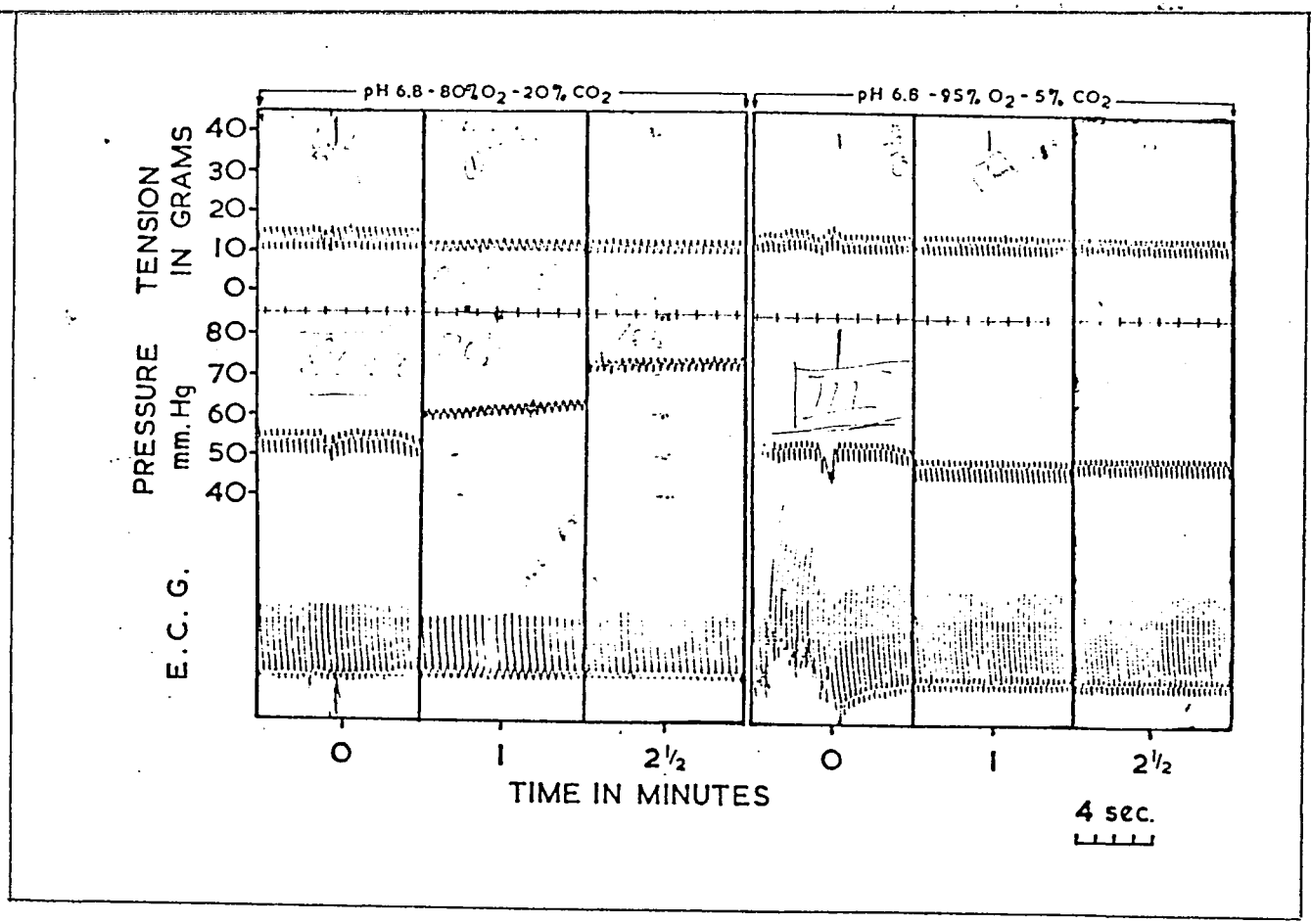

FIGURE 31. A Recorded Comparison of the Effect of pH and Carbon Dioxide

0 time is the point of change from control to experimental perfusate. Experiment 57 C.

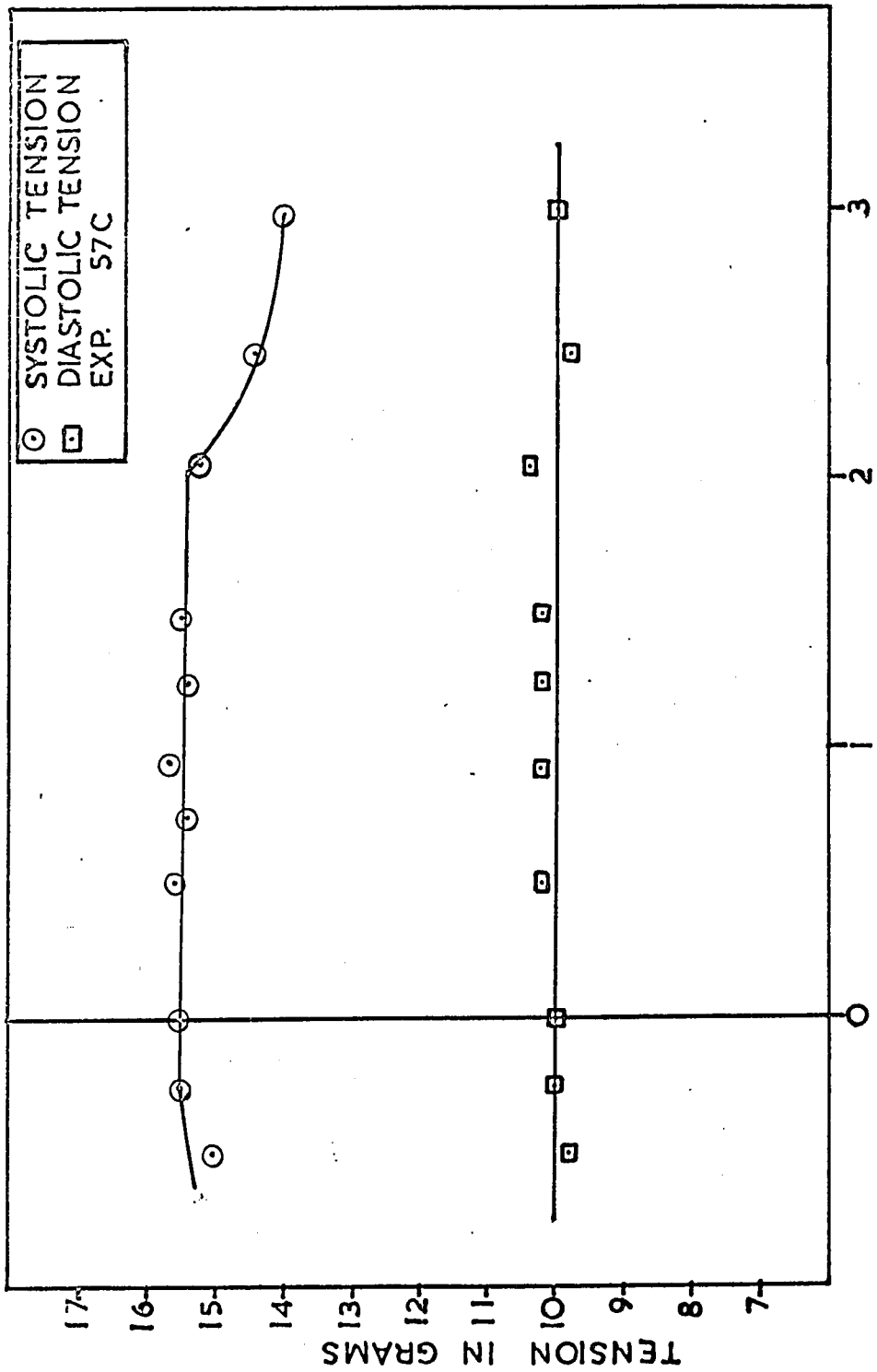

FIGURE 32. A Typical Tension Response to Reducing Perfusate pH

until near the end of the perfusion period when systolic tension begins to decrease slightly.

Figure 33 shows the change in rate and the change in coronary resistance at systole and diastole with the onset of experimental perfusion in Experiment 57 c. The effect on rate is small and reversible, but the significance of the change will have to be determined statistically. The changes in coronary resistance are smaller and of a different nature from those observed by lowering pH with a concurrent increase in pCO_2.

A graph of the statistical comparison of the mean values of systolic tension changes with the various experimental perfusates is shown in Figure 34. The tension change at pH 6.8-20% CO_2 is that previously described for the typical response to perfusion with 80% O_2 and 20% CO_2. The change in tension, following treatment with low pH without the increase in pCO_2, is very small compared to that with the concomitant increase in pCO_2. The sodium methyl sulfate control values are added, since, as previously described in Methods, it is used to replace the decreased bicarbonate, and a control for its possible pharmacological action was devised as described in Methods.

Figure 35 shows the mean values for the changes in diastolic tension with the various experimental treatments. The changes seen, when examining the sampled population of rat hearts, are all very small for each treatment.

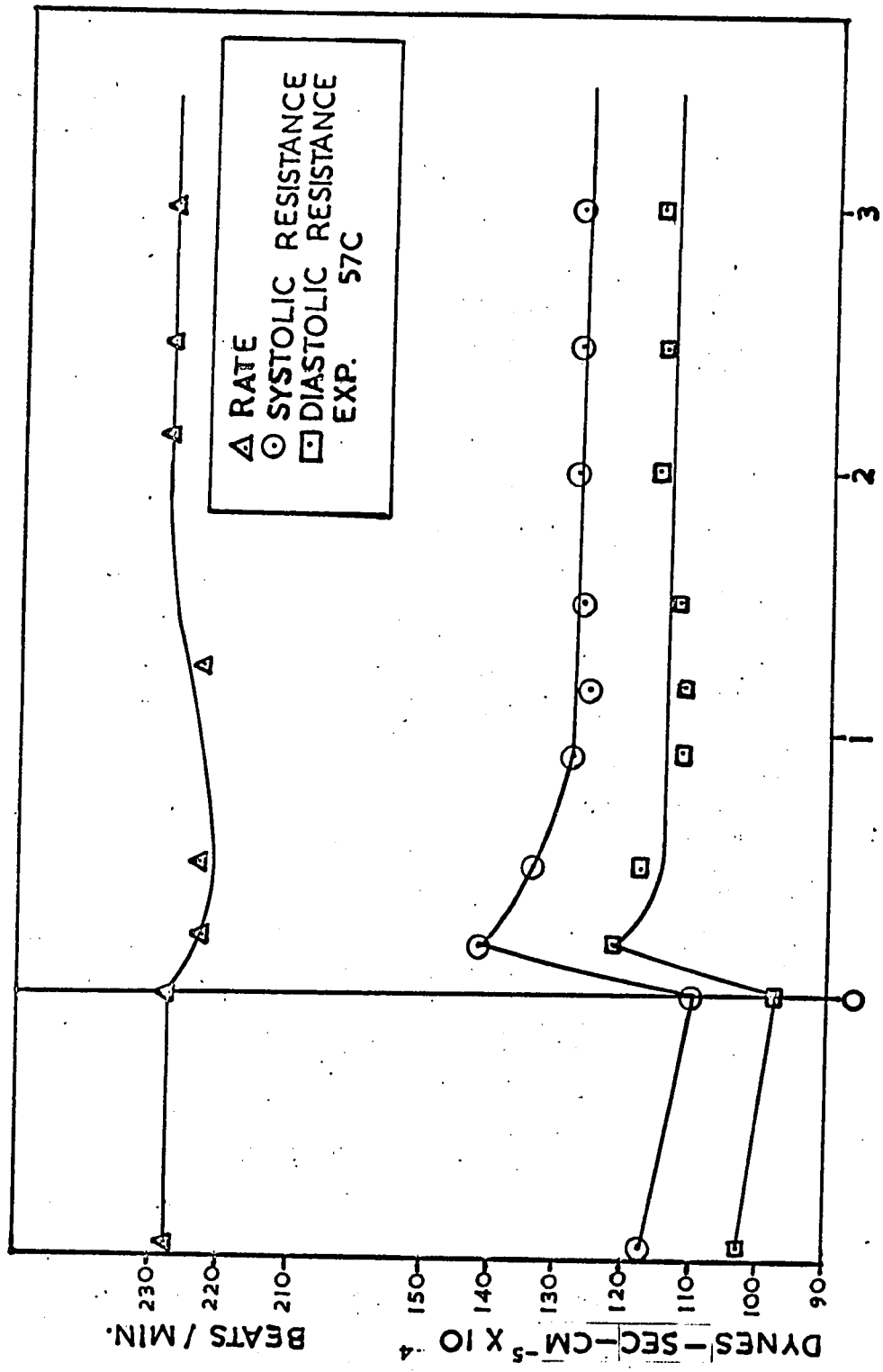

FIGURE 33. Typical Rate and Coronary Resistance Responses to Reducing Perfusate pH

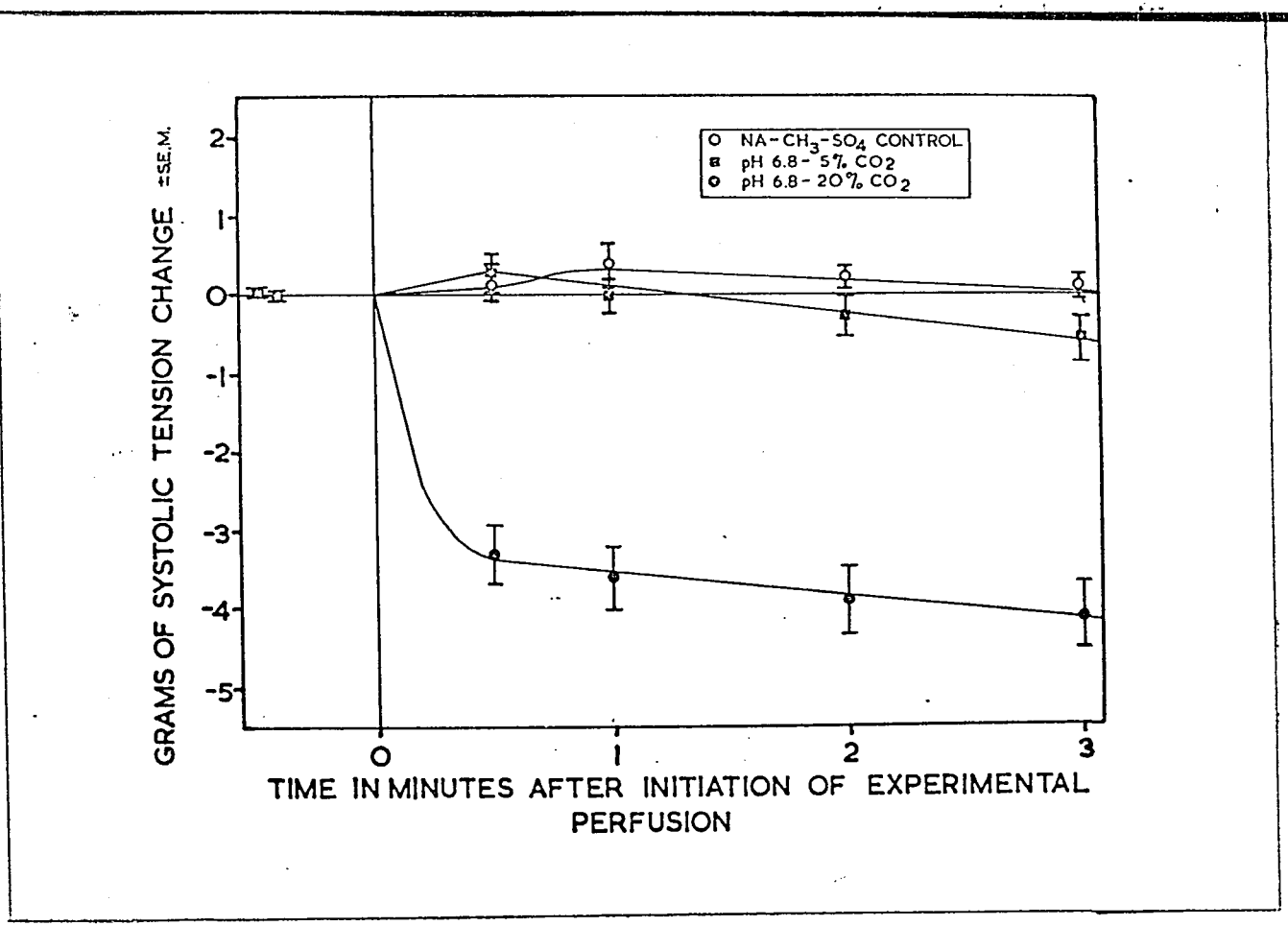

FIGURE 34. A Statistical Comparison of the Effect of pH and Carbon Dioxide on Systolic Tension

Mean control systolic tensions were as follows: Na-CH_3-SO_4 Control, 11.15 g, pH 6.8-5% CO_2, 20.45 g; pH 6.8-20% CO_2, 18.02 g.

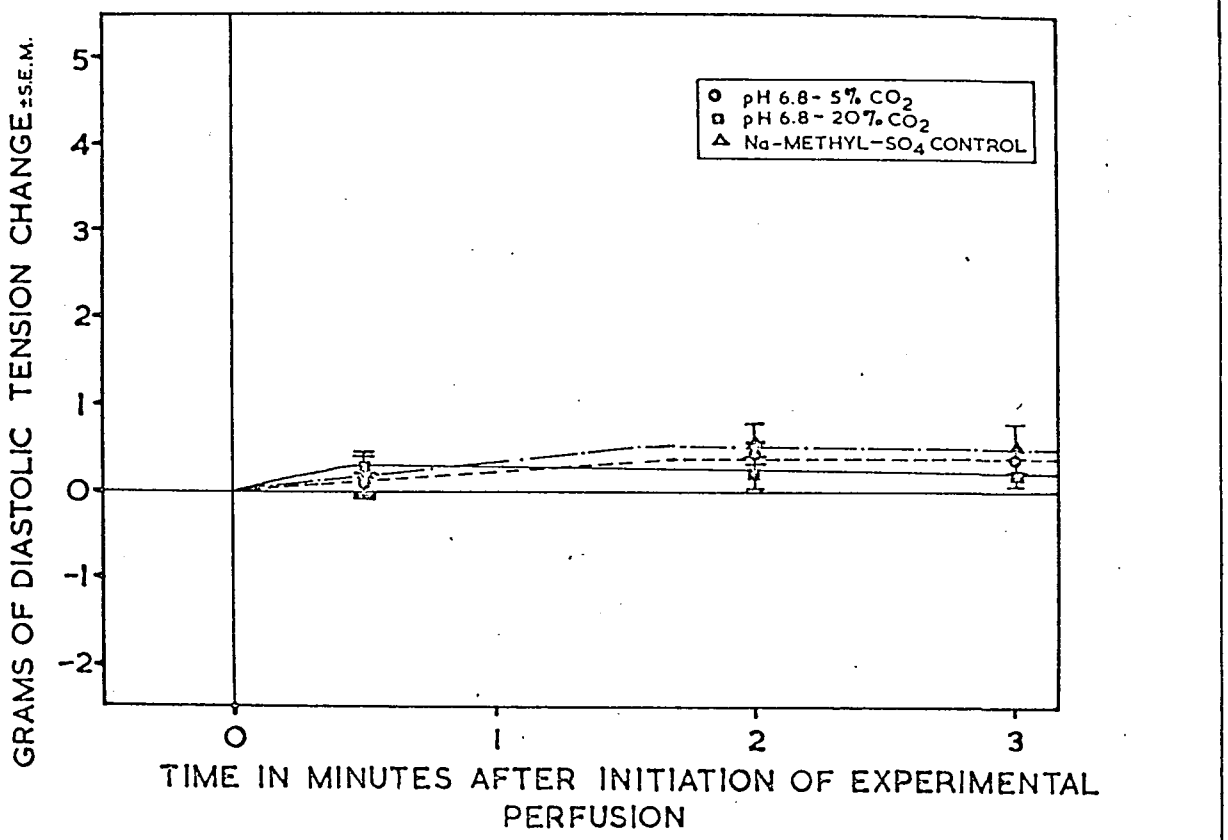

FIGURE 35. A Statistical Comparison of the Effect of pH and Carbon Dioxide on Diastolic Tension

Mean control diastolic tensions were as follows: pH 6.8-5% CO_2, 11.15 g; pH 6.8-20% CO_2, 8.86 g; Na-Methyl-SO_4 Control, 10.78 g.

