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WATER AND ELECTROLYTE BALANCE
IN NORMAL AND HYPERTROPHIED HEARTS

by

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Submitted in partial fulfilment
of the requirements for the degree of
Master of Science

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August 1968



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Date: September 10, 1968.

This investigation was supported by a grant to Professor M. V. Beznak from the Medical Research Council of Canada. The author wishes to express her appreciation for support to the Medical Research Council.

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ACKNOWLEDGEMENTS

The author wishes to express deep gratitude to all members of the Department of Physiology who helped in any way.

First and foremost thanks is expressed to Dr. M. Beznak for wise counseling based on vast experience in this field and also for making it possible for me to fulfil a lifelong ambition to play even a very small part in this field of heart research.

Special thanks is due the following;- a) Dr. I. French for suggesting the method used in the radioactive tracer work, a method which saved countless hours over previously tried methods and proved efficient as well, b) Dr. G. Mainwood who divulged the intricacies of flame photometry and c) Dr. B. Hunt who supervised the sodium and potassium recovery studies.

Sincere thanks also to other members of the Staff and especially to the graduate students who were always willing to discuss some point of the work.

Last but not least, sincere appreciation is expressed to the technicians who helped in countless ways; to the Medical Illustrator; and to my husband for his patience and encouragement.

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ABSTRACT

The condition of arteriosclerosis was simulated in male, Wistar-strain rats by sub-diaphragmal aortic constriction. Two days after constriction, a significant ($P < 0.05$) increase in cardiac mass had occurred. Significant changes in extracellular fluid volume and electrolyte content of the left ventricle, right ventricle and atrium were found much sooner than changes in cardiac mass and may thus play a role as instigating factors in the mechanism of cardiac hypertrophy.

The extracellular fluid volume was measured by a radioactive isotope dilution method employing S^{35} -sulphate. Significant increases in sulphate space and sodium content were found to occur in the left ventricle within 24 hours after aortic constriction. These increases became even more significant ($P < 0.001$) at 10 days, then both parameters decreased to within normal range between 2 and 3 weeks after aortic constriction. Close correlation ($r = 0.915$) was found between sulphate space and sodium content in both ventricles of hypertrophied hearts. Total water remained remarkably constant in the left ventricle but rose significantly in the right ventricle of hearts with the greatest degree of hypertrophy (over 66% increase). Potassium content remained unchanged in the left ventricle but dropped significantly in the right ventricle, as did total phosphate.

The same parameters were measured in different regions of normal hearts of unoperated, 150 to 450 gram rats. It was found that sulphate space, total water, sodium content, intracellular potassium, etc. all reached maximal values in the 250 to 300 gram rats and then all (except sodium) decreased in larger rats. Because this weight group occurs at the

steepest part of the normal growth curve, these changes in water and electrolyte values are thus associated with rapid normal growth as well as with the accelerated growth of cardiac hypertrophy.

Possible reasons for the observed changes in water and electrolyte balance are discussed from the point of view of hormonal, metabolic, hemodynamic and physical changes.

CHAPTER I

Introduction

General Review of the Literature

The response of the heart to a chronic increase in work load results in an increase in myocardial mass. This biological phenomenon is known as cardiac hypertrophy or cardiomegaly. It is believed by many to be a useful mechanism of adaptation but this view has been opposed by others who consider hypertrophy to be an indication of the inability of the heart to cope with demands made by the circulation. In any case it is agreed that when the degree of hypertrophy exceeds a certain limit, its usefulness is lost.

Where does this boundary of useful hypertrophy occur? The gross anatomical changes between normal and hypertrophied human hearts were summarized and classified by Linzbach (1960). He coined the term "physiologic hypertrophy" to describe cardiac enlargement of athletes or labourers from a normal 300 g. to 500 g. with fibres of both left and right ventricles becoming thicker and longer. "Pathologic hypertrophy" he classified as either "eccentric" or "concentric" depending on whether the pathological mechanism produced a volume or pressure hypertrophy. The former occurs when valvular insufficiencies cause a larger residual blood volume and hence larger ventricular chambers. On the other hand, when increased work is due to resistance to ejection, as in aortic stenosis or hypertension, a pressure hypertrophy results with no change in ventricular chamber size and residual blood