This agrees with the typical responses so far characterized for the two types of experimental changes.

Table 5 summarizes the changes in diastolic and systolic tension which occur with perfusion of the heart with saline whose pH is decreased without an increase in pCO_2. When compared with the results of Table 4, the most striking observation is that the decrease in pH alone will not cause a significant decrease in systolic tension, whereas a decrease in pH in conjunction with an increase in pCO_2 will cause a striking and significant decrease in systolic tension. This also is illustrated graphically in Figure 34. Diastolic tension increases gradually by a small, though significant, amount when exposed to a change in pH alone, but increases significantly only in a transitory fashion during the first minute of perfusion with 80% O_2 and 20% CO_2 (see Table 4). Therefore, in the case of perfusion with 5% CO_2 bubbled saline with a pH of 6.8, a consideration of change in contraction tension might provide additional information since systolic tension decreases slightly and diastolic tension increases significantly.

The use of sodium methyl sulfate as a substitute for sodium bicarbonate in the solution which changes pH without changing pCO_2 was tested for its effect on tension by control perfusions wherein the sodium methyl sulfate replaces sodium chloride (see Methods). The results of

TABLE 5. Summary of Diastolic and Systolic Tension Changes Upon Perfusion with 95% O_2 - 5% CO_2 Bubbled Saline, pH 6.8

Number of Hearts	Time of Determination	Mean Change in Grams	± Standard Deviation	± Standard Error	p	Percent Change
			DIASTOLIC TENSION			
26	0	11.15				Control
26	1/2	+0.08	0.60	0.12	>0.40	+0.76
26	1	+0.35	0.63	0.12	<0.01	+3.17
26	2	+0.39	0.61	0.12	<0.05	+3.52
24	3	+0.37	0.73	0.15	<0.03	+3.32
			SYSTOLIC TENSION			
26	0	20.45				Control
26	1/2	+0.29	1.25	0.25	>0.20	+1.43
26	1	-0.03	1.18	0.23	>0.50	-0.17
26	2	-0.25	1.24	0.25	>0.20	-1.24
24	3	-0.55	1.44	0.29	>0.05	-2.71

these control perfusions are summarized in Table 6. It can be seen that sodium methyl sulfate has no significant effect on systolic or diastolic tension.

The contraction tension changes for perfusate changes to pH 6.8 with and without pCO_2 changes are shown in Figure 36. The hypothesis that the combined effect of the increase in diastolic tension and the decrease in systolic tension upon exposure to a perfusate which lowers pH would combine to produce a significant change in contraction tension, is confirmed in Figure 36 and Table 7. It can be seen that contraction tension decreases slowly and significantly after an initial non-significant rise. The change of contraction tension with a combined decrease of pH and increase of pCO_2 can be seen from Figure 36 and Table 7 to be quick, marked, and significant. The magnitude of change is much larger in this case; also it should be noted that tension decrease reaches an equilibrium level quickly.

Figures 37 and 38 show that diastolic and systolic tension changes, though small and non-significant, are corrected back to the control values by initiation of perfusion with the control saline. This occurs in both the typical response example (Figure 37) and in the population response (Figure 38).

TABLE 6. Summary of Diastolic and Systolic Tension Changes Upon Perfusion with Sodium Methyl Sulfate Containing Saline

Number of Hearts	Time of Determination	Mean Change in Grams	± Standard Deviation	± Standard Error	p	Percent Change
		DIASTOLIC TENSION				
6	0	10.78				Control
6	1/2	+0.23	0.62	0.25	>0.20	+2.16
6	1	+0.48	0.58	0.24	>0.05	+4.48
6	2	+0.55	0.56	0.23	>0.05	+5.10
6	3	+0.48	0.65	0.29	>0.10	+4.48
		SYSTOLIC TENSION				
6	0	17.67				Control
6	1/2	+0.23	1.04	0.42	>0.50	+1.32
6	1	+0.38	0.67	0.28	>0.20	+2.17
6	2	+0.40	0.76	0.31	>0.20	+2.26
6	3	+0.20	0.73	0.30	>0.50	+1.13

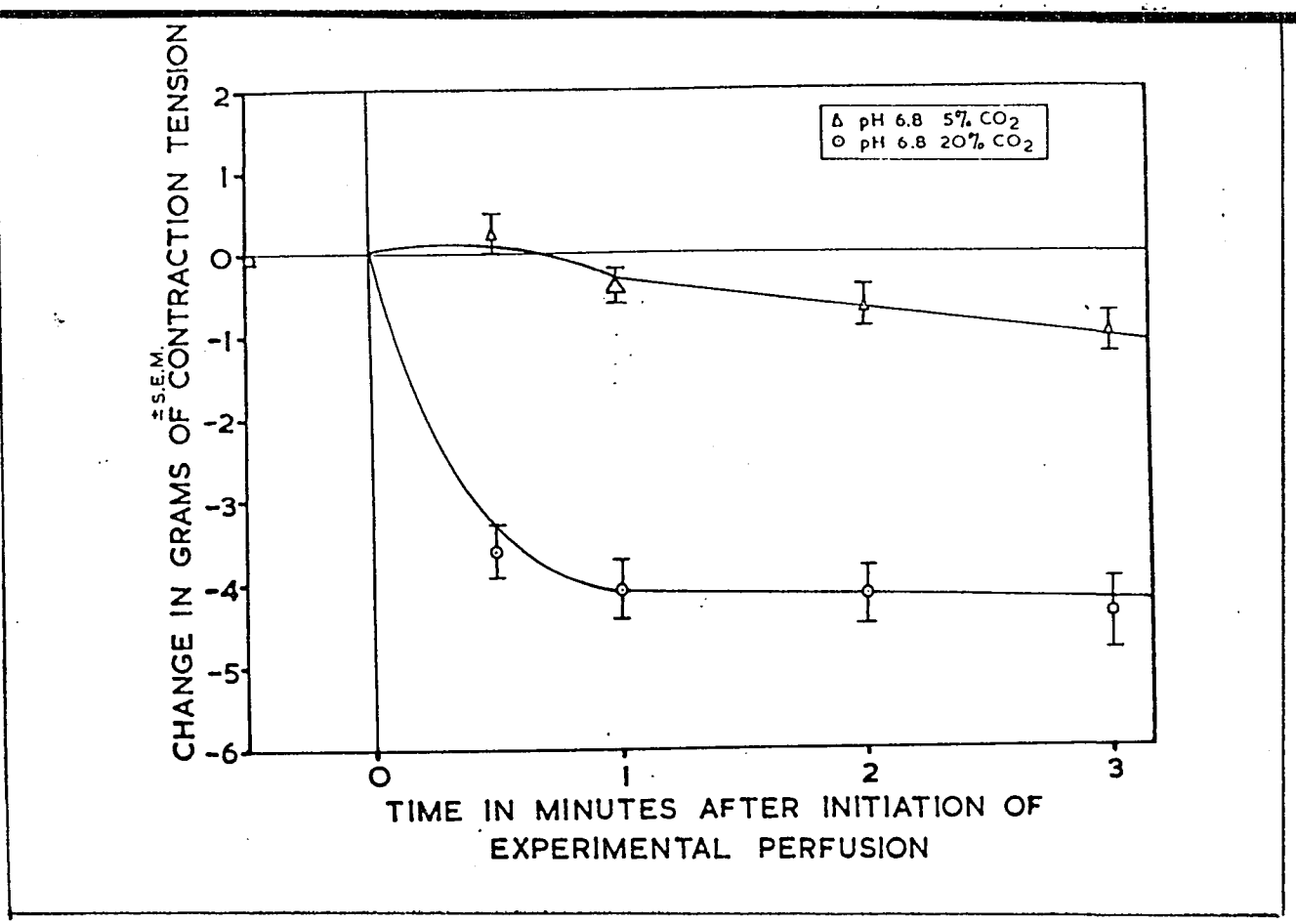

FIGURE 36. A Statistical Comparison of the Effect of pH and Carbon Dioxide on Contraction Tension

Mean control contraction tensions: pH 6.8-5% CO_2, 9.28 g; pH 6.8-20% CO_2, 9.16 g.

TABLE 7. Summary of Contraction Tension Changes

Upon Perfusion with Two Different Experimental Perfusates

Number of Hearts	Time of Determination	Mean Change in Grams	Standard Deviation	Standard Error	p	Percent Change
		95% O_2 - 5% CO_2: pH 6.8				
26	0	9.28				Control
26	1/2	+0.26	1.22	0.24	>0.20	+3.22
26	1	-0.37	1.05	0.21	>0.05	-3.55
26	2	-0.68	1.23	0.24	<0.01	-7.27
24	3	-0.98	1.25	0.26	<0.001	-11.36
		80% O_2 - 20% CO_2: pH 6.8				
56	0	9.16				
56	1/2	-3.59	2.23	0.30	<0.001	-38.87
56	1	-4.04	2.68	0.35	<0.001	-44.02
56	2	-4.12	2.70	0.36	<0.001	-44.69
56	3	-4.13	2.86	0.40	<0.001	-46.40

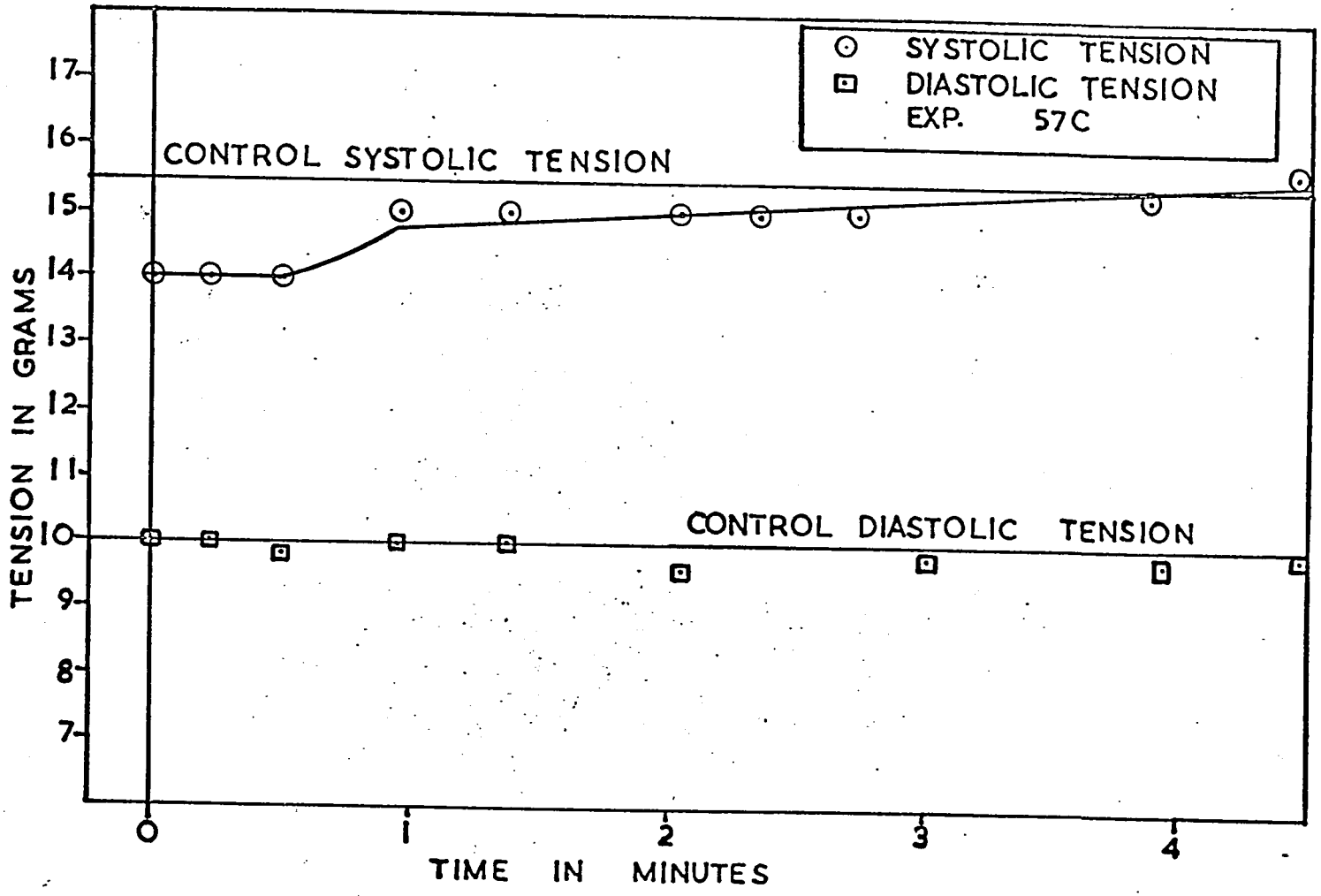

FIGURE 37. A Typical Tension Recovery from the Effects of Low pH

The control tensions are those observed during the previous control perfusions.

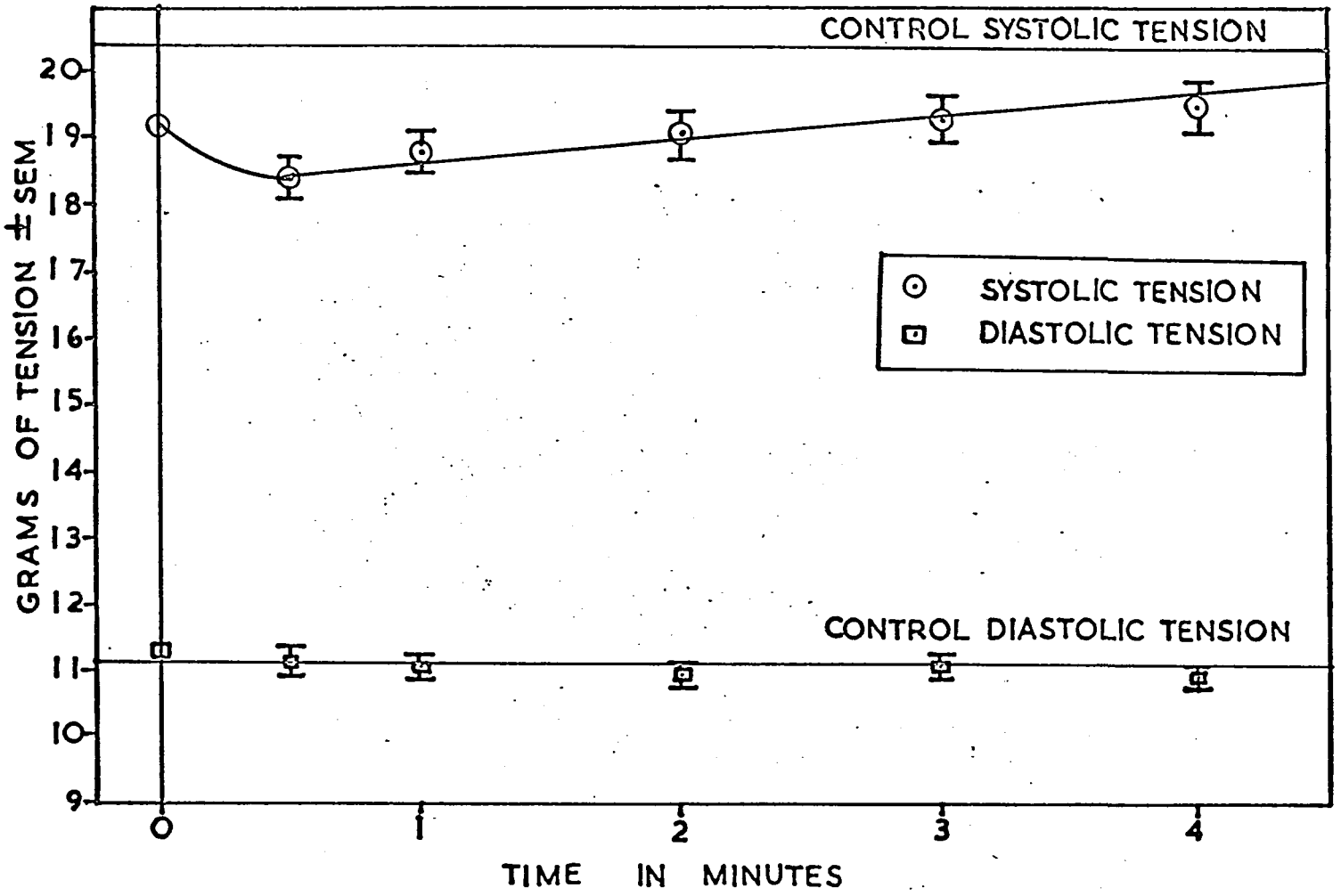

FIGURE 38. A Statistical Analysis of the Recovery of Tension from the Effects of Low pH

The control tensions are means of the tensions observed during control perfusions prior to each of the sampled experimental perfusions.

The effect of decreased pO_2 on the negative inotropic effect of 80% O_2 - 20% CO_2. One of the other factors alluded to at the beginning of the previous section, which still might be operative in the production of the negative inotropic effect in the isolated heart perfused with 80% O_2-20% CO_2, is the reduction in pO_2. The change from the control perfusate with 95% O_2 to the experimental perfusate with 80% O_2 represents a considerable decrease in O_2 content. This change in oxygen concentration does not occur in those experiments where pH alone decreases since the gas mixture used is the same as that used to bubble the control perfusate, 95% O_2-5% CO_2. In this section will be described the results of perfusion designed to determine the effect of decreased O_2 on tension when the pH and CO_2 are kept constant, i.e., the same as during control perfusion.

To produce the change in pO_2 without a concurrent change in the other variables, the normal control saline was bubbled with a gas mixture containing 80% O_2 - 5% CO_2 - 15% N_2. This introduces a new unknown, the effect of N_2. However, this will be considered later in this section. A comparison of the effect on tension of solely decreasing O_2 with the effect on tension of 80% O_2 - 20% CO_2 is shown in Figure 39. There results a positive inotropic effect from perfusion in which only a reduction in O_2 has occurred. Referring to Table 8, it can be seen that this positive inotropic effect is statistically significant.

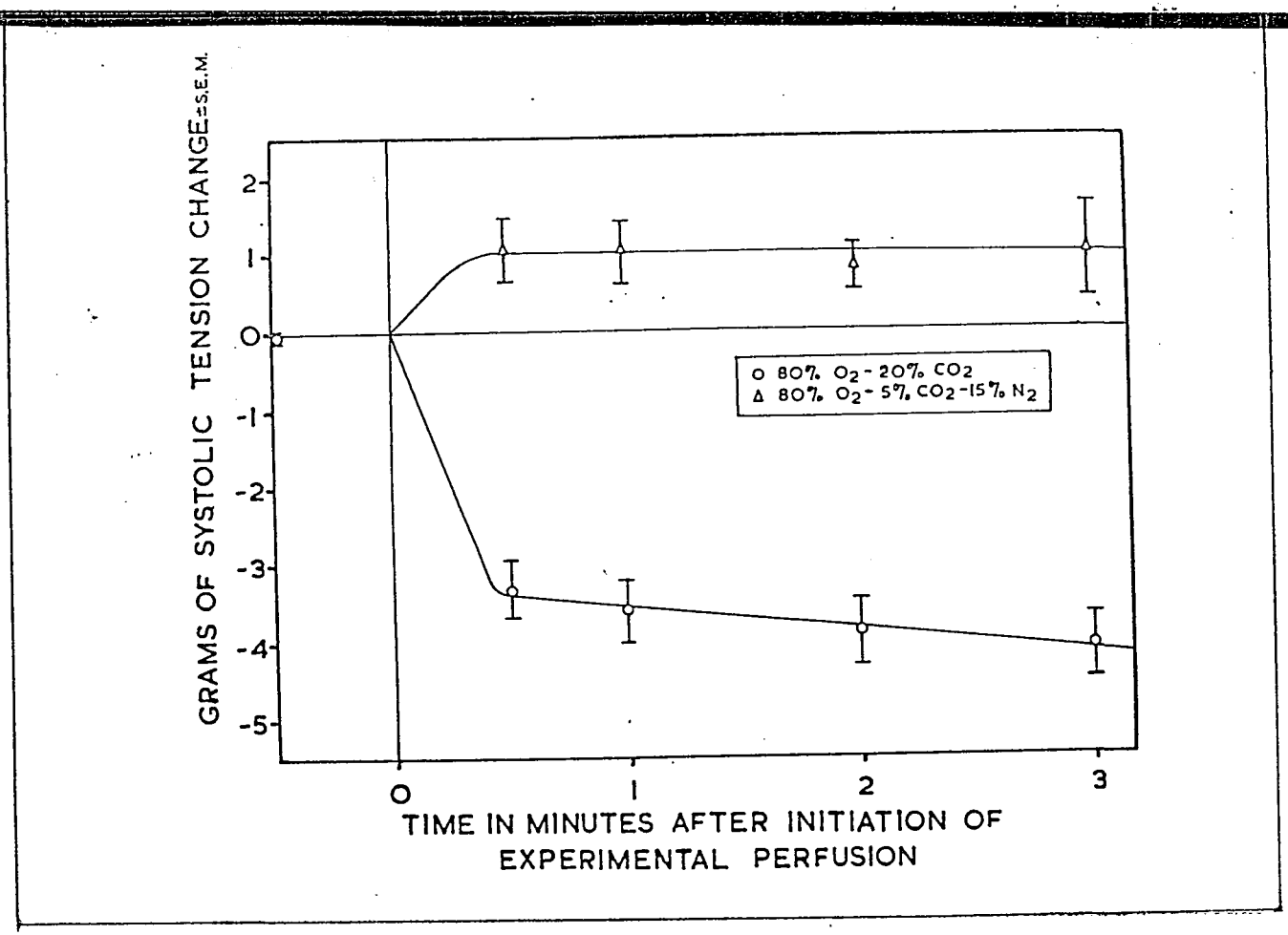

FIGURE 39. A Statistical Comparison of the Inotropic Effects of Decreased Oxygen and Increased Carbon Dioxide

Mean control systolic tensions: 80% O_2-20% CO_2, 18.02 g; 80% O_2-5% CO_2-15% N_2, 18.86 g.

TABLE 8. Summary of Diastolic and Systolic Tension Changes

Upon Perfusion with Saline Bubbled by 80% O_2 - 5% CO_2 - 15% N_2

Number of Hearts	Time of Determination	Mean Change in Grams	± Standard Deviation	± Standard Error	p	Percent Change
		DIASTOLIC TENSION				
9	0	10.53				Control
9	1/2	-0.45	0.41	0.14	<0.03	-3.91
9	1	-0.56	0.60	0.20	<0.03	-5.35
9	2	-0.64	0.51	0.17	<0.01	-6.08
8	3	-0.73	0.66	0.23	<0.03	-6.92
		SYSTOLIC TENSION				
9	0	18.86				Control
9	1/2	+1.07	1.31	0.43	<0.05	+ 5.69
9	1	+1.03	1.21	0.40	<0.05	+ 5.48
9	2	+0.78	0.96	0.32	<0.05	+ 4.15
8	3	+0.97	1.77	0.63	>0.10	+ 5.12

To further check that this positive inotropic effect was not a random peculiarity of the hearts used, experiments were done wherein both the change to low pH and the maintenance of constant pH accompanied the decrease in pO_2. The results of one of these experiments (Experiment 42) is shown in Figure 40. The positive inotropic effect of low pO_2 occurs as well as the usual negative inotropic effect of 80% O_2 - 20% CO_2. Since the perfusion with low pO_2 alone occurred 137 minutes after the characteristic negative inotropic response, it was felt necessary to have some control to prove that the positive inotropic effect had not occurred because of fatiguing of the preparation. It was reasoned that if a negative inotropic response from perfusion with 80% O_2 - 20% CO_2 was again obtained at the end of the experiment, then the positive inotropic response was truly an effect of decreased pO_2. From the negative inotropic response at 250 minutes, it can be seen that the preparation had not acquired peculiar inotropic characteristics due to aging of the preparation. The response was slower, but it reached the same equilibrium level as that achieved earlier in the experiment. It already has been mentioned that a slower tension response is characteristic of experimental perfusions as the preparation ages.

Figure 41 compares the effect of low pO_2 on diastolic tension when it occurs with a pH change (80% O_2 - 20% CO_2) and without a pH change (80% O_2 - 5% CO_2 - 15% N_2). As

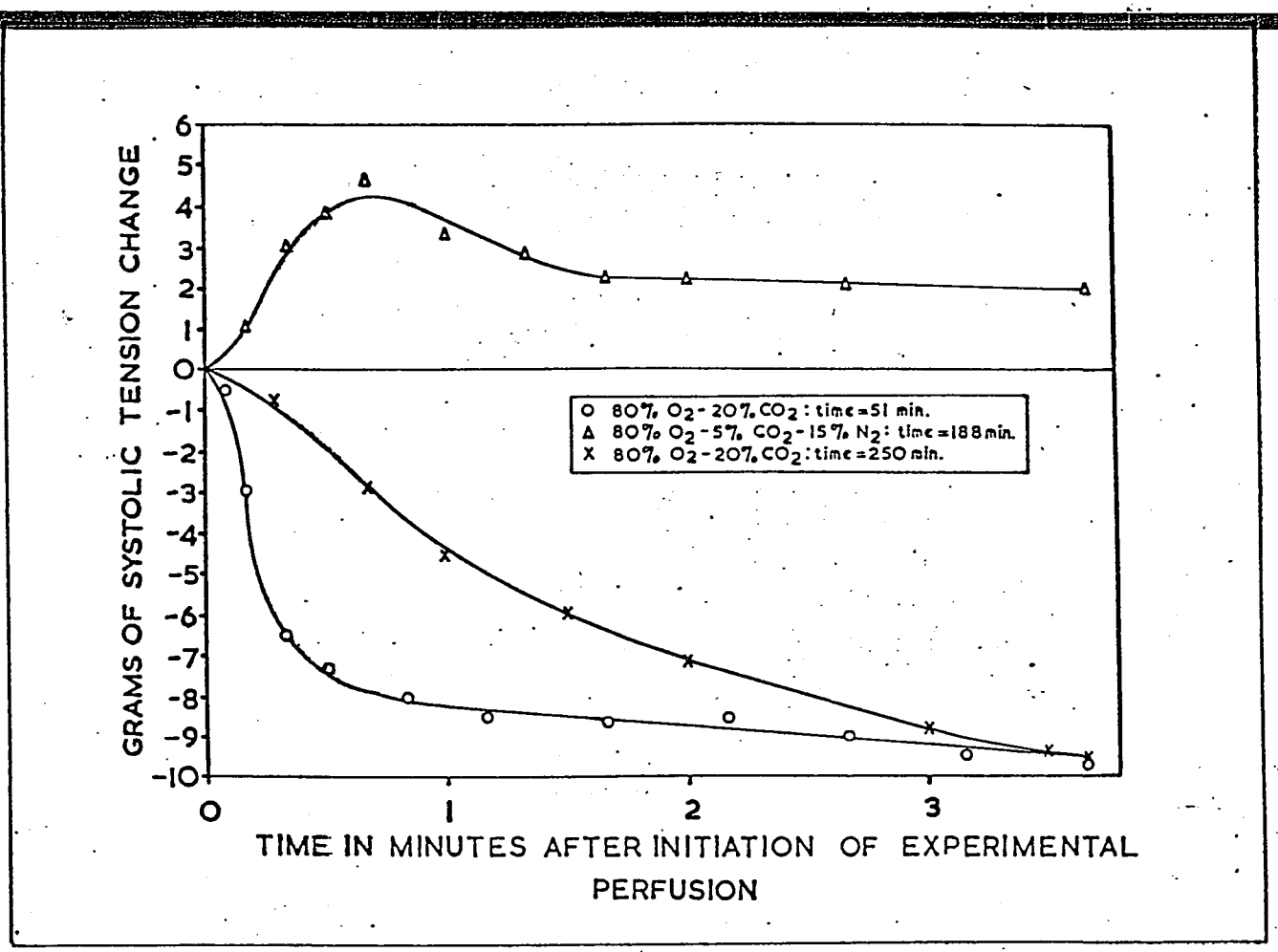

FIGURE 40. A Typical Systolic Tension Response to 80% O_2

Control systolic tensions: Time 51 min, 15.50 g; 188 min, 30.00 g; 250 min, 25.10 g. Experiment 42.

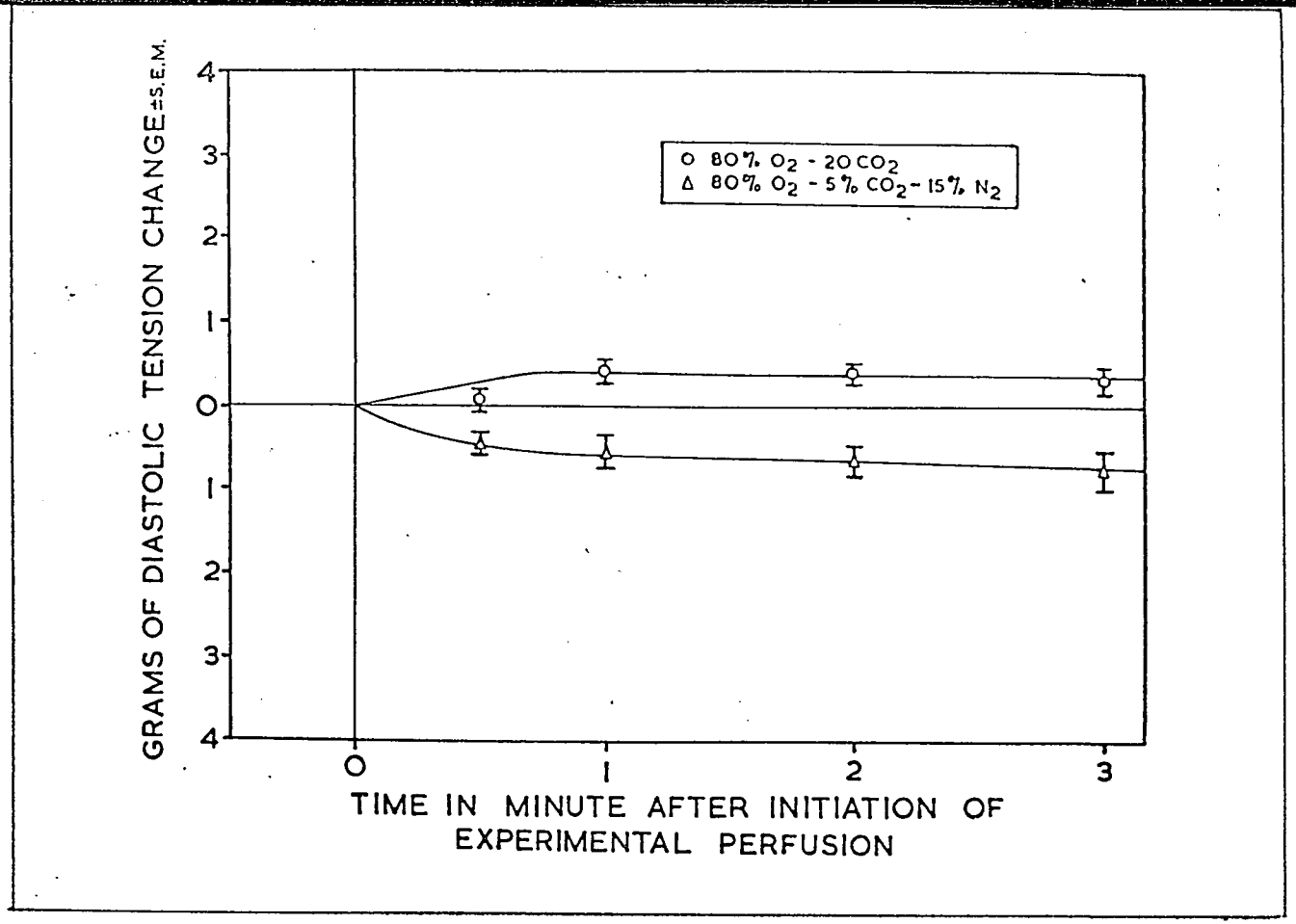

FIGURE 41. A Statistical Analysis of the Effect of 80% O_2 on Diastolic Tension

Control diastolic tensions: 80% O_2-20 CO_2, 8.86 g; 80% O_2-5% CO_2-15% N_2, 10.53 g.

previously described, the increase in diastolic tension with 80% O_2-20% CO_2 is not significant. With the decrease in pO_2 alone the decrease in diastolic tension is significant as can be seen from Table 8. This is the first experimental perfusion which has produced a significant decrease in diastolic tension.

The problem of the effect of N_2 which was used as a "filler" gas in the experiments on the effect of decreasing pO_2 alone must now be considered. However, because of the large standard deviations occurring in the statistical determination of the rate changes, it was decided to conduct further control experiments. To assess the possible effect of N_2 on rate which, in turn, might affect tension, the heart was driven at a rate slightly faster than its natural in vitro rate; this was usually four beats per minute. Figure 42 shows the result of such an experiment. Despite the constant rate, the positive inotropic response still occurs. As indicated on the graph, an additional type of control experiment was done in which the change to low pO_2 was allowed to occur with the heart beating at its natural rate. This produced the usual positive inotropic effect. While perfusing with the low pO_2 saline, the heart was then driven at a rate slightly greater than the natural in vitro rate. This produced no change in the tension development.

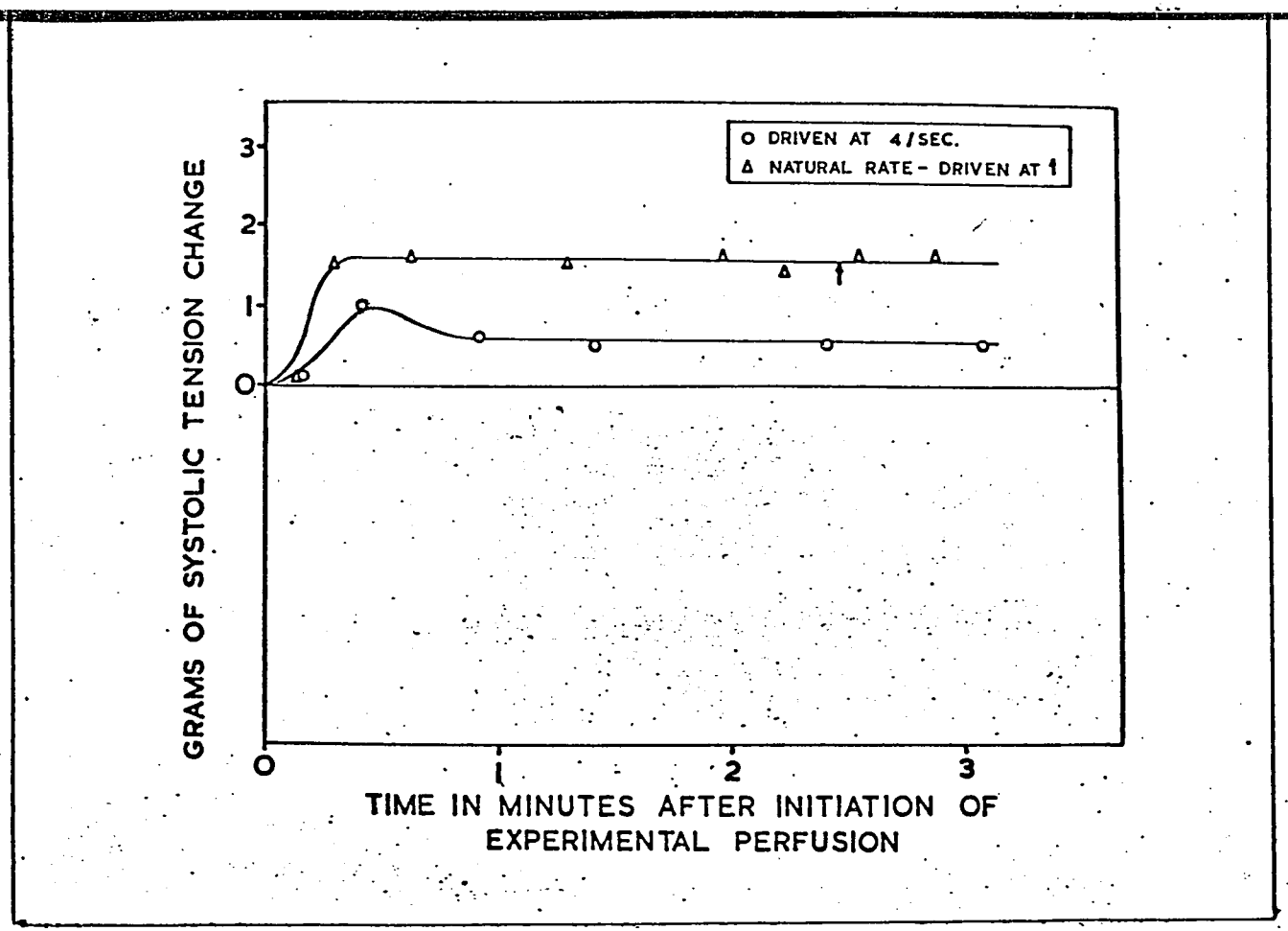

FIGURE 42. A Typical Experiment Demonstrating the Effect of Constant Rate on the Positive Inotropic Effect of 80% O_2

Saline saturated with 80% O_2-5% CO_2. Control systolic tensions: Driven (Experiment 32 d), 22.5 g; Natural rate (Experiment 32 c), 23.4 g.

The relationship of the negative chronotropic effect of 80% O_2 - 20% CO_2 to the simultaneous negative inotropic effect. The negative chronotropic effect of perfusion with 80% O_2 and 20% CO_2 was demonstrated previously in Figure 16. According to the findings of BURGEN and TERROUX (1953) this could be responsible for a positive inotropic response. Although a negative inotropic response is observed with 80% O_2 - 20% CO_2, it was felt that the rate effect should be controlled in order to eliminate the possibility that decreased inotropism was, somehow, the resultant of altered rate. A series of experiments was done in which the change to the experimental perfusate was made while the heart was driven at a constant rate, which was slightly faster than its natural rate in vitro. The results were analyzed statistically and are compared with results from the previously characterized non-driven change in Figure 43. It can be seen from this graph that the negative inotropic effect is still present, and that it is of the same order of magnitude as the change in the non-driven hearts.

The 1/2-time for rate change is 40 seconds, and the 1/2-time for the tension change is 2 seconds. From this large difference between the two values, it seems reasonable to conclude that the two changes are not related.

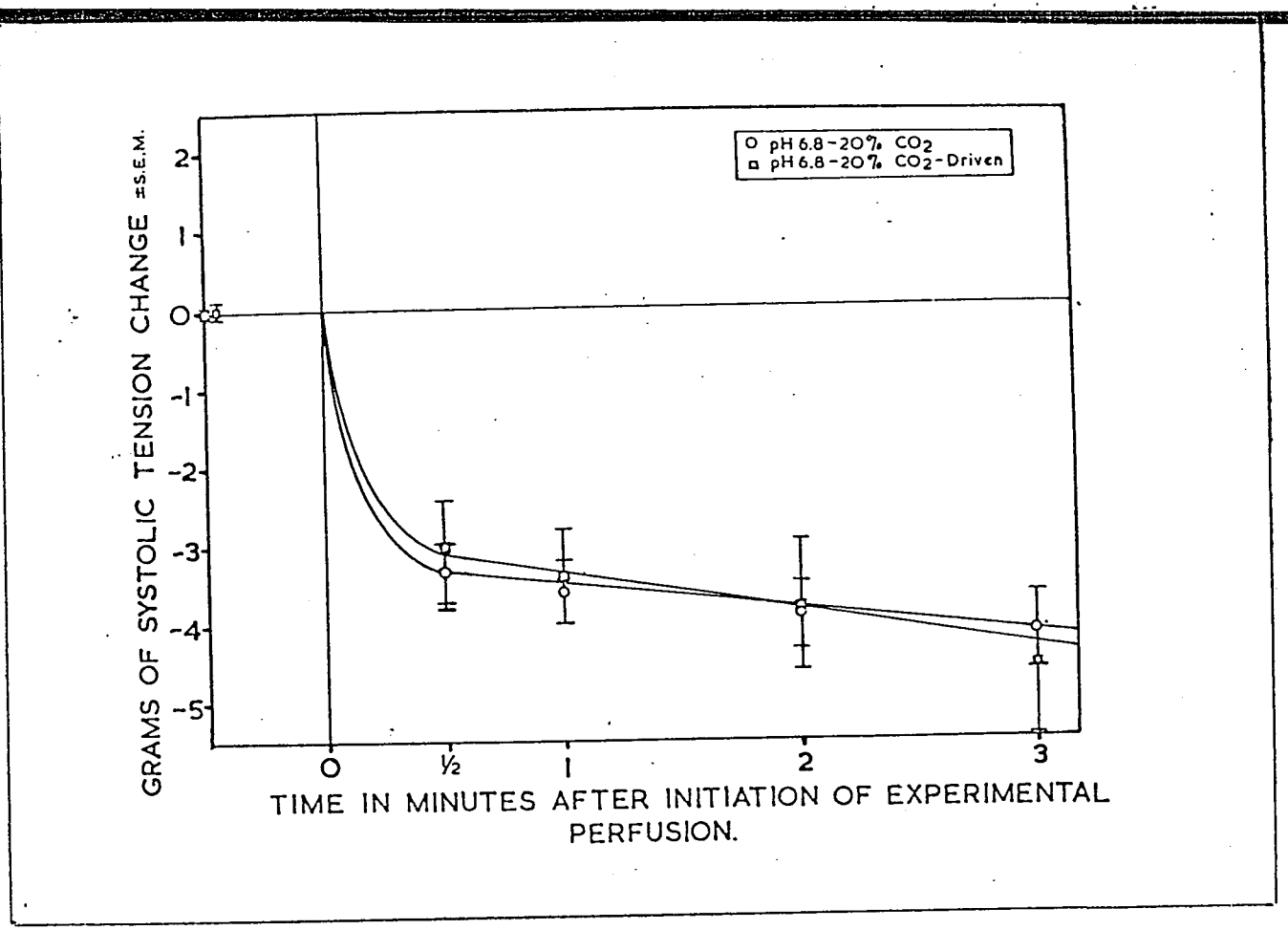

FIGURE 43. A Statistical Analysis of the Effect of Constant Rate on the Negative Inotropic Response to 80% O_2-20% CO_2

Mean control tensions: pH 6.8-20% CO_2, 18.02 g; pH 6.8-20% CO_2-Driven, 25.97 g.