volume. Above a critical level of 500 g. Linzbach showed that "concentric" hypertrophy could develop into "eccentric" hypertrophy, all hearts becoming hypoxic above a 66% increase in size, not because of any change in capillary-to-fibre ratio (Shipley, et al, 1937) but because the diameter of the coronary vessels no longer increases linearly with heart mass. As this discrepancy increases, the coronary reserve is finally exhausted leading to disturbances of myocardial nutrition and areas of focal necroses. Because of areas of necrosis and scarring, the force per unit of effectively contracting muscle must be greater. In this type of hypertrophy the right ventricle also shows hypertrophy and dilatation due to the elevated pressure in the pulmonary artery resulting from failure of the left ventricle.

The immediate effect of an increased work load on the heart is (a) stretching of the muscle fibres (b) increased tension per unit fibre length or (c) both. Since the muscle fibres run parallel, when stretched they become thinner and the interstitial spaces become wider to a corresponding degree. Hort (1951) demonstrated this in the right ventricular wall of the guinea pig, showing that the number of muscle layers decreases with greater degrees of dilatation, apparently because muscle fibres of the adjacent layers would slip into the spaces. Although the right ventricle is only half as heavy as the left ventricle, it has roughly the same number of fibres and nuclei, although the fibres are thinner and arranged in fewer fibre layers than in the left ventricle.

Histological examination of hypertrophied heart muscle (Linzbach 1947) indicated that in physiologic hypertrophied hearts, at least, the increase in weight is not due to an increase in the number of fibres, the fibres simply become thicker and longer. However, in pathologic hypertrophies he believed there to be an absolute increase in the number of fibres, nuclei and capillaries, due to a longitudinal cleavage between points of anastomosis of the myocardial syncitium, resulting in a marked increase in the number of fibre layers. The question of hypertrophy (myocardial cell enlargement) vs. hyperplasia (increase in number of myocardial cells) still remains debatable.

One of the most important findings morphologically, concerned the distance between Z-bands in myocardium. Linzbach (1951) showed that there was no significant difference from Z-band to Z-band (sarcomere length) in normal, hypertrophied or pathologically dilated hearts nor between left and right ventricular fibres. This was confirmed by Hort (1957) whose sarcomere measurement (2.05μ) is generally accepted, and found to be true in many animals as well.

Thus cardiomegaly is accomplished by addition of sarcomeres in series. That the cross-sectional area is increased by uniform addition of the contractile protein, actomyosin, and not by increase in nuclear proteins (and DNA) was shown by Grimm, et al (1963).

Experimental Models

Since the number of functional studies which could be performed on human hearts is quite limited, experimental models for studying

hypertrophied hearts were produced, usually by one, or a combination, of the following methods;-

<u>Type of Hypertrophy</u>	<u>Method of production</u>
Pressure hypertrophy	Partial nephrectomy, Aortic stenosis
Anoxic hypertrophy	Anaemia, High altitude hypoxia, Coronary artery ligation, Exercise.
Nutritional hypertrophy (dietary deficiencies or excesses)	Salt loading, B ₁ avitaminosis.
Hormone-induced hypertrophy	Thyroxine
Volume hypertrophy	Valvulotomy

Physiological studies

Various experimental models have provided functional studies not only of hypertrophied myocardium but of prehypertrophied myocardium i.e. after the hemodynamic load has been applied but before actual hypertrophy has set in. For example, Beznak (1958) has shown that between one and two hours after narrowing, the cardiac output and heart rate drop sharply and so does the reserve force of the heart (the maximum to which the cardiac output can be raised by infusion with 3.5% polyvinylpyrrolidone into the right side of the heart via the jugular vein). By the end of 24 hours, tachycardia develops and by the end of one week when the weight of the heart has increased by about 30%, the cardiac output has returned to normal, so has the heart rate and reserve force of the heart. Many have suggested that enlarged myocardium is capable of increased cardiac work capacity. Beznak (1958) showed that a higher

maximum output and work per gram left ventricle was obtained when the hypertrophy was due to thyroxine treatment. When hearts hypertrophied by aortic constriction, chronic anaemia and thyroxine treatment were tested for maximum output in heart-lung preparations, it was shown that in no case was the maximum output per gram left ventricle above that of normal rat hearts (Korecky, et al 1966). No change in resting and developed length-tension curves between normal and hypertrophied rat myocardium was observed by Grimm, et al (1963). Neither did they find any change in the quality of the excitation membrane phenomenon; electrical parameters were the same in normal hearts and those hypertrophied by aortic constriction. Thus it is concluded from these and many other studies that hypertrophied hearts (at least up to the critical heart weight increase of 66%) function just as well, if not better than normal hearts "in vivo".