The possible inter-relationship of increased coronary resistance observed with 80% O_2 - 20% CO_2 and the concurrent negative chronotropic effect. A decrease in flow rate was observed with 80% O_2 - 20% CO_2 and is graphed for all experiments in Figure 44. The net 1/2-time for the flow rate change was 43 seconds. This is much longer than the 1/2-time for the tension change, 2 seconds, as shown in Figure 14.

A second bit of evidence against the hypothesis that the change in flow rate was responsible for the change in tension can be found in Experiment 29 b where flow rate was manually maintained constant at 3.08 ml/minute while changing to the experimental perfusate. The same tension response was produced despite the flow rate's constancy.

The effect of graded pCO_2 changes on systolic tension. From the evidence presented, it seems that the increase in CO_2 from 5% to 20% was responsible for the negative inotropic effect which occurs quickly and which is significant and reproducible. It was thought that this indicated a very precise control on contractility and that intermediate values for CO_2 content might produce reproducible, intermediate decreases in contractility. To test this hypothesis, gas mixtures were constituted in a spirometer with oxygen kept constant at 80%. CO_2 usually was varied

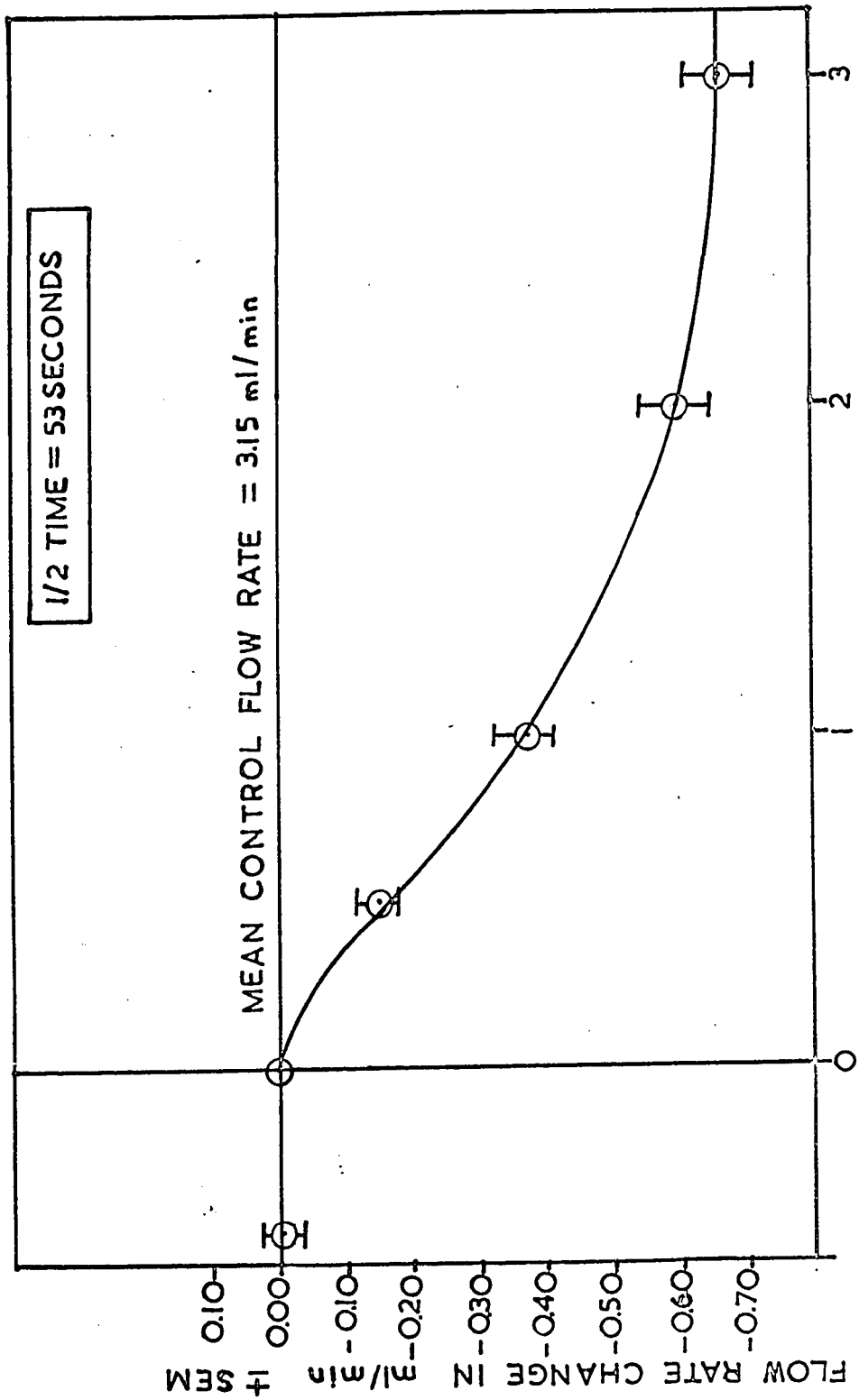

FIGURE 44. A Statistical Analysis of the Effect of 80% O_2-20% CO_2 on Coronary Flow Rate

in 2% steps, and N_2 was used to fill in the remaining volume. The perfusates were bubbled for ten minutes prior to experimental perfusion. Ten minutes had been found, by pH determinations, to be adequate to achieve equilibrium solution of gases. Figure 45 is a graph of the responses to and the recoveries from such a series of changes in CO_2 concentration as observed in Experiment 40. Due to the large number of experimental changes, the graph has been divided into three smaller graphs to keep the changes well separated and distinct. Each tension curve is labelled as to the gases bubbling the perfusate in the following sequence, O_2 - CO_2 - N_2. The last curve from the preceding graph is repeated in the following graph to serve as a reference point permitting continuity of interpretation. The initial recovery point is not necessarily coincident with the last point showing the experimental effect, and this lack of coincidence can be readily seen on some curves. Four minutes was considered to be adequate to demonstrate all of the important changes.

It can be seen from these graphs that there is a progressive increase in positive inotropic response as the CO_2 is increased from 1% to 6%; then there is a gradually decreasing inotropic effect as the percentage of CO_2 is increased from 8% to 20% which becomes negative at 12% CO_2.

Figure 46 shows the effect of, and recovery from, 80% O_2 - 16% CO_2 - 4% N_2 in two successive changes within

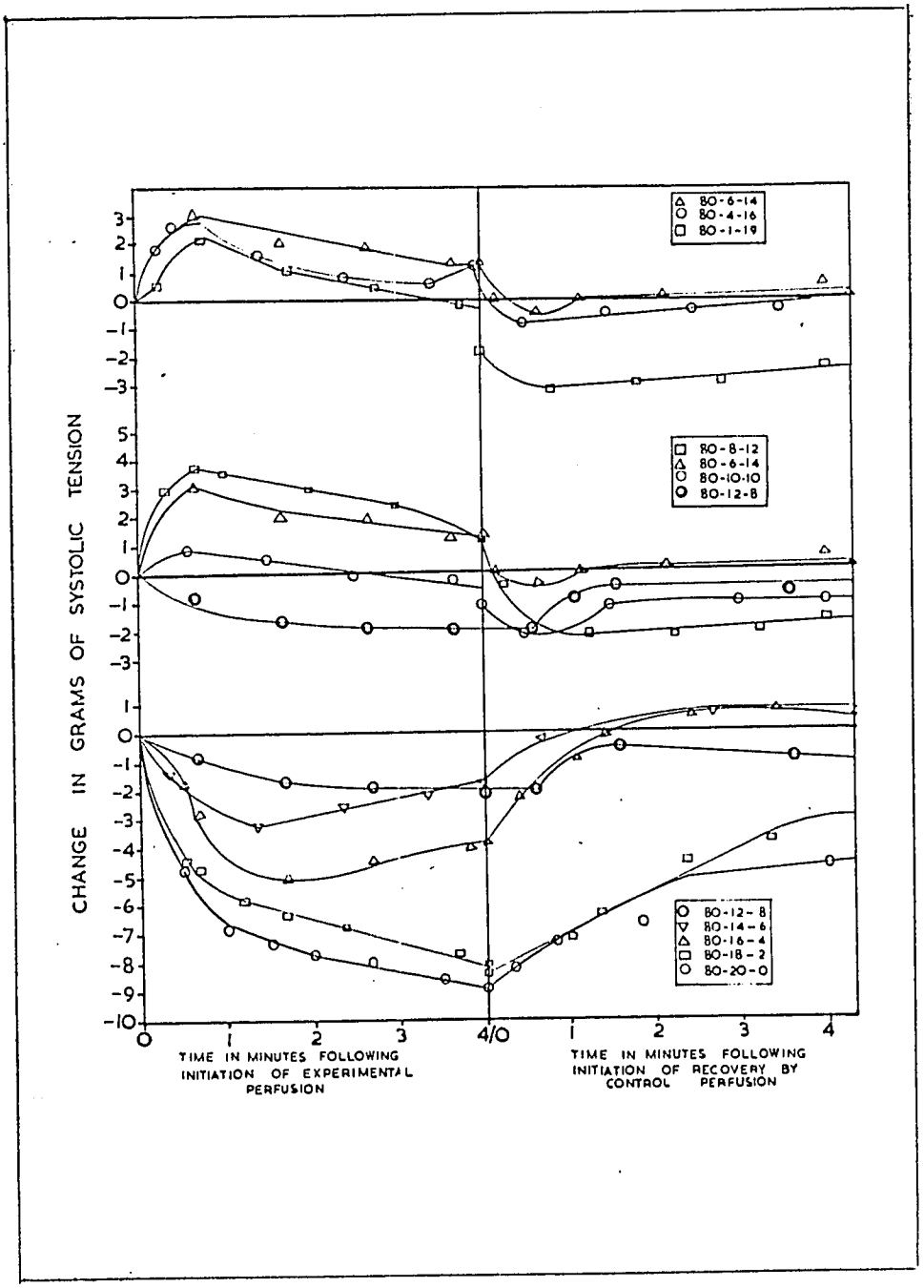

FIGURE 45. Systolic Tension Changes and Recoveries with Stepwise CO_2 Increases

The first number of each sequence labelling a curve, i.e., 80-6-14, refers to the percentage of oxygen, the second to carbon dioxide, and the third to nitrogen. Control systolic tension was kept approximately constant at 90 g. Experiment 40.

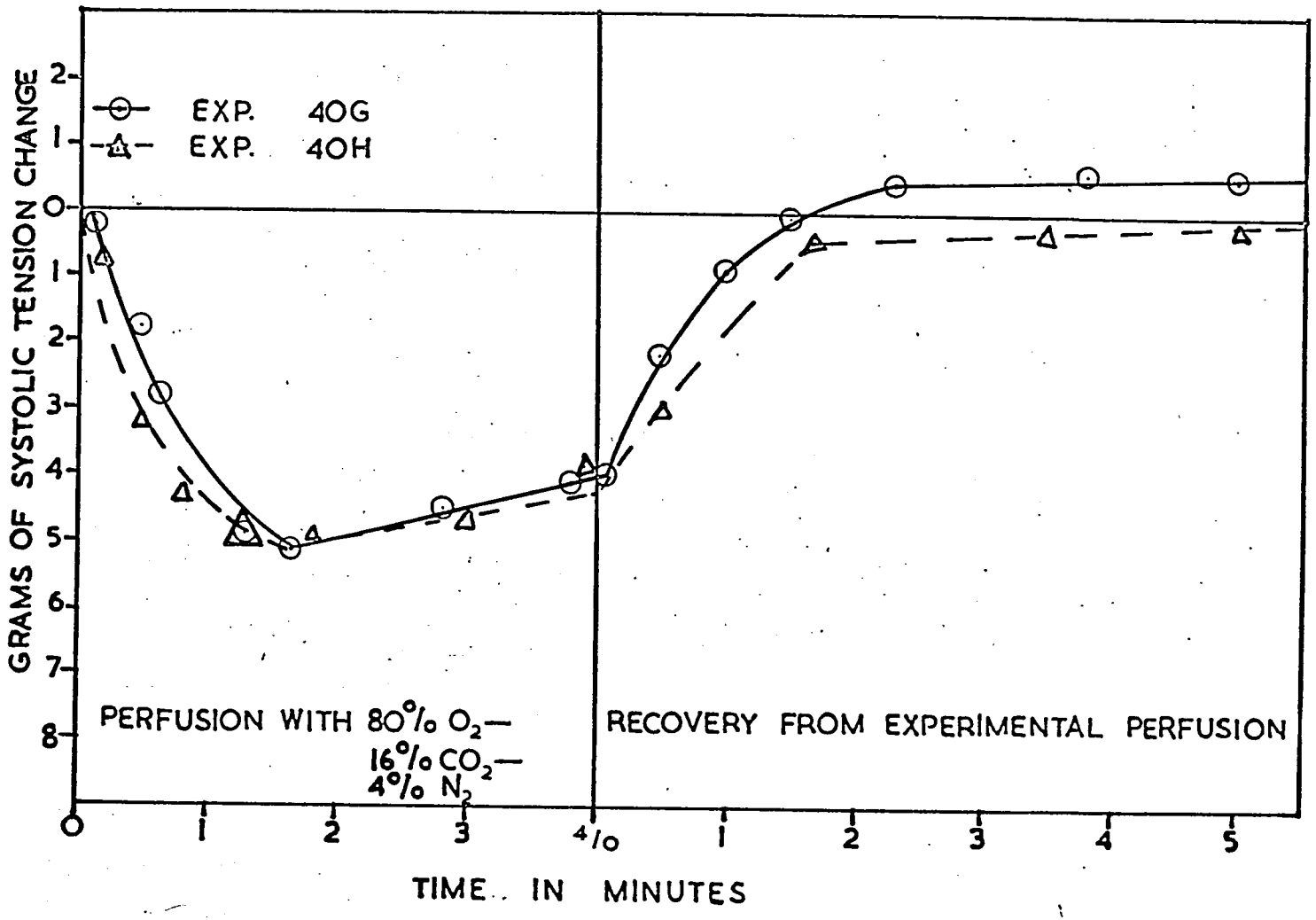

FIGURE 46. An Example of the Reproducibility of the Tension Effect Resulting from an Intermediate CO_2 Concentration

Control tensions: Experiment 40 G, 13.1 g; Experiment 40 H, 13.9 g.

Experiment 40. This is presented as evidence that these changes are reproducible.

In Figure 45 it can be seen that the approximate time of maximum tension change is about 1/2 minute following the onset of experimental perfusion. Figure 47 shows a comparison of the grams of tension change at 1/2 minute for two experiments. This again indicates that the effect of graded pCO_2 changes on tension are reproducible. The dotted line on this figure is a mean value for the positive inotropic effect of decreased pO_2 obtained by interpolating between the values for tension change with 4% and 6% CO_2 to estimate the effect of 5% CO_2 in each experiment. This should equal the effect of 80% O_2 - 5% CO_2 - 15% N_2 and should be equal to the effect of changing pO_2 alone. This represents the amount of positive inotropic effect which is imposed on all of the experiments in this series as a result of reduced pO_2. This establishes a new baseline at the level of the dotted line, and any significant decrease from this level of systolic tension represents a negative inotropic effect of CO_2 in excess of the positive inotropic effect of reduced pO_2. It can be seen that at approximately 10% CO_2, the effect of increased CO_2 causes a net negative inotropic effect.

The sequence of presentation of gas mixtures to the isolated heart was not a determining factor. Experiment 41 was done with the order of presentation exactly reversed

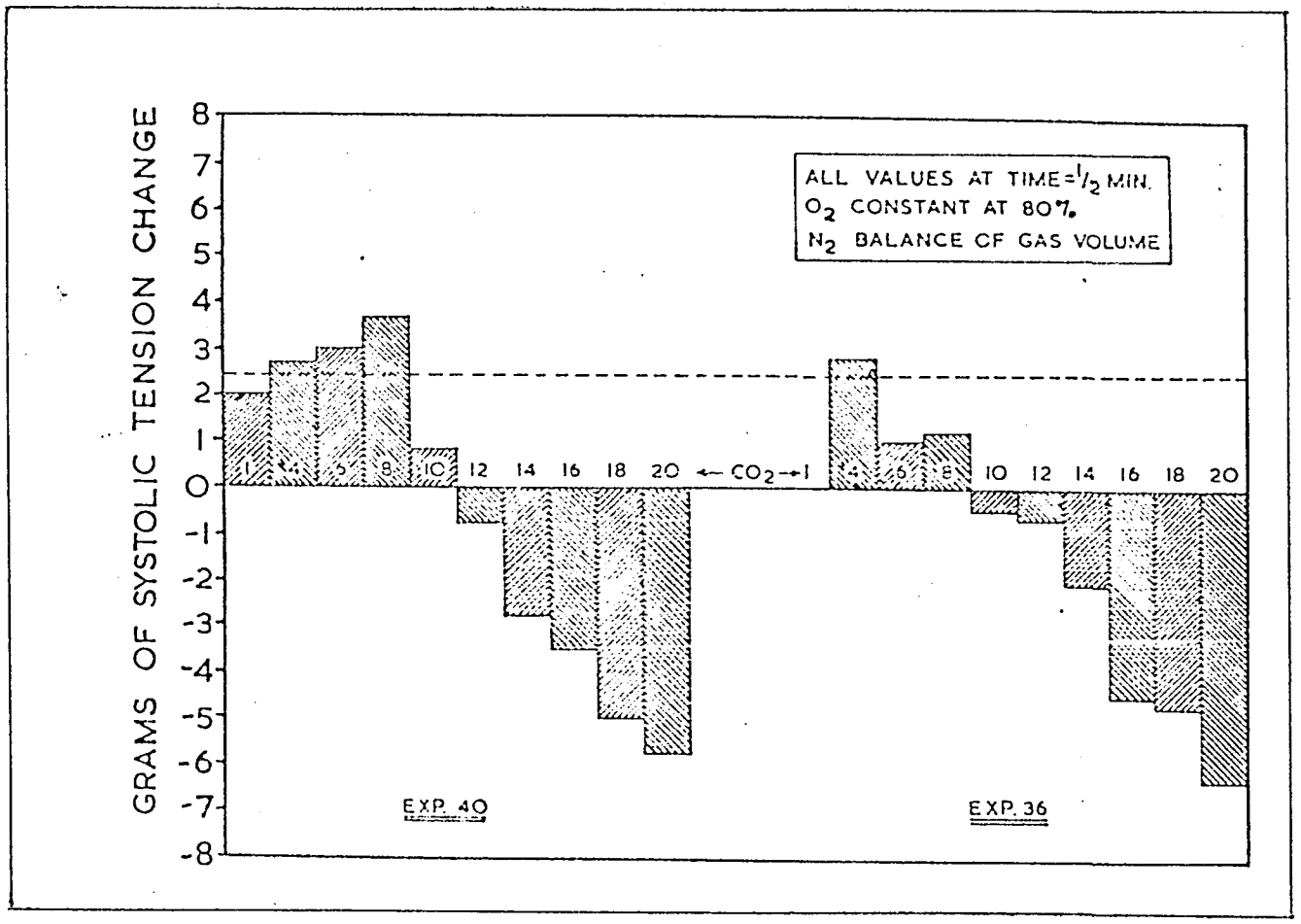

FIGURE 47. The Graded Effect of CO_2 Concentration Changes on Systolic Tension

Control systolic tensions were approximately 11 g for Experiment 40, and approximately 17 g for Experiment 36.

from that of the two experiments in Figure 47, yet the tension changes are in the same direction and of the same magnitude.

III. THE EFFECT OF VARIOUS EXPERIMENTAL PERFUSATES ON HEART RATE

General. The scheme of presenting the results for the effects of perfusate change on heart rate will be the same as used for the tension results. The logic used in developing the experimental sequence, described in detail in the section on tension, will not be repeated here.

The typical response to a perfusate bubbled with 80% O_2 and 20% CO_2. The change from the control perfusate to an experimental perfusate bubbled with 80% O_2-20% CO_2 and containing 28 mM HCO_3 (pH 6.80) caused a decrease in the heart rate of the isolated preparation. This may be seen in a qualitative way by examining the record of a typical experiment in Figure 13. Figure 16 shows a graph of the total response for Experiment 37 b, the typical experiment. The experimental perfusate caused a 31.70% decrease in rate from the control level.

The statistical analysis of the response by all of the experimental hearts is shown in Figure 48 as the curve labelled pH 6.80, 20% CO_2. These results are also summarized in Table 9. When both the graph and Table 9 are examined, it can be seen that the decrease in rate is very significant at all of the times analyzed. When the decreased rate had reached an equilibrium level, the rate

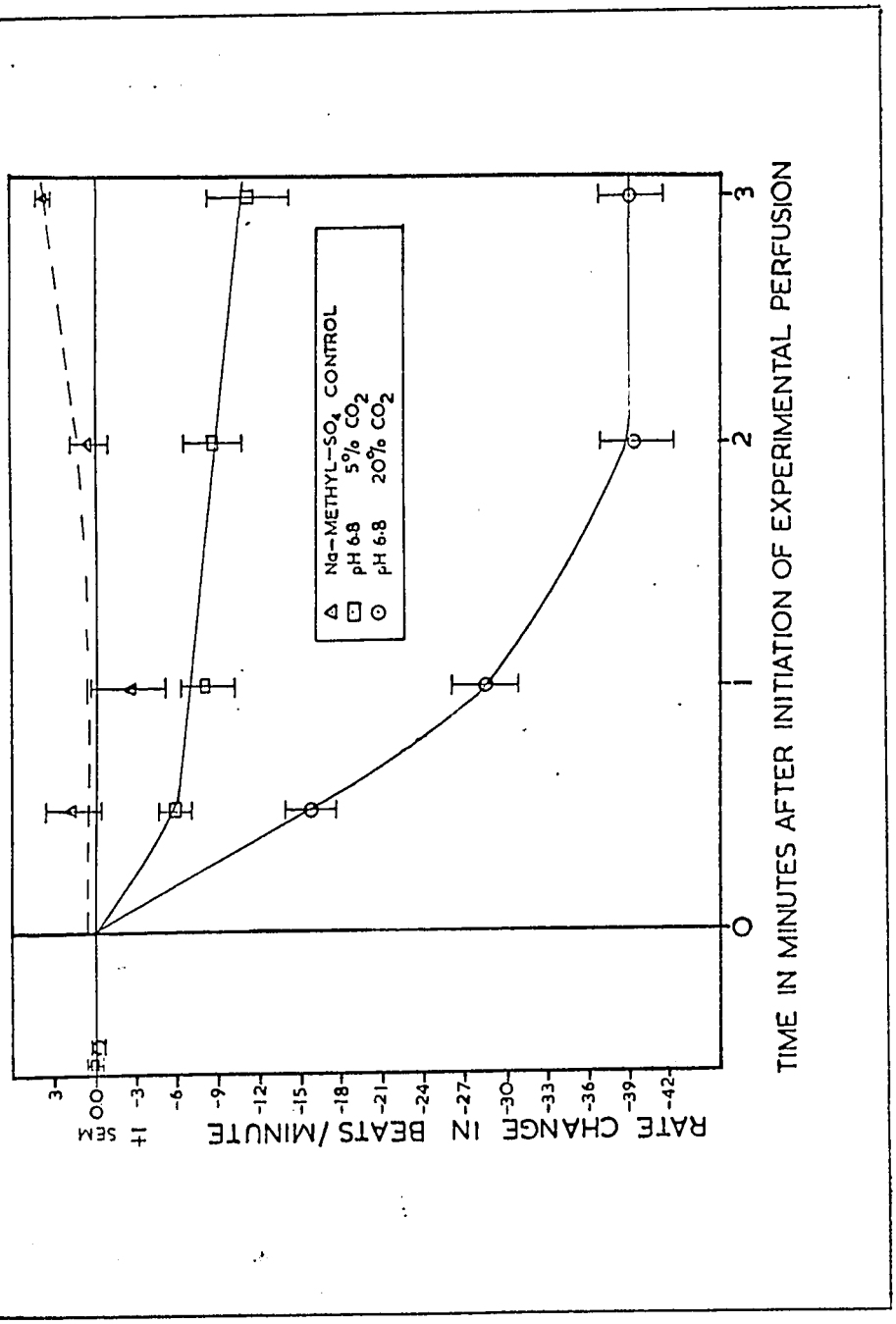

FIGURE 48. A Statistical Analysis of the Effect of Three Experimental Perfusates on Heart Rate

Mean control rates in beats/minute were: $Na\text{-}CH_3\text{-}SO_4$ control, 200; pH 6.8-5% CO_2, 201; pH 6.8-20% CO_2, 203.

TABLE 9. Summary of Heart Rate Changes with Various Experimental Perfusates

Number of Hearts	Time of Determination	Mean in Beats/min.	± Standard Deviation	± Standard Error	p	Percent Change
		80% O_2-20% CO_2, pH 6.80				
64	0 min.	202.9				Control
64	1/2 "	-15.9	14.2	1.8	<0.001	-7.81
64	1 "	-28.7	18.9	2.4	<0.001	-14.14
64	2 "	-39.6	20.7	2.6	<0.001	-19.51
60	3 "	-39.2	19.0	2.4	<0.001	-19.31
		95% O_2-5% CO_2, pH 6.80				
25	0 min.	201.3				Control
25	1/2 "	-5.9	6.1	1.2	<0.001	-2.92
24	1 "	-8.4	9.4	1.9	<0.001	-4.16
23	2 "	-8.7	10.7	2.2	<0.001	-4.30
20	3 "	-11.2	14.1	3.2	<0.005	-5.54
		80% O_2-5% CO_2-15% N_2, pH 7.40				
9	0 min.	191.6				Control
9	1/2 "	-2.6	2.6	0.9	<0.025	-1.33
9	1 "	-3.3	4.8	1.6	>0.050	-1.74
9	2 "	-4.3	5.9	2.0	>0.050	-2.26
9	3 "	-2.4	8.2	2.8	0.400	-1.27

had decreased by approximately 19% from the mean control rate of 203 beats per minute. The typical response of a 31.70% decrease is within one standard deviation of the population's mean decrease.

Recovery from the effects of perfusion with 80% O_2 and 20% CO_2. The return to the control perfusate bubbled with 95% O_2-5% CO_2 (pH 7.40) caused a return to the former control frequency of contraction. This is indicated by inspection of the record from a typical experiment as shown in Figure 23. Figure 26 shows the complete recovery made by heart rate when the complete results from the typical experiment are plotted. The 1/2-times of experimental response and recovery, 50 seconds and 44.5 seconds respectively, are not appreciably different.

The statistical analysis of the recovery by all the hearts is shown in Figure 49. The 0 point is the mean heart rate at the end of all the experimental changes to 80% O_2-20% CO_2. The response by the population of hearts also indicates that the heart recovers completely from the effects of the experimental perfusion.

General questions relative to the performance of the preparation. The effect of the pre-perfusion protocol on the negative chronotropic effect of 80% O_2-20% CO_2 was

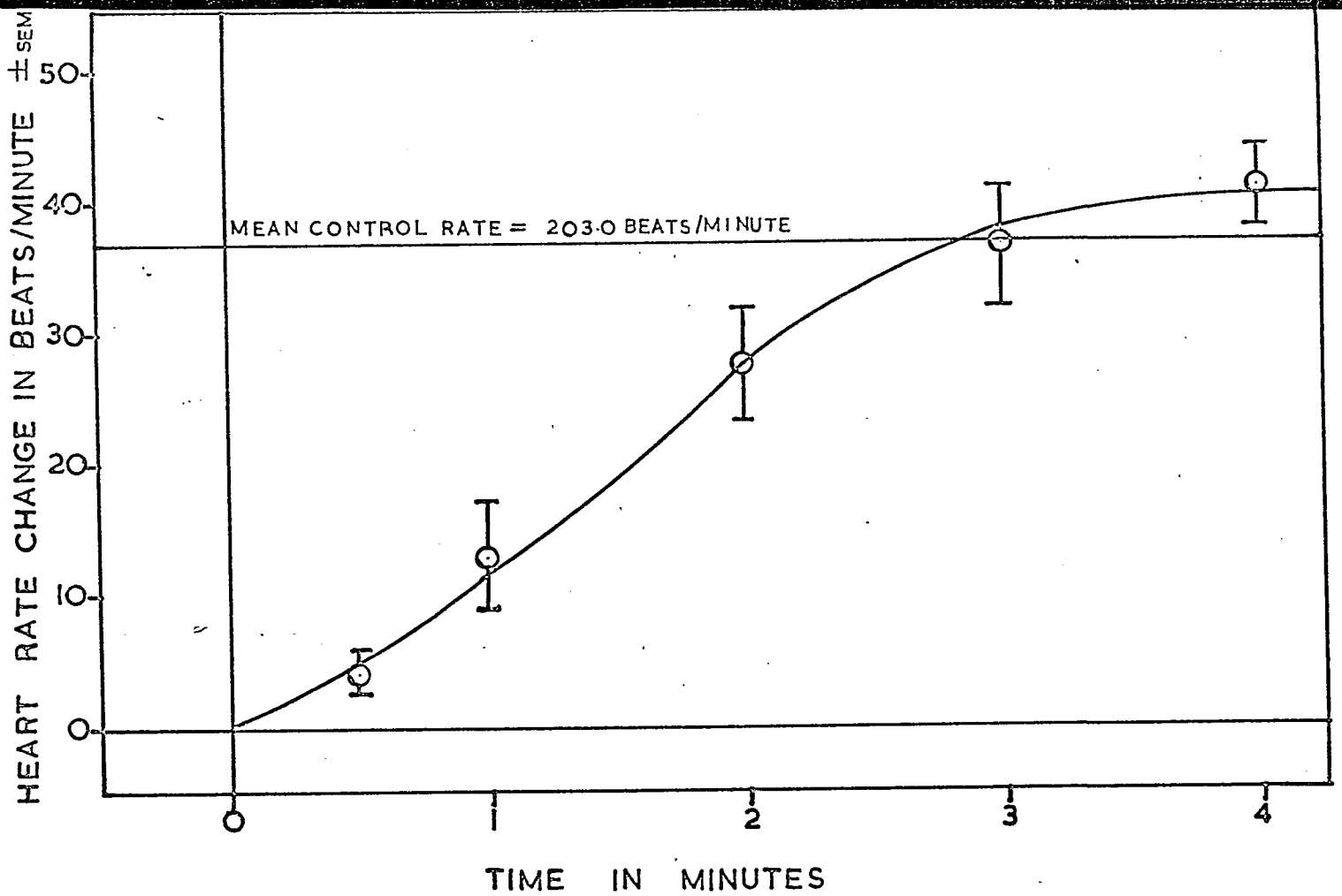

FIGURE 49. A Statistical Analysis of the Recovery of Heart Rate from the Effects of 80% O_2-20% CO_2

Mean control rate is derived from the control period preceding each of the experimental perfusions analyzed. 0 heart rate change is the mean of the heart rates at the end of the perfusion periods.

analyzed and is shown in Figure 50. The effect of the experimental perfusion on the rate response by the heart from a rat stunned by a blow on the head, is not significantly different from that of the grouped responses of those hearts undergoing the total pre-perfusion protocol.

The effect of reduced temperature, as used in the routine preparations on the negative chronotropic effect of 80% O_2 and 20% CO_2, was checked by effecting the experimental changes at 37 C. Since the control heart rate was higher than at 32 C, the percentage decrease in rate was calculated. The percentage decreases for 1/2, 2, and 3 minutes were 10.04%, 23.91%, and 25.76% respectively. This agrees well with the values listed in Table 9 for the usual response to the perfusion with 80% O_2-20% CO_2 at 32 C.

The effect of decreased pH versus increased pCO_2 on the characteristic negative chronotropic effect observed with 80% O_2-20% CO_2. Figure 31 compares the recorded effect of decreased pH versus increased pCO_2 on heart function in a typical experiment. Rate appears to remain constant when the pH is decreased without a simultaneous increase in pCO_2. Figure 33 shows that complete analysis of the results reveals that heart rate does decrease by 2.5% for approximately 1.5 minutes and then returns to its former control level.

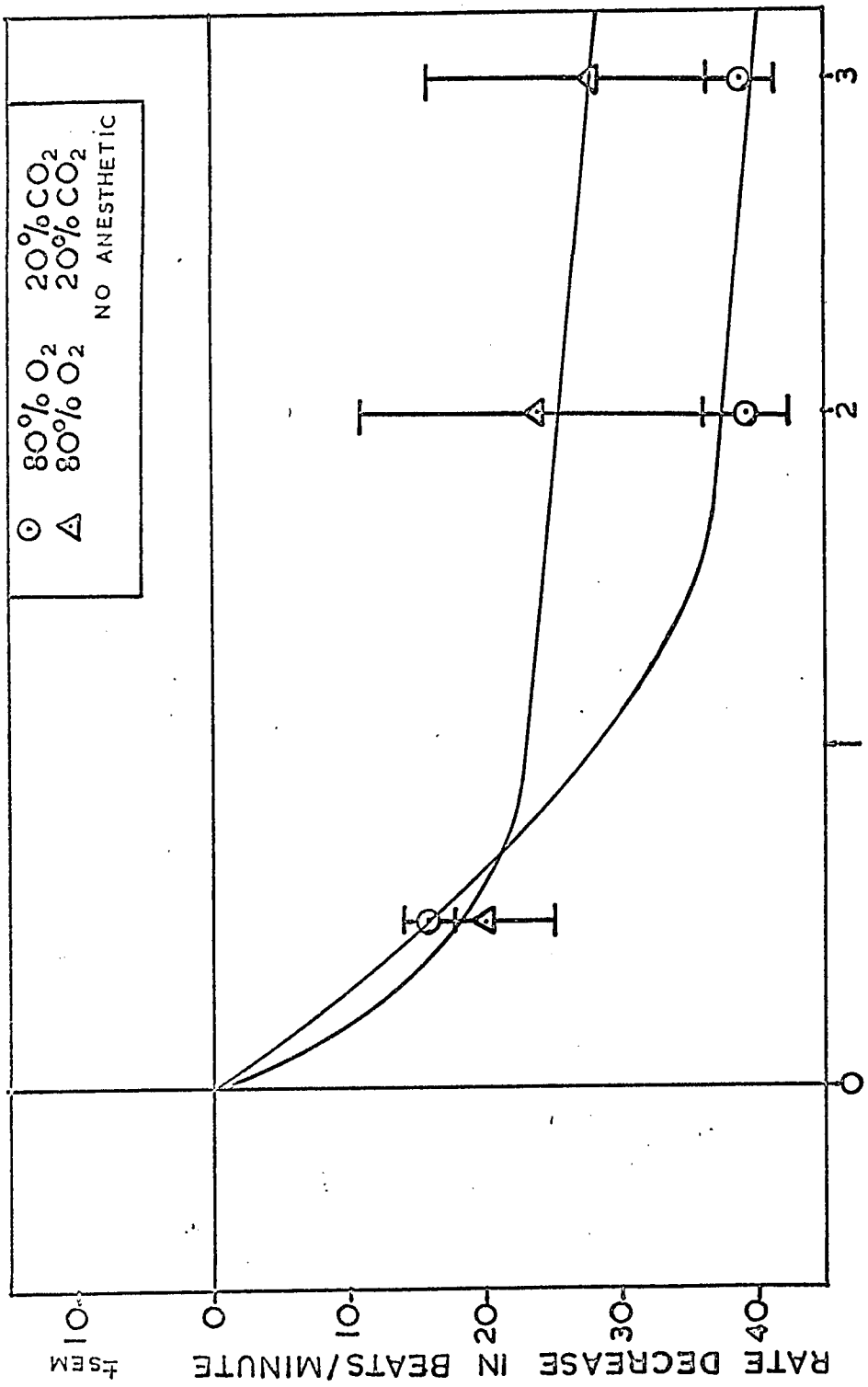

FIGURE 50. The Effect of Heparin, Cold Saline and Anesthesia on the Negative Chronotropic Effect of 80% O_2-20% CO_2

Mean control rates were 231 and 203 beats/minute for the non-anesthetized and anesthetized rats respectively.

The response of the heart population to the effect of pH change alone is graphed in Figure 48. There is a decrease in rate which is significant at all times considered. This also can be seen from the data in Table 9. Figure 48 indicates that pH and CO_2 have negative chronotropic effects of different magnitudes on heart rate, with the major depressive effect displayed by 80% O_2-20% CO_2. Sodium methyl sulfate does not have a significant effect on rate as shown by the curve of Figure 48.

The effect of decreased pO_2 on the negative chronotropic effect of 80% O_2-20% CO_2. Table 9 describes the effect of decreasing the concentration of oxygen only, by using an 80% O_2-5% CO_2-15% N_2 gas mixture to bubble the perfusate. The decrease in rate reaches a maximum of 2.26% which is non-significant. The 1/2 minute value reaches the level of significance, but the other values are non-significant. These changes may be considered non-significant when compared with the much larger effect of 80% O_2-20% CO_2.

The relationship of control tension to heart rate. The possible relationship between control resting (diastolic) tension to heart rate was checked by increasing the diastolic tension and comparing the heart rate, after the

increase, with the heart rate before the increase. The tension increases were then grouped according to resting tension and according to the amount of increase in tension. A statistical evaluation of the changes showed that the changes in rate were non-significant in every case. The largest mean change was approximately 2%.

The possible inter-relationship of coronary resistance and the negative chronotropic effect. A possible relationship between the decreased coronary flow rate and the decreased heart rate is indicated by the similarity of the 1/2-times for the two responses following the initiation of experimental perfusion with 80% O_2-20% CO_2. Figure 44 shows a net 1/2-time for the flow rate change of 43 seconds, and Figure 48 shows a net 1/2-time for the rate change of 27 seconds. A more complete analysis will be made following examination of coronary resistance results.

Another bit of evidence indicating that a relationship between rate and flow rate may exist was gained by examining the mean heart rate at different control flow rates. Those hearts subjected to mean control flow rates of 0.50 to 3.49 ml/min had a mean heart rate of 172.6 beats per minute. Those with flow rates of 3.50 to 4.49 ml/min had a mean heart rate of 219.2 beats per minute. However, those hearts perfused at flow rates of 4.50 to 9.49 ml/min had an

equivalent mean heart rate of 820.7 per minute. Individual experiments at different flow rates on the same heart showed that increasing the flow rate caused an increase in rate. This was done in Experiments 14 and 19. Therefore, it appears that there is an optimal rate of perfusion and decreases below that rate will result in decreased heart rate.

The effect of graded pCO_2 changes on heart rate. Figure 51 illustrates that a fine control is exerted on heart rate by stepwise increases in the concentration of carbon dioxide. This effect of CO_2 on rate appears to be a linear one. However, decreasing the CO_2 concentration from the control value causes an increased heart rate.