Biochemical studies

Since protein synthesis is preceded by and definitely related to an increase in ribonucleic acid (RNA) whereas cell division is preceded by and usually follows doubling of deoxyribonucleic acid (DNA) therefore many investigations into the amounts and ratio of RNA to DNA in normal and hypertrophied hearts have been prompted by an effort to settle the question of hypertrophy vs. hyperplasia, or both. Norman and Carter (1961) found a considerable increase in the RNA:DNA ratio in anaemic and hyperthyroid hearts. This indicates that cellular enlargement is predominant in these types of enlargement. However, the degree of hypertrophy may be a factor as Korecky and French (1967)

showed an increase in total DNA, RNA and protein in hearts hypertrophied 100% by chronic anaemia suggesting that hyperplasia occurs above the critical weight. No significant differences in the actomyosin concentration or characteristics of the contractile protein in the enlarged fibres can be found (Grimm, et al 1963).

The few biochemical changes generally noted, are included in a summary by Meerson (1962) of studies by Russian workers on hearts of rabbits and dogs hypertrophied by aortic constriction. Meerson divides the developments of compensatory hypertrophy into three stages. First, or "transient breakdown stage" - symptoms of left ventricular insufficiency are observed with chemical changes consisting of decrease of the glycogen and phosphocreatine content of the myocardium. The weight of the heart increases steadily each day. The rate of protein synthesis, as measured by incorporation of S^{35} -methionine, doubles, RNA increases 32%. Second, or "protracted stage of relatively stable hyperfunction" - characterized by hypertrophy of the muscular fibres and absence of signs of cardiac insufficiency. The content of glycogen, phosphocreatine and ATP (adenosine triphosphate) is normal. The heart weight is approximately twice that of normal. Moderate anoxia is indicated by the accumulation of lactic acid in the myocardium. An increase in oxidation-reduction enzymes such as succinic dehydrogenase and increased myoglobin content may tend to overcome the development of hypoxia. Third, or "protracted stage of progressing cardiosclerosis" - in this stage cardiac insufficiency develops with myocardial fibrosis, focal

fatty degeneration and decrease of DNA concentration, rate of protein synthesis and ATP level.

Histological studies

As in the microscopic studies described on page 3 by Linzbach and Hort (1951), Norman (1962) could find no significant alterations in myocardium of hyperthyroid or anaemic rats even with an electron microscope. However changes have been observed in ischemic hearts before any changes could be observed by light microscope. Caulfield and Klionsky (1959) noted the following changes in myocardium within five minutes after onset of ischemia;- decrease in glycogen, clear spaces in various portions of the muscle which they ascribed to influx of fluid and clumping of nucleoplasm. After twenty minutes, swelling of the mitochondria occurred with development of coarser cristae and after five hours an increase in the number of lipid droplets was evident. Since ischemia is thought to be one of the factors to cause hypertrophy these observations may be significant.

Possible factors controlling Cardiac Hypertrophy

The ultimate question asked by all researchers on the topic, whether in the field of Physiology, Biochemistry or Histology is, - "What is the exact mechanism to stimulate cardiac hypertrophy and which factor, or combination of factors, controls it?" A short summary of the possible factors follows:

1. Oxygen utilization - Since so many of the conditions leading to hypertrophy are associated with hypoxia (anaemia, hyperthyroidism, high

altitude hypoxia, coronary artery occlusion, etc.) it has been concluded by many that hypoxia or increased oxygen utilization may be the actual stimulus that induces myocardial cell growth. Beznak (1958) showed that immediately after aortic constriction in rats, the arteriovenous oxygen difference increased at the same time that the cardiac output was down. Although the total oxygen consumption was slightly lower than normal, the tissues evidently compensated for the diminished cardiac output by removing more oxygen from the blood.