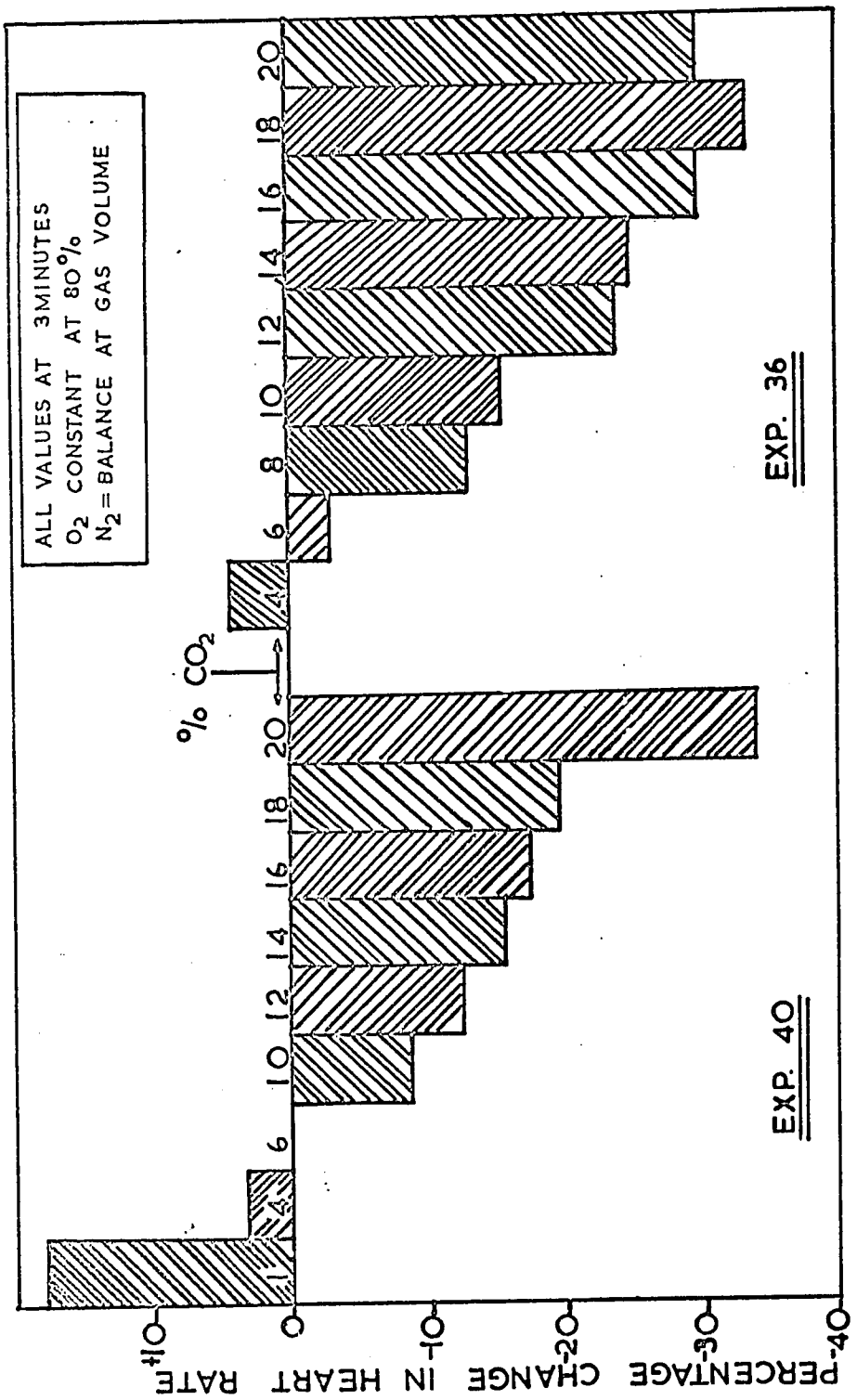

FIGURE 51. The Effect of Stepwise CO_2 Changes on Heart Rate

IV. THE EFFECT OF VARIOUS EXPERIMENTAL PERFUSATES ON CORONARY RESISTANCE

General. As previously discussed, changes in coronary resistance are indicated by changes in flow rate and changes in perfusion pressure. Changes in either of these two variables give an indication of resistance changes. The evidence from the records will be observable as changes in perfusion pressure. The information from the manually recorded flow rate changes will be demonstrated and incorporated into a single datum, the coronary resistance.

The units used in all cases are dynes-sec-cm^{-5} x 10^{4}. For the sake of simplicity these units will not be written out, but will be referred to as resistance units.

The sequence of result presentation will be the same as used for tension and heart rate.

The typical response to a perfusate bubbled with 80% O_2 and 20% CO_2. The change from the control perfusate to an experimental perfusate bubbled with 80% O_2-20% CO_2 and containing 28 mEq HCO_3^- (pH 6.80) caused a biphasic pressure response. This is very evident in Figure 13. First there was a decrease in perfusion pressure which was of short duration, followed by a marked and persistent increase in pressure. The perfusion pressure rose to

levels well above the control perfusion pressure.

The term pulse pressure will be used in reference to the difference between systolic and diastolic pressure for the sake of brevity. Although the term as used to apply to this preparation has the same mathematical definition as its use in the whole organism, no claims are made that the two uses are synonymous.

From Figure 13 it should also be noted that the pulse pressure decreases to zero with the onset of the experimental perfusion.

Flow rate gives the response predicted from the pressure response. When the values for flow rate from Experiment 37 b were analyzed, flow rate had increased by 0.10 ml/min at 1/3 minute, but by 3 minutes, the flow had decreased by 1.20 ml/min. The control flow rate was 3.80 ml/min. Combining the pressure and flow data into a resistance analysis gives the results shown in Figure 15. This again shows a biphasic change in resistance which equilibrates at a coronary resistance much greater than the control resistance. Comparing the control diastolic resistance and the equilibrium diastolic resistance shows that there has been a 107.7% increase in coronary resistance. Figure 15 also shows that the resistance at systole and diastole are equal following experimental perfusion.

Diastolic coronary resistance will be used in the subsequent analyses since it gives much the same information

as systolic resistance due to the decrease of systolic resistance to the level of diastolic resistance. Furthermore, when the preparation is undergoing control perfusion, and a pulse pressure is present, the coronary resistance at diastole is not complicated by extramural pressure applied to the vessels by myocardial contractions.

The statistical analysis of the change in diastolic coronary resistance, following initiation of perfusion with 80% O_2-20% CO_2, is plotted in Figure 52. Here the pattern of change is approximately equivalent to that seen in the typical experiment with the exception that the initial fall in resistance is absent.

Table 10 demonstrates that all increases in diastolic coronary resistance are significant. The typical experiment increase of 140 units of resistance is within one standard deviation of the population response.

Recovery from the effects of 80% O_2 and 20% CO_2. Figure 23 indicates that perfusion pressure, hence coronary resistance, tends to return to the control level following initiation of control perfusion. When the data from Experiment 37 b, the typical experiment, is plotted as shown in Figure 25, it can be seen that coronary resistance recovers completely to its former control level. The 1/2-time for recovery is 20 seconds, and the 1/2-time for

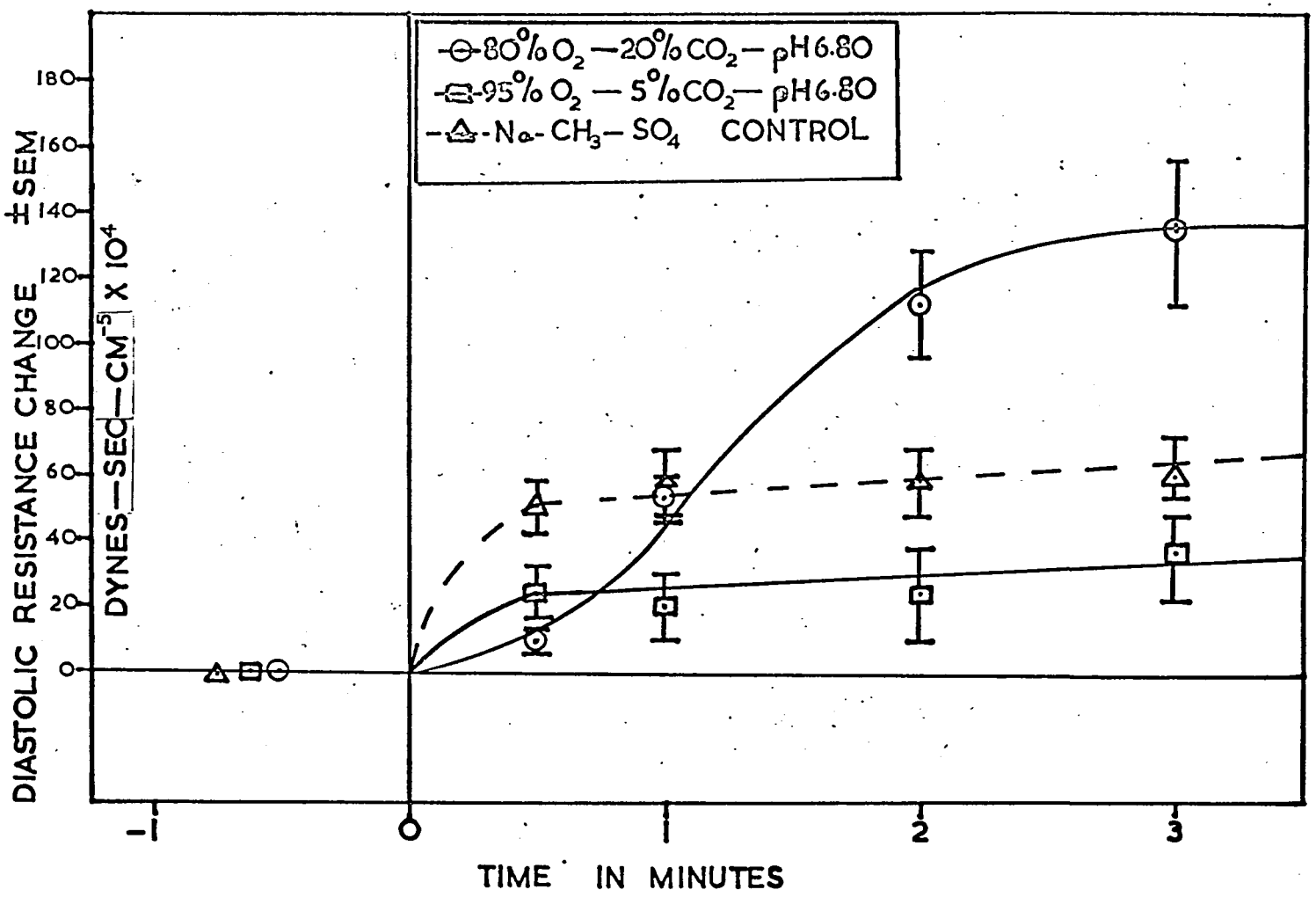

FIGURE 52. A Statistical Analysis of the Effect of Experimental Perfusates on Coronary Resistance

Mean control resistances were: 80% O_2-20% CO_2-pH 6.80, 191.37; 95% O_2-5% CO_2-pH 6.80, 199.14; Na-CH_3-SO_4 control, 126.62.

TABLE 10. Summary of Diastolic Resistance Changes with Various Experimental Perfusates

Number of Hearts	Time of Determination	Mean in $\frac{\text{dynes-sec}}{\text{cm}^5} \times 10^4$	± Standard Deviation	± Standard Error	p	Percent Change
		80% O_2-20% CO_2, pH 6.80				
60	0 min.	191.37				Control
60	1/2 "	+ 9.64	24.59	3.17	<0.005	+ 5.04
60	1 "	+ 53.43	57.34	7.40	<0.001	+27.92
59	2 "	+112.22	127.79	16.63	<0.001	+ 58.64
55	3 "	+134.45	176.25	23.77	<0.001	+ 70.26
		95% O_2-5% CO_2, pH 6.80				
26	0 min.	199.14				Control
26	1/2 "	+ 23.08	36.96	7.25	<0.005	+11.59
26	1 "	+ 19.66	47.69	9.35	<0.050	+ 9.87
26	2 "	+ 23.75	72.96	14.31	>0.100	+11.92
24	3 "	+ 37.08	85.60	17.47	<0.050	+18.62
		Methyl sulfate control, pH 7.40				
6	0 min.	126.62				Control
6	1/2 "	+ 51.03	20.33	8.26	<0.005	+ 40.31
6	1 "	+ 56.85	25.08	10.24	<0.005	+ 44.90
6	2 "	+ 58.55	26.41	10.78	<0.005	+ 46.24
6	3 "	+ 60.30	29.49	12.04	<0.005	+ 47.62

the resistance increase is 75 seconds. Also, it should be noted that the presence of a pulse pressure returns with the return to control resistance levels.

When the recovery of the grouped hearts is statistically evaluated, the mean diastolic coronary resistance is 198.73 units at 4 minutes following initiation of recovery. This is in good agreement with the mean diastolic resistance of 191.37 for the control period preceding the experimental perfusion. As in the typical experiment, recovery takes longer than the onset of the effect of experimental perfusion.

General questions relative to the performance of the preparation. The effect of the pre-perfusion protocol on the coronary resistance response to perfusion with 80% O_2 and 20% CO_2 was analyzed as described for the other variables. The results for the unanesthetized animal were of the same biphasic type as those with the anesthetized animals. Again using diastolic resistance for comparison, the equilibrium value at 3 minutes for the stunned animal was an increase of 38.50% above the control value. The grouped hearts had increased 70.26% at 2 minutes. One standard deviation includes the response by the stunned animals.

At a temperature of 37 C, the response to experimental perfusion with 80% O_2-20% CO_2 was again a biphasic

resistance change. The mean increase in diastolic resistance at 3 minutes was 27.30%.

Therefore, the pre-perfusion protocol did not alter the resistance response to perfusion with 80% O_2-20% CO_2.

The effect of decreased pH versus increased pCO_2 on the characteristic coronary resistance response. The effect of decreasing pH only, as compared with decreasing pH and simultaneously increasing CO_2 concentration, is shown in Figure 31. The perfusate with both the high concentration of CO_2 and the low pH caused the already characterized marked increase in coronary resistance. However, the perfusate with the pH decrease alone caused no marked change in pressure, hence no change in coronary resistance. The resistance change following perfusion with decreased pH alone is graphed in Figure 33. The diastolic coronary resistance had increased by 14.20% from the control value at 3 minutes. The response to a decrease in pH with a simultaneous increase in pCO_2, as displayed by the left portion of Figure 31, caused a 96.70% increase in coronary resistance. Both perfusions were done on the same heart.

The response of the coronary circulation to a change in perfusate pH alone is indicated in Figure 32 by the curve labelled 95% O_2-5% CO_2, pH 6.80. The grouped hearts

responded with an increase in coronary resistance which was significantly different from the control resistance. This is shown in Table 10 also. However, when control experiments were done to determine the effect of sodium methyl sulfate alone, they showed a significant increase in resistance (Figure 52 and Table 10).

Since the curves for the effect of pH change alone and for the effect of methyl sulfate control were not very far apart, it was considered necessary to analyze the two groups of results to check the possibility that they were not significantly different from each other. This analysis was carried out according to the method for comparing two groups of unequal size (SNEDECOR, 1962). The response to methyl sulfate was not significantly different from the response with the pH change alone ($p = 0.50$). Therefore, pH change alone caused no significant change in coronary resistance.

The effect of decreased pO_2 on the coronary resistance response of 80% O_2-20% CO_2. Perfusion with 80% O_2-5% CO_2-15% N_2 saturated saline caused no significant change in coronary resistance. In seven such control experiments, there was a decrease in coronary resistance which did not quite reach the level of significance.

The relationship of control tension to coronary resistance. Experiments were done wherein the diastolic tension was increased and the resistance change was observed. The experiments were grouped according to the tension increases and analyzed statistically. For increases in tension of 1-15 grams, the resistance change was non-significant.

The effect of rate changes on coronary resistance. The possible inter-relationship between the negative chronotropic effect of 80% O_2-20% CO_2 and the concurrent characteristic increase in coronary resistance was analyzed by driving a group of hearts at a constant rate while undergoing experimental perfusion. The rate used was just slightly greater than the normal rate, in order to keep conditions as near to control levels as possible. The pattern of coronary resistance change was the same as with the non-driven hearts.

The effect of graded pCO_2 changes on coronary resistance. Figure 53 shows the effect of progressively increasing the pCO_2 on diastolic coronary resistance. Since it had been demonstrated previously that the coronary resistance changes had reached equilibrium at 3 minutes, the changes analyzed were those at 3 minutes following initiation of

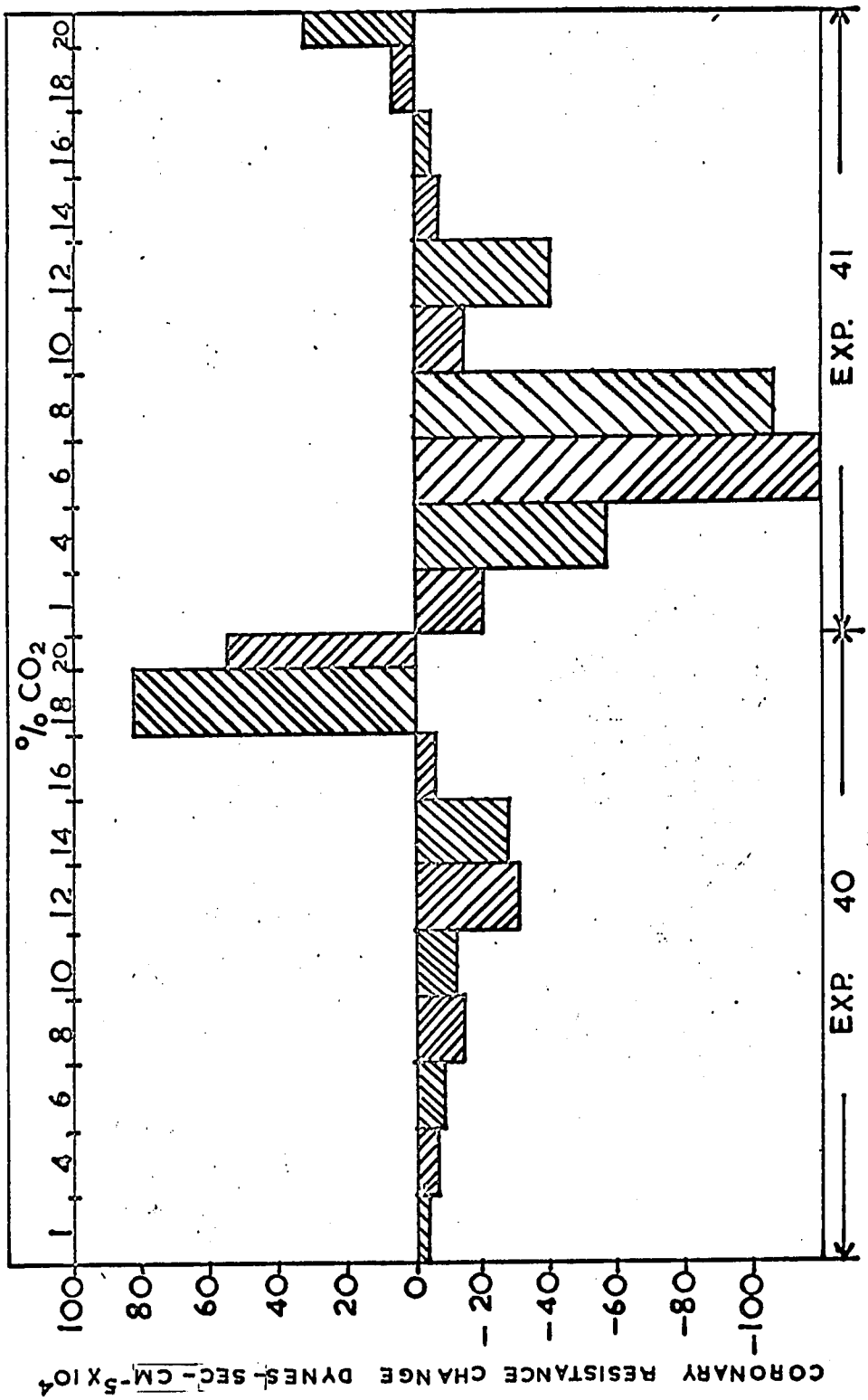

FIGURE 53. The Effect of Stepwise CO_2 Changes on Coronary Resistance

All values taken at 3 minutes following initiation of experimental perfusion.

experimental perfusion. Experiment 40 was done with a sequence of decreasing pCO_2 values, whereas Experiment 41 followed a sequence of increasing pCO_2 concentrations. Up to a point, increasing CO_2 concentration reduces coronary resistance; beyond that point, further increases in CO_2 concentration increase coronary resistance.

V. THE EFFECT OF VARIOUS EXPERIMENTAL PERFUSATES ON HYDROGEN AND BICARBONATE ION EXCHANGE

Hydrogen ion exchange. The mean pH of the perfusates bubbled with 95% O_2-5% CO_2, 28 mEq bicarbonate was 7.3698 ±0.0041 (SEM) from 59 determinations. The mean pH of the 80% O_2-20% CO_2 bubbled perfusate with 28 mEq bicarbonate was 6.8381 ±0.0046 (SEM) from 52 determinations. The mean pH of the 95% O_2-5% CO_2 bubbled saline with 7 mEq bicarbonate was 6.7978 ±0.0036 (SEM) from 37 determinations. The saline used for control determinations of the effect of sodium methyl sulfate replacing an equivalent amount of sodium chloride had a mean pH of 7.3867 ±0.0030 (SEM). The perfusate pH values taken at the beginning of the experiment were compared statistically with the perfusate pH values from the end of the experiment and were found to be statistically equivalent in all cases.

When the hearts were perfused with the control saline bubbled by 95% O_2-5% CO_2, the effluent from the isolated hearts had a mean pH difference of -0.0803 ±0.0081 (SEM) from the perfusate pH as determined by 56 experiments. This pH difference between the effluent and the perfusate was statistically very significant. This indicates a constant loss of acidic substances by the heart or the loss of bases from the saline.

Perfusing the hearts with perfusates bubbled by 80% O_2 and 20% CO_2 and containing 28 mM bicarbonate caused

the effluent to reach pH values which were greater than those of the perfusate. The time course of the mean pH changes is plotted in Figure 54. The pH values were grouped according to their time of occurrence. For the first minute following initiation of perfusion, 30 second intervals were used, and for all other times, one minute intervals were used. The pH values were analyzed statistically and plotted at the midpoint of their time intervals. The 0.00 pH line represents the mean experimental perfusate pH values which were defined above. All mean pH differences between effluent and perfusate were statistically significant. This difference between effluent and perfusate pHs was observed for as long as nine minutes following onset of experimental perfusion. The consistently higher effluent pH during perfusion with 80% O_2-20% CO_2, indicates that the heart was constantly losing basic substances, or that the saline was constantly losing acidic substances.

Perfusion of hearts with saline containing 7 mEq bicarbonate bubbled by 95% O_2-5% CO_2 caused the effluent to reach a level which was consistently and significantly below the pH of the perfusate (Figure 54). After 5 minutes the effluent pH apparently approached the perfusate pH. This last point is the mean of only two determinations, however. These pH determinations indicate that with this perfusate the heart loses acidic substances, or the perfusate loses basic substances. It should be noted that

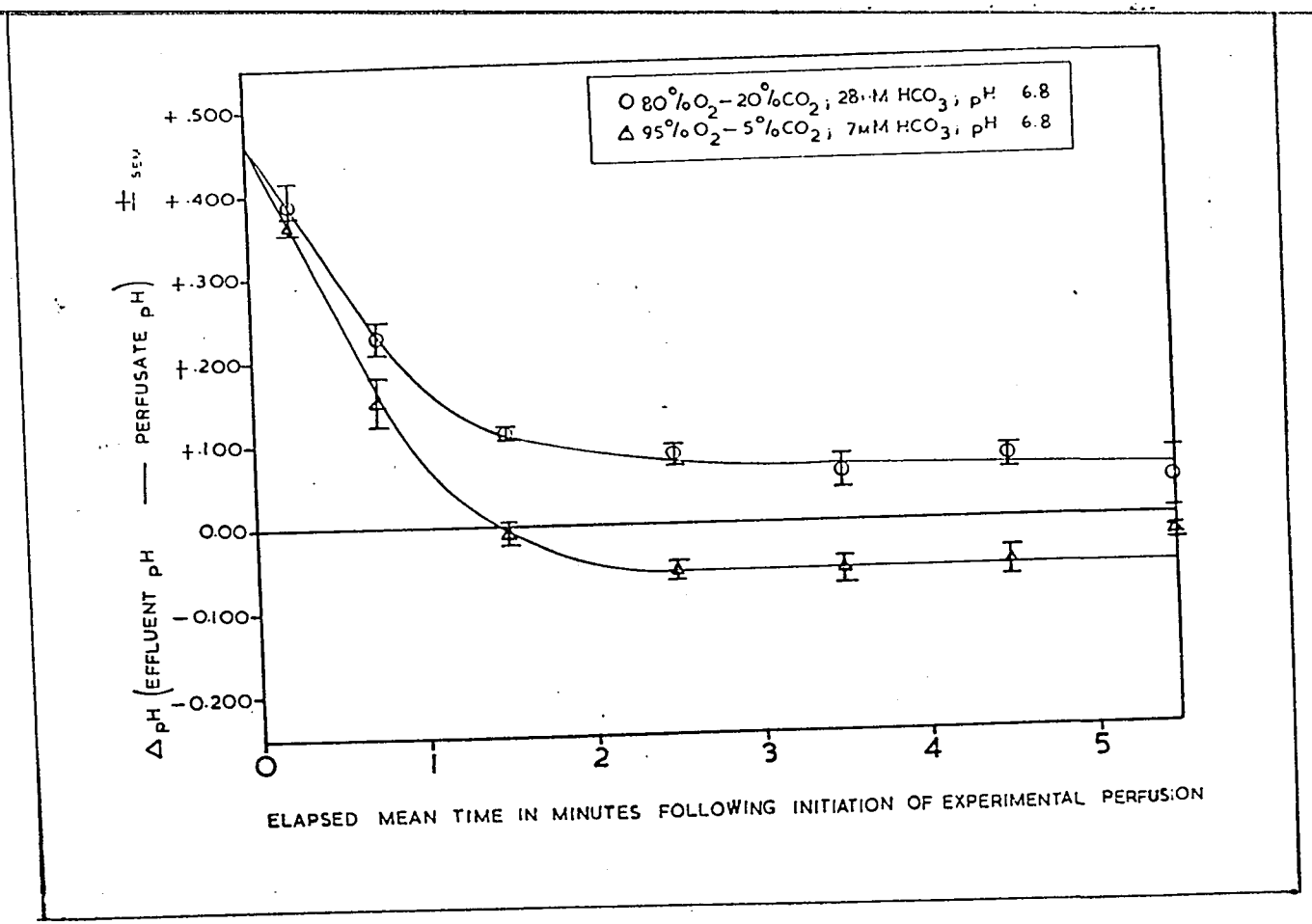

FIGURE 54. The Effect of Experimental Perfusates on the Perfusate-Effluent pH Difference

The 0.00 value is equivalent to the perfusate pH. The mean perfusate pH with 80% O_2-20% CO_2 was 6.8381 and with 95% O_2-5% CO_2 was 6.7978.

this behavior of pH is identical with that observed during the control perfusions. In both cases the saline was bubbled with 95% O_2-5% CO_2.

The saline used to check the effect of sodium methyl sulfate alone was made by replacing 21 mEq of sodium chloride with 21 mEq of sodium methyl sulfate. This saline was bubbled by 95% O_2-5% CO_2. The pH difference between perfusate and effluent was equivalent to that observed with the control perfusate. The effluent pH was consistently and significantly lower than the perfusate pH.

Recovery from the experimental perfusions was complete in all cases. Recovery from perfusion by the low bicarbonate (7 mEq) saline bubbled by 95% O_2-5% CO_2 was complete in approximately 1-1/2 minutes. Its approximate 1/2-time was 1 minute. Recovery from perfusion by 80% O_2-20% CO_2 bubbled saline containing 28 mEq bicarbonate took much longer, however. Recovery was not complete until approximately 9-1/2 minutes following initiation of control perfusion. Its 1/2-time was approximately 3 minutes.

Bicarbonate ion exchange. Since the perfusate-effluent pH difference could be due to bicarbonate ion exchange, bicarbonate concentration determinations were done on the perfusate and the effluent.

The previously described method of bicarbonate determination was used in an attempt at detection of bicarbonate fluxes. The milliequivalents of HCO_3^- gained or lost by the effluent from the perfusate concentration level, was calculated for each effluent sample analyzed. The results were grouped according to whether the samples of effluent were taken during control perfusion with 95% O_2-5% CO_2 bubbled saline, or during experimental perfusion with 80% O_2-20% CO_2 bubbled saline. These two groups of results were analyzed statistically.

The 29 effluent samples collected during control perfusion of the hearts were found to contain a mean increase of 1.349 $\pm$0.816 (SEM) mEq/liter of bicarbonate. The effluent collected during experimental perfusion (29 samples) demonstrated a mean increase of 0.769 $\pm$0.816 (SEM) mEq/liter of bicarbonate. The increase during control perfusion was statistically significant, whereas that during experimental perfusion was not.

A statistical comparison of the bicarbonate gained during control perfusion with that gained during experimental perfusion showed that the two were not significantly different.

Interpretations from these results must be made only with extreme caution since errors were introduced by the required storage of the samples. Despite storage at low temperatures, there was evidence of bicarbonate concentration

changes. These changes could have been effected by CO_2 loss from the sample, coupled with metabolite production by a growing microorganism population. The glucose in the perfusate enhanced the growth of microorganisms even under refrigerated conditions.

VI. THE EFFECT OF TWO PERFUSATES ON POTASSIUM AND SODIUM EXCHANGE

General. There is evidence that potassium moves into cardiac tissues during prolonged respiratory acidosis (YOUNG et al, 1954; BROWN and MOWLEM, 1960), and that potassium leaves the myocardium upon reversal of respiratory acidosis (LADE and BROWN, 1963; BROWN and MOWLEM, 1960). There is evidence also that the transmembrane distribution of sodium and potassium may be determinants of vascular smooth muscle tension in the rat tail vessels (FRIEDMAN et al, 1963). This also might be the case in the vessels of the coronary system.

Evidence of potassium and sodium ionic exchanges could serve as the functional connecting link between myocardial ionic fluxes and vascular smooth muscle tension changes to form a unified hypothesis of the interrelationship of their function. For this reason, the analysis of sodium and potassium in the various solutions was attempted.

Results. Sodium and potassium concentrations were determined by flame photometry according to the methods previously described. The samples used were the same as for the bicarbonate determinations. The results were grouped as described for the bicarbonate determinations.

During perfusion with saline bubbled with 95% O_2 and 5% CO_2 bubbled saline containing 28 mEq bicarbonate (pH 7.40), the effluent showed a mean increase of 0.070 ± 0.050 (SEM) mEq of potassium per liter, which was statistically non-significant. In the same effluent, sodium showed a mean decrease of 0.480 ± 0.779 (SEM) mEq/liter. This decrease was also non-significant.

When the heart was perfused with 80% O_2-20% CO_2 bubbled saline containing 28 mEq/liter of bicarbonate (pH 6.80), the effluent showed a mean increase of 0.080 ± 0.039 (SEM) mEq/liter of potassium. This was a significant increase. In the same effluent there was a mean increase of 0.366 ± 0.312 (SEM) mEq/liter of sodium. This was a non-significant change.

A statistical comparison of the sodium concentration changes during control and experimental perfusions was carried out. This showed that the changes were not significantly different one from the other. The same analysis was done for potassium with the same results.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

I. THE ISOLATED HEART PREPARATION

The heart from an animal stunned by a blow on the head, responded to experimental perfusion in much the same manner as did the hearts from animals subjected to heparinization and cold saline infusion. This raises the question of the value of the extra time and experimental complexity resulting from the utilization of this method.

When hearts were not prepared by heparinization and cold saline infusion, there was evidence that the resulting preparation was of poorer quality. Upon opening the thoracic cavity, the vigorously beating heart had a mottled appearance. This seemed to be the result of coagulated blood. With the completion of cannulation, the heart usually had stopped beating and had to be started by squeezing when perfusion was initiated. These hearts characteristically failed more rapidly than did the prepared hearts.

Hearts prepared by the heparinization-cold saline infusion sequence were opalescent when exposed and showed

no evidence of residual blood. These hearts initiated strong contractions immediately upon perfusion with 31 C saline. At the end of experiments, the prepared hearts consistently displayed strong contractions. It was concluded that a better preparation resulted when isolation was preceded by the heparin-cold saline sequence.

Another question of importance concerns the degree of anoxia which perfusion imposes on the preparation. FISHER and WILLIAMSON (1961) reported that the mean oxygen uptake (Q_{O_2}) of isolated rat hearts perfused with Krebs-bicarbonate medium, with or without glucose, was 43.2 μ liters O_2/mg dry wt/hr at 38.5 C. From this determination and another at 35 C, they calculated a Q_{10} of 2.3 for oxygen uptake. Their calculated oxygen uptake agrees well with a calculation for a similar preparation by CHALLONER and STEINBERG (1965).

Using Fisher and Williamson's temperature coefficient and oxygen uptake values for 38.5 C, the oxygen uptake for isolated hearts at 31 C was calculated using the van't Hoff Equation for Q_{10} (GIESE, 1962). The calculated oxygen uptake for hearts at this temperature was 23.67 μ liters O_2/mg dry wt/hr. The mean dry weight of the experimental hearts was 110 mg. Therefore, the mean oxygen requirement of the experimental hearts was 2.60 ml O_2/hr. This was compared with the oxygen available from the perfusing solution. The oxygen available depends on the absorption

coefficient for oxygen at 31 C (0.02574 ml O_2/ml H_2O) (HODGMAN, 1950) and the flow rate through the coronary circulation. Using the mean flow rate during control perfusion, 3.15 ml/min, the mean oxygen supplied by the 95% O_2 saturated solution was 4.356 ml O_2/hr. Therefore, this exceeds the calculated requirement by a comfortable margin.

Perfusion with 80% O_2-20% CO_2 saturated saline represents a decrease in oxygen concentration and causes a secondary decrease in coronary flow rate. Using the mean flow rate under experimental conditions (2.50 ml/min) and calculating oxygen absorption based on the presence of 80% O_2, the experimental perfusate provides 3.2537 ml O_2/hr. This also exceeds the calculated requirement. Since contractility decreases during experimental perfusion, it is reasonable to assume that the myocardial metabolic requirements also decrease. This further increases the safety margin of oxygen available to oxygen required.

ZACHARIAH (1961) calculated that rat hearts perfused with saline saturated with 95% O_2 at 37.5 C, required a flow rate of 5 ml/g wet wt/hr. Using the temperature coefficient of 2.3 yields a calculated flow rate requirement at 31 C of 2.910 ml/g wet wt/hr. The mean wet weight of the experimental rat hearts used was less than a gram, therefore, the flow rates employed provided adequate oxygen by Zachariah's criterion also.

Thus it may be concluded that the oxygen requirement of the perfused hearts is satisfied by perfusion saline saturated with 95% or 80% oxygen.

II. TENSION

Increased carbon dioxide concentration caused a rapid and pronounced decrease of contraction tension. The decrease in tension from a pH change alone was much smaller and slower in its development than was the tension change from CO_2. This would appear to reflect carbon dioxide's well known ability to penetrate membranes rapidly, and bicarbonate's less easy penetration. This apparent correlation between the tension response and the ability to penetrate membranes suggests that the effect on tension is exerted intracellularly.

Assuming that the effect on tension by CO_2 is exerted intracellularly, is this control effected by carbon dioxide as the molecular entity or is it effected by the resulting hydrogen ion concentration increase? An examination of tension's response to an extracellular bicarbonate concentration decrease may provide some indirect evidence on this question.

When bicarbonate concentration is decreased in the perfusate, it is reasonable to assume that a decreased bicarbonate concentration also results in the extracellular space. As was previously discussed, the majority opinion is that muscle cell membranes are practically impermeable to bicarbonate ions. However, ADLER et al (1965 a, b) have concluded from their experiments with the rat diaphragm

that these muscle cells display a small but significant permeability to bicarbonate. The very slow decrease in contraction tension, when the only variable changed was extracellular bicarbonate, indicates that myocardial cells also are permeable to bicarbonate. During control perfusions with 28 mEq bicarbonate, the intracellular bicarbonate concentration would tend to equilibrate with the extracellular bicarbonate concentration. Consequently, when the experimental perfusate containing 7 mEq bicarbonate replaces that containing 28 mEq, an outwardly directed concentration gradient for bicarbonate is established. As the bicarbonate leaves the intracellular compartment, the pH of that compartment decreases. As the intracellular pH decreases from bicarbonate loss, the tension response approaches that observed with increased intracellular carbon dioxide concentration. Since the common requirement to a tension decrease in both types of perfusion is an intracellular hydrogen ion concentration increase, it would appear logical to conclude that tension changes are mediated by intracellular pH changes.

The possibility exists that the extracellular hydrogen ion concentration affects myocardial tension development. When extracellular hydrogen ion concentration is increased by carbon dioxide alone, there is a rapid decrease in tension. However, when extracellular pH is decreased by a bicarbonate decrease alone, there is only a slow decrease

in tension. Extracellular hydrogen concentration increases are present in both cases, yet tension decreases rapidly only when CO_2 also is increased. Therefore, increased extracellular hydrogen ion concentration does not appear to cause decreased tension, and the evidence indicates that it is the intracellular hydrogen ion concentration change which ultimately alters tension.

What is the locus of increased intracellular hydrogen ion concentration's effect on tension? These experiments provide no evidence as to the mode of action of intracellular pH changes. However, observations by other workers indicate that a controlling effect by pH changes is feasible. DUBUISSON (1954) considered it possible, as a result of his study of intracellular pH changes, that molecular rearrangements which accompany shortening may produce changes in the hydrogen ion concentration. Conversely, it may be reasoned that hydrogen ion concentration changes may produce molecular rearrangements which, in turn, alter the degree of shortening. It is also possible that enzymes, critical to energy liberation or utilization, respond to a departure from optimum pH by decreased activity which, in turn, results in decreased contraction tension.

Neither experimental perfusate caused a significant change in diastolic tension. Therefore, it would appear that the effect of intracellular pH decreases are on the

mechanisms of contraction tension development and not on those of resting tension maintenance. This suggests that the effect of intracellular hydrogen ion changes may be on the energy releasing or utilization mechanisms rather than on molecular rearrangements resulting in shortening.

An exact and reproducible control of contraction tension was exerted by stepwise increases in CO_2 concentration. Increasing CO_2 by 2% steps from 8% to 30% caused contraction tension to decrease by reproducible and significant increments. The lack of significant tension decreases, when CO_2 concentration is increased to the 6% and 8% levels, may be due to a masking of the negative inotropic effect by the positive inotropic effect of decreasing the oxygen concentration. The effect of reducing oxygen from 95% to 80% will be discussed below.

That the negative inotropic effect of increased CO_2 concentration was not the result of concurrent negative chronotropic effect, was demonstrated by using hearts driven at constant rates. The characteristic negative inotropic effect of increased CO_2 concentration occurred despite the maintenance of a constant rate.

The dissimilarity of the 1/2-time of carbon dioxide's negative inotropic effect compared with the 1/2-time of the coronary resistance increase indicates that the two events are not related. This conclusion was supported by experiments wherein flow rate was manually kept constant;

increased carbon dioxide again had a negative inotropic effect.

The possible effect of changing the concentration of N_2 and O_2 in the gas mixtures saturating the saline was investigated. From indirect evidence, it was concluded that N_2, which was used as a filler gas, did not affect tension development. This agrees with the conclusion of PENNA et al (1965). These authors reported that the stimulant effect of hypoxia persisted, in isolated guinea pig atria or cat papillary muscles, when nitrogen was replaced by helium.

The increased contraction tension observed with decreased oxygen concentration has also been observed by BEAN and BOHR (1938) in frog skeletal muscle, by PENNA et al (1965) in isolated guinea pig atria and cat papillary muscle, and by SOMA et al (1965) for the dog heart-lung preparation. Diastolic tension decreases significantly with decreased oxygen concentration. Since diastolic tension decreases were not present with pH or pCO_2 changes, a different mechanism for the oxygen effect is indicated. STADIE et al (1944) considered that the effects of higher than optimum oxygen was most likely due to depression of enzyme activity. Bean and Bohr also concluded that oxygen's effect may be on certain enzyme systems. The experiments reported here do not provide evidence as to the site of oxygen's effect. The positive inotropic effect of decreased

oxygen may be an important stimulant to increased cardiac output during hypoxia (PENNA et al, 1965).

III. HEART RATE

Increased carbon dioxide concentration and decreased pH both decrease heart rate, however, the effect of increased CO_2 is much greater than that of decreased pH. The negative chronotropic effect of carbon dioxide is slower than its negative inotropic effect. The net 1/2-time for the tension decrease is 2 seconds, whereas the net 1/2-time for the rate decrease is 27 seconds.

It is tempting to attribute the delay in the appearance of the negative chronotropic action of carbon dioxide to the time required for the release of a chemical mediator. Such a substance could be acetylcholine which has been shown to be plentifully and persistently present in the sino-atrial node area of isolated rat hearts (VLK and TUČEK, 1964). That a very small concentration of acetylcholine (10^{-15} to 10^{-8} g/ml) was adequate to produce a profound negative chronotropic effect on perfused frog hearts was shown by BOYD and PATHAK (1965).