2. Dilatation - The classical idea based on Starling's law of the heart is that the ventricles adjust in fibre length to expel whatever volume is put into them even if there is a change in inflow or diastolic pressure. So the first response of the heart to increase in blood pressure is thought by many to be dilatation. Most of the pathological conditions encountered by the heart would thus be capable of producing a change in fibre length as a regulatory mechanism. A theory based on dilatation causing myocardial injury was proposed by Eyster in 1927.

3. Tension-time index - Another school of thought believes that myocardial fibres do not stretch but maintain isometric contraction with adjustments in tension only produced per unit cross-sectional area. Rushmer, (1959) stated "when increased oxygen consumption was not accompanied by rapid heart rate, the stroke volume increased." Tension-time index refers to the total area under the systolic pressure curve.

4. Tachycardia - Sarnoff (1958) related tension-time index and increased oxygen utilization to an increase in heart rate rather than stroke volume. Beznak (1964) suggests that a period of tachycardia might be the triggering mechanism in every case for a weight increase of the heart.

5. Hypertension - Genetically hypertensive rats are shown to have relatively larger hearts than normotensive rats and this suggests to many that hypertension may play a leading role. Most of the methods which produce experimental hypertrophy also cause hypertension, but the fact that hypertension and hypertrophy are not always correlated (Beznak 1954) seems to indicate the participation of other factors.

6. Hormonal control - Hormones are necessary to control normal growth in animals and plants. Usually a combination of hormonal factors is required. Beznak (1952) showed that hypertrophy only occurred in hypophysectomized, constricted aorta, animals with growth hormone and thyroxine. A humoral factor has been demonstrated to be necessary for the control of liver regeneration (Glinos 1958).

7. Neurogenic control - In 1948 Beznak and Hajdu showed that extirpation of the stellate ganglia causes a gradual increase in the heart weight of albino rats. Nerve tissue is known to play an important part in control of regeneration of tissue in lower animals (Singer 1960). The role of the nervous system in control of the heart (e.g. vagal stimulation and bradycardia) was re-emphasized by Sarnoff and Mitchell in 1961.

8. Water Balance - That tissue water alterations might in some way stimulate cardiac enlargement is not a new idea. It has long been known that exercised, striated muscle takes up water and Loeb (1894) assumed that this was due to increased osmotic pressure leading to absorption of water within the muscle fibres. The volume of muscle fibres was thereby increased and this was thought to be followed (or accompanied) by deposition of new material.

In 1911, Stewart reported a distinct, though small, increase in water content of the heart in early stages of hypertrophy in dogs on whom aortic valvulotomy had been performed.

Hastings, et al (1939) reported an increase of 10% in the extracellular phase of heart muscle following a short ligation of a coronary artery. This was believed to be the result of both an increase in interstitial fluid and changes in permeability due to injury. A few years later, Hitchings, et al (1942) concluded that cardiac hypertrophy began to occur within the first three days after aortic valvular rupture in rabbits. It was characterized within this period by an increase in sodium chloride and water in the extracellular phase of a magnitude amounting to interstitial edema. Hitchings et al compared this transient increase in extracellular phase and other chemical changes in early hypertrophy to changes which occur in other tissues which have been stimulated to rapid growth by means of hormones.

Beznak (1954) noted that in a group of rats, five days after narrowing the aortae, the percentage dry-matter content of the hearts had decreased compared to normal controls. Earlier (1944) Hajda and

Beznak observed that the right ventricles behave somewhat differently than the left (in which no appreciable change in per cent dry weight or ash content pro mille was noticed). Regarding the right ventricle, they observed, "Though its weight does not change there is a significant diminution of dry elements. At the same time the ash content is unchanged, the only possible explanation is that the right ventricle lost organic materials which were replaced by some fluid having the same ash content as the heart."

Krakower and Heino (1949) who produced hypertrophy in chickens within 24 hours by feeding excess NaCl, claimed an increase in total nitrogen and potassium as well as total water and total chloride. To them this indicated increases in both extra- and intracellular water, but differences were not statistically significant. Skeletal muscle showed similar increases, therefore, interstitial edema may have been present throughout the system.

Although a few careful studies, such as the above mentioned, included measurements of dry weight and total water in normal and hypertrophied hearts, the slight changes noted were not usually significant, especially in comparison to other changes. Thus water, as a factor involved in hypertrophy, became disregarded.

It is the purpose of the present investigation to assess any changes occurring in water balance in cardiac muscle preceding cardiac hypertrophy. Distribution of water between extra- and intracellular phases will be examined in normal and in hypertrophied hearts prior to

and during the onset of hypertrophy.

The main model used to produce hypertrophy is that of aortic constriction in the rat.

A variation of the radioisotope dilution method is employed to measure extracellular fluid volumes and many reasons are given supporting S^{35} -sulphate as the choice of radioactive isotope for estimation of ECF volume in myocardium.

Because changes in water balance are closely related to changes in electrolyte balance, the final section of the work is entirely devoted to measurements of sodium, potassium and phosphate in the same regions of normal and hypertrophied hearts.

At each stage of the investigation, emphasis is placed on changes in the measured parameters which occur in normal hearts due to normal growth and development. Wherever possible, values for normal rats, covering the entire range of size of experimental animals i.e. 150 to 450 g, are given. Changes in hypertrophied hearts are recorded as differences over and above normal, unoperated controls so that normal growth factors and differences entirely due to normal growth, are not included in changes recorded.

The review of the literature is divided into three main categories:- 1) a general review of cardiac hypertrophy investigations 2) a review of extracellular fluid volume research and 3) theories regarding ion distribution in muscle tissue. The latter two appear at the beginning of the chapters devoted to these topics.

CHAPTER II

General Methods

1. Preparation of experimental model

Male albino rats, Wistar strain, with initial weights of 180 to 250 grams were used for all experiments. Rats were received at 7 weeks of age, weighing 160 to 200 grams. They were placed in individual wire cages and fed Master Laboratory Cubes and tap water, ad libitum.

Method used to produce cardiac hypertrophy:-

Aortic constriction - The constricting rings were made by winding silver wire around a hypodermic needle of suitable diameter (0.8 mm). The spiral was then sawed longitudinally with a very thin blade to give a number of separate rings which are opened slightly and filed smooth under magnification. The rats were anaesthetized with ether, an incision made in abdomen and viscera displaced. The ring was then slipped under the aorta, just below the diaphragm, and closed with forceps above the aorta. The viscera were replaced and incision sutured.

Collection of Samples

All rats used in the radioactive tracer experiments were anaesthetized with 2% Nembutal (0.2 ml per 100 g. body weight) injected intraperitoneally. Ether anaesthetic was used for other groups.

a) Blood samples -

For radiotracer experiments - blood was withdrawn from the abdominal aorta using a heparinized syringe. The needle was unscrewed

from syringe and blood allowed to drain into a 10 ml test tube. After standing 10 - 20 minutes, a few polystyrene crystals were dropped into each test tube (to help prevent hemolysis) and sample centrifuged for 15 minutes. For electrolyte experiments - the abdominal cavity was wiped, then aorta clipped below renal arteries in two places, approximately 1/2 inch apart. Then aorta was cut, clip released and blood collected in 20 ml test tube. This sometimes produced mixed (venous and arterial) blood samples but exsanguination was considered to be a better procedure when electrolytes were to be measured in tissues.

b) Tissue samples -

The complete heart was excised, cleared of pericardium, attached fat, etc., placed on slightly moist filter paper (no. 1) and dissected into the following samples:- atria (combined), right ventricle wall (separated from interventricular septum), left ventricle wall (separated from interventricular septum), septum and a sample of skeletal muscle from upper thigh region. Samples for the tracer studies ranged from 30 to 80 mg., samples for electrolyte and total water determinations consisted of remaining tissue. Each sample, as dissected, was lightly blotted on no. 1 filter paper and immediately weighed on a torsion balance and weight recorded. Weights of all samples were totalled, to give the total heart weight in each case.

2. Radioactive analysis of C¹⁴ and S³⁵ by liquid scintillation counting

Cocktail composition - Scintillator counting solution was prepared