The larger delay required for the chronotropic effect to appear than for the inotropic effect has a more basic explanation, however. The rat heart has a dual blood supply (HALPERN, 1957). The atria are supplied almost wholly by the cardiac branches of the cardiomediastinal arteries which arise from the right internal mammary artery. Therefore, perfusion of the coronary vessels through the aorta

does not provide an intimate supply of saline to the sino-atrial node. Halpern has shown that a few terminal branches of the right coronary artery do approach the region of the sino-atrial node, but they do not supply this area at all thoroughly.

Therefore, the delay in the appearance of the negative chronotropic effect is most probably due to the longer diffusion pathway which the experimental perfusate must travel before reaching the pacemaker cells. Since no direct perfusion of the sino-atrial node occurs, the perfusion fluid must reach the cells by diffusing through the wall of the atrium after issuing from the coronary sinus, or by diffusing toward the node from the few, and fairly remote, branches of the right coronary artery.

These experiments cannot distinguish whether the effects of CO_2 affect the pacemaker cells directly or affect undegenerated nerve endings which, in turn, release acetylcholine which causes the negative chronotropic effect. Again some indirect evidence may be gleaned from the various responses. The negative chronotropic response of the pacemaker cells resembles the negative inotropic response of the myocardial cells: Both cell types give a quick and marked response to the presence of increased carbon dioxide, but both cell types give a slower and smaller response to decreased bicarbonate (decreased pH). The response by both cell types to graded pCO_2 changes is very similar. Based on the similarity

of response and the similarity of ultrastructure (RHODIN et al, 1961) of the myocardial and pacemaker cells, the hypothesis that CO_2 acts directly on the pacemaker cells may be justified.

Assuming that the carbon dioxide effect is exerted directly upon the pacemaker cells, is there an ionic basis for such a control? The ionic basis of excitation as developed by Hodgkin and Huxley (HODGKIN, 1951) can be applied to heart muscle with very little modification (HECHT, 1965). Application of these concepts to pacemaker function and to the evidence obtained in the experiments reported herein may provide a reasonable explanation for the rate alterations by CO_2 or pH.

HECHT (1965) concluded that the characteristic gradual depolarization of the cell membranes in pacemaker tissue during diastole, depends on a high resting sodium current and a gradual increase in the membranes' resistance to the passage of potassium ions. HUTTER and NOBLE (1961) reported that mammalian Purkinje fibers were permeable to chloride and that abolition of the chloride current slowed the rate of depolarization during diastole and the rate of repolarization during the plateau phase. These authors also reported that resting cardiac fibers, and presumably pacemaker fibers, have a smaller chloride current than do skeletal muscle fibers. They concluded that this smaller chloride current of cardiac cells makes them more sensitive to

changes in cation permeability. Therefore, factors altering heart rate may operate through effects on chloride, sodium, or potassium permeability.

HODGKIN and HUXLEY (1952) demonstrated that the sodium conductance of the squid axon membrane decreased as the resting membrane potential decreased. WEIDMAN (1955) reported that in sheep Purkinje fibers the amount by which sodium permeability increases during depolarization similarly depends on the value of the potential difference which had been acting on the membrane before depolarization. Are ionic conditions established, in the isolated rat heart experiments herein reported, which could cause depolarization of the pacemaker membranes and a consequent decrease in sodium conductance?

A small permeability to bicarbonate was postulated from the slow development of a tension decrease in the presence of low bicarbonate (7 mEq). This was in agreement with the findings of ADLER (1965 a, b) in skeletal muscle. Assuming that pacemaker cells also exhibit a small permeability to bicarbonate, the slow development of a negative chronotropic effect in the presence of a perfusing solution with low bicarbonate should be related to intracellular bicarbonate loss. With a lower concentration on the outside of the cell, the diffusion gradient should promote the loss of intracellular bicarbonate. As intracellular bicarbonate is lost the pacemaker cells are depolarized. As discussed

above, a decreased resting membrane potential decreases sodium conductance. Decreased sodium conductance then results in a slower development of the pacemaker prepotential and a slower repolarization.

When the evidence for chloride's contribution to the depolarization leading to the development of the pacemaker prepotential is coupled with evidence for a pH effect on chloride conductance, as shown by BROOKS and HUTTER (1962, 1964), a hypothetical basis for carbon dioxide's negative chronotropic effect may be developed. Brooks and Hutter demonstrated that reducing pH by a THAM buffered solution caused a decreased chloride current through skeletal muscle membrane. If this information is linked with the findings of HUTTER and NOBLE (1961), that cardiac tissue also displays a permeability to chloride, the extrapolation that a similar mechanism of effect could cause the negative chronotropic effect of CO_2 appears to have validity. Brooks and Hutter postulated that a channel for chloride exists in the membrane which is opened wide at a basic pH but which is almost closed at acidic pH values. This agrees with the description by HECHT (1965) of a "tighter" membrane in an acid medium. HECHT and HUTTER (1964) reported that rise time and repolarization of Purkinje fibers was suppressed by solutions with decreased pH.

COMROEUF and BOISTEL (1953) reported that increasing CO_2 reduces the cardiac resting potential. This would

cause a decreased sodium conductance as discussed above. Therefore, it is possible that carbon dioxide reduces rate by a double effect--one affecting chloride conductance and one affecting sodium conductance.

The evidence from the literature provides a basis whereby both molecular CO_2 and pH may cause rate decreases. The difference in magnitude of the CO_2 and pH effects reported here for the isolated heart experiments remain to be explained.

As previously discussed, the diffusion pathway between the nearest capillary supply and the pacemaker tissue must be long in the isolated heart of the rat, due to the dual blood supply. Carbon dioxide's ability to diffuse rapidly would permit it to reach the site of its effect more quickly than the less mobile bicarbonate ion. This would partially explain the difference in rate of onset of the negative chronotropic effect. However, even allowing additional time for the extracellular bicarbonate concentration to reach the new equilibrium levels, the negative chronotropic response with 7 mEq bicarbonate (pH 6.8) gives no indication of a marked rapid response such as is observed with increased CO_2. This suggests that the rate limiting factor may be a semi-permeable barrier--the pacemaker cell membrane. This would further argue that the effect of pH or CO_2 on ionic conductance is exerted from within the pacemaker cell membrane. Carbon dioxide, with its capacity

for rapid permeation of membranes, would then exert its effect more rapidly than extracellular hydrogen ions or bicarbonate ions.

HARRIS (1965) concluded that the sensitivity of chloride conductivity to pH changes, and to anion concentration changes, indicated that chloride movement was, in part, controlled by passage through an ion exchange region. Harris further postulated that a pH reduction increased the number of cationic steps in the path of chloride through an amphoteric substance such as protein. This harmonizes well with the above developed hypothesis that a semipermeable barrier favors the more rapid effect of CO_2, providing that it is further stipulated that the effect of CO_2 is by way of the resultant hydrogen ion increase. Therefore, it seems reasonable that CO_2 is able to diffuse quickly through the pacemaker cell membranes, and increases the intracellular concentration of hydrogen and bicarbonate ions. The newly arrived hydrogen ions are taken up by proteins with the resultant formation of more cationic steps in the chloride pathway. This increases the membranes' resistance to chloride which decreases the rate of diastolic depolarization.

The operation of the negative chronotropic effect according to the above discussed ionic mechanism, would leave an intracellular excess of bicarbonate after the proteins immobilize some of the hydrogen ions. This intracellular excess of bicarbonate ions could then additionally

contribute to the negative chronotropic effect by decreasing the resting membrane potential as previously discussed.

The operation of the CO_2 effect on rate from within the cell by way of a hydrogen ion increase, would explain the observed slower response to an extracellular pH or bicarbonate ion concentration change. Both the hydrogen ion and the bicarbonate ion can permeate membranes more slowly and consequently, could only alter intracellular hydrogen ion concentration more slowly than CO_2. Once the hydrogen ion concentration changed, the sites which add steps to the chloride pathway would begin their effect on chloride conductance and rate.

Some idea of the time required for a large non-permeating ion to reach the pacemaker cells can be gleaned from the experiments with the sodium methyl sulfate control perfusate. HUTTER and NOBLE (1961) found that replacing chloride with methyl sulfate caused an initial transient positive chronotropic response. This rate increase was, according to the authors, most likely due to the flow of a depolarizing current which was, in turn, due to the replacement of some of the extracellular chloride by the non-permeating methyl sulfate anion. This initial depolarizing current exceeded the natural depolarizing current and thereby increased the rate. Figure 48 shows that in the isolated heart a positive chronotropic effect is also exhibited at approximately 2 minutes following onset of

perfusion. Subtracting a minimum of 10 seconds for clearing the dead space of the saline distribution system indicates that approximately 1-1/2 minutes may be required for the solution with the new ionic species to reach the pacemaker cells.

HOFFMAN and CRANEFIELD (1960) concluded that CO_2 had the same effect on Purkinje fibers as on the sino-atrial node. This links the literature findings relative to Purkinje fibers with the postulated pacemaker effects. Conversely, a similar mode of action of the experimental perfusates on the conductile elements of the heart adds another locus at which the negative chronotropic effect may be effected.

Within the range of tension changes observed as resulting from the experimental perfusates, the negative inotropic effects could not have caused the negative chronotropic effects.

The previously described minimum flow rate to achieve optimum heart rate appears to be a logical consequence of the dual circulation to the heart discussed above. Since the diffusion pathway is quite long for the pacemaker cells, a minimal flow rate is required to maintain the concentration gradients for oxygen diffusing to the cells and carbon dioxide diffusing away from the cells. Both inadequate removal of carbon dioxide and inadequate supply of oxygen could contribute to slowing the function of the

pacemaker.

The 1/2-times of flow rate change and heart rate change were previously described as being of the same order of magnitude, hence it was tentatively concluded that the two parameters might be related. When coronary resistance was calculated, the 1/2-times became significantly different. The net 1/2-time for coronary resistance increase is 57 seconds while that for rate is 27 seconds. Therefore, the heart rate decrease is not dependent on the coronary resistance increase.

IV. CORONARY RESISTANCE

When the perfusate was saturated with 20% CO_2, a marked and persistent coronary vasoconstriction occurred. Taking account of the inherent vasoconstrictive effect of sodium methyl sulfate, lowering pH to an equivalent level without increasing the pCO_2 caused no significant change in coronary resistance.

The effect of carbon dioxide was observed, in the typical experiment, to be biphasic. However, when all the experiments were grouped together for analysis, the initial decrease of resistance was not apparent. Since the sampled response was at 1/2 minute following initiation of perfusion, the maximum decrease in coronary resistance was not always sampled due to its short duration. A check of the individual records revealed that only 0.53% of the resistance responses did not show an initial decrease.

The biphasic response of coronary resistance can be related to the dual action of CO_2 on resistance as demonstrated by the results of graded CO_2 increases. At lower concentrations carbon dioxide has a vasodilatory effect, but at approximately 18% the vasodilatory effect changes to a vasoconstrictor effect. Therefore, the biphasic response is possibly due to the experimental saline equilibrated with 20% CO_2 replacing the control saline equilibrated with 5% CO_2. As this happens, the CO_2

concentration increases and passes through its vasodilatory spectrum before reaching maximum saturation and expressing its vasoconstrictive effect.

What is the site of carbon dioxide's effect on coronary resistance? The observed effect might result from the imposition of extramural forces on blood vessel walls, from swelling of capillary endothelial cells, or from an intrinsic effect of carbon dioxide on smooth muscle cells. Increased extramural pressure on the blood vessels, imposed by an increase in diastolic tension resulting from increased CO_2, was initially postulated as a mechanism of coronary resistance increase. This possibility was eliminated when diastolic tension was shown not to change significantly with increased CO_2.

Swelling of endothelial cells due to altered membrane permeability in the presence of CO_2 could account for the increased resistance. The permeability of endothelial cells might be so altered that the volume of extracellular fluid increased to such a point, that extramural pressures were applied to the walls of the blood vessels, with a resultant resistance increase.

The possibility that the resistance response is due to permeability changes causing either endothelial cell swelling or increased extracellular fluid pressure, cannot be directly eliminated by these experiments. There are observations which argue against this sort of control,

however. The biphasic response to increasing CO_2 concentrations tends to indicate a sensitive cellular control mechanism imposing its effect on coronary resistance. The rapid recovery of resistance with return to controllevels of CO_2 argues against a process dependent on diffusion alone. Also, if an increase in carbon dioxide concentration caused an increased permeability to the saline, then conversely the decrease in carbon dioxide concentration should decrease permeability. Thus the fluid would be trapped in the endothelial cells or in the extracellular spaces and recovery would be a very slow process.

Therefore, it is reasoned that the precise nature of the control exerted by CO_2 on coronary resistance is evidence for control by vascular smooth muscle. The logical locus for this control would be the smooth muscle cells of the arterioles.

The action of carbon dioxide on coronary resistance, herein demonstrated, is reminiscent of its action on respiratory control as discussed by GRAY (1949). Increasing carbon dioxide increases respiration until a critical concentration is reached (approximately 9%). Further increases of carbon dioxide result in a decrease in respiration. This is much the same type of response demonstrated by graded increases of carbon dioxide on coronary resistance (Figure 58).

V. HYDROGEN ION EXCHANGE

The efflux of acidic materials during perfusion with saline saturated with 5% CO_2, is most probably due to the constant production of acidic products of oxidative metabolism. This release occurs regardless of the extracellular pH.

When carbon dioxide is increased to 20%, some additional process appears to supervene so that the pH of the effluent now becomes basic as compared to the perfusate. No attempt was made to identify the basic material causing this pH rise. However, it is interesting to speculate that the release of a basic substance, possibly ammonia, could be related to Berne's hypothesis that autoregulation of coronary blood flow is due to adenosine release by myocardial cells during hypoxia (BERNE, 1964). He concluded that for the hypothesis to be valid, it must be demonstrated that adenosine is not deaminated before it leaves the myocardial cells. The increased pH of the effluent indicates that a basic substance such as ammonia is released constantly when CO_2 is increased to 20%. This agrees with the findings of CONWAY and MOORE (1945) that 15% carbon dioxide, at constant pH, markedly increased the penetration of ammonia through muscle or yeast cell membranes. Elevated carbon dioxide concentration is the only experimental condition which causes the pH of the effluent to be higher than the perfusate

pH. This may indicate an increased release of ammonia which may be due to deamination of adenosine. If this is the case, the adenosine of Berne's theory is deaminated intracellularly and this argues against its function as a vasodilator. Although these experiments do not identify the basic substance, they indicate that such an analysis might be worthwhile.

VI. SODIUM, POTASSIUM, AND BICARBONATE EXCHANGES

No evidence of potassium or sodium loss from cardiac tissue, as the result of an increased CO_2 concentration, was observed. YOUNG et al (1954) and BROWN and MOWLEM (1960) had reported myocardial potassium loss in the presence of elevated CO_2 concentrations.

The absence of significant increases in effluent sodium or potassium during periods of increased CO_2 concentration, argues against their role as agents of smooth muscle tension changes in the coronary system. This had been hypothesized to be a link between smooth muscle function and myocardial ionic exchange, based on the findings of FRIEDMAN et al (1963) on the effect of sodium and potassium on the smooth muscles of rat tail vessels.

The possibility that loss of potassium by the myocardium can influence rate by accumulating increased concentrations of potassium around the cells of the pacemaker must be discarded in light of the lack of a significant potassium increase in the effluent.

Bicarbonate concentration changes observed were not adequate to explain the pH differences observed between perfusate and effluent.

VII. GENERAL CONCLUSIONS

The results reported herein disagree with those of McELROY et al (1958) and VAUGHAN WILLIAMS (1954) who found that carbon dioxide had an effect on cardiac function only when a pH change was concurrently present. This difference of results may be due to the practice of these workers, of allowing the hearts to equilibrate with the experimental perfusate for extended periods of time (15-30 minutes), before analyzing its effect on a particular variable. This practice would miss the quick effect of increased CO_2. From the magnitude of the response to increased CO_2, it seems reasonable that this quick effect may have the most severe consequences on cardiac function.

Also, the change of pH by altering the concentration of bicarbonate, a rather poorly permeating anion, and balancing the change by altering the concentration of chloride, a freely permeating anion, could have cellular consequences which mask the effect of carbon dioxide or pH.

As discussed in the literature survey, there have been indications that increased carbon dioxide may also play a role as a vasoconstrictor agent in addition to its vasodilatory effect. The demonstration herein of its dual effect reconciles the two points of view.

Extrapolation from the observations on isolated organs to the functioning of these organs in situ may only be made

with extreme caution. However, the marked effect of increased carbon dioxide on the heart's three cell types may provide the link in the explanation of why the heart's function irreversibly fails after reaching a certain point.

As previously discussed, high concentrations of CO_2 have been suspected as the causative agents in various clinical states. BING et al (1964) have stated that "despite the large volume of studies, the fundamental cause of heart failure, so commonly encountered in clinical practice, still remains obscure". This statement emboldens one to advance the hypothesis that simple, ubiquitous, and elusive carbon dioxide is the agent which trips cardiac mechanisms down the hill of irreversible failure. As metabolically derived carbon dioxide builds up within the tissues of the heart due to impaired coronary circulation, the resulting negative inotropic and chronotropic effects decrease cardiac output. This further reduces coronary circulation which, in turn, promotes additional CO_2 accumulation. As CO_2 concentration increases, the beneficial vasodilation is replaced by vasoconstriction. Vasoconstriction causes even greater CO_2 accumulation; this makes recovery from the effects of this cycle difficult or impossible.

VIII. SUMMARY

The problem formulated in Chapter I, concerning the relative effects of low pH and high CO_2 on three areas of heart function, has been resolved with the demonstration of the following:

(a) Carbon dioxide has a rapid negative inotropic effect which cannot be accounted for by the fall in pH of the perfusing fluid.

(b) Carbon dioxide has a rapid negative chronotropic effect which may be due partly to the concurrent lowering of pH.

(c) Decreasing pH by decreasing bicarbonate concentration causes slower and smaller negative chronotropic and inotropic effects.

(d) Carbon dioxide has a double effect on coronary resistance: A vasodilatory effect results when concentrations are increased up to approximately 16%, and a vasoconstrictor effect is exhibited by 18% and 20% concentrations.

(e) Decreasing pH by decreasing bicarbonate concentration has no effect on coronary resistance.

(f) Decreased oxygen saturation of the perfusion fluid caused a positive inotropic effect but was without effect on rate or coronary resistance.

(g) A study of the possible interactions of the three variables showed that carbon dioxide had a

direct effect on tension, rate, and coronary resistance.

Models were proposed to explain the mode of effect of carbon dioxide and pH changes on the three areas of heart function.

The possible significance of these findings to the intact organism is considered.

CLAIMS TO ORIGINAL RESEARCH

Using the principle of minimal qualitative and quantitative changes in the anionic constituents of the perfusate, the effects of CO_2 and hydrogen ion changes on the isolated rat heart were analyzed with the following results:

(a) CO_2 has a negative inotropic effect which is not dependent on the perfusate pH change, the concurrent negative chronotropic effect, or on the concurrent changes in coronary resistance.

(b) CO_2 has a negative chronotropic effect which is due, in part, to the concurrent pH change, but which is not related to the concurrent negative inotropic effect or the concurrent increase in coronary resistance.

(c) CO_2 causes an increase in coronary resistance at high concentrations (18% and 20%) which is not mediated through the concurrent tension change, rate change, or pH change.

(d) Neither CO_2 nor pH changes alter the myocardial tension at diastole.

(e) CO_2 concentrations intermediate between the control level (5%) and the highest

concentration (20%) cause intermediate decreases of contraction tension and heart rate.

(f) CO_2 concentrations intermediate between the control level (5%) and the highest concentration (20%) cause a coronary resistance decrease between 6% and 16%, and a coronary resistance increase at 18% and 20%.

(g) A decrease in the O_2 concentration from the control level (95%) to the experimental level (80%) causes a positive inotropic effect which is not mediated by rate or coronary resistance changes.

(h) A decrease in the O_2 concentration from the control level (95%) to the experimental level (80%) has no effect on rate or coronary resistance.

(i) The heart loses a basic substance at high CO_2 concentrations (20%), but loses acidic substances at low CO_2 concentrations (5%). The loss of acidic substances is independent of perfusate pH.

These are, to the best of my knowledge, original items of research.

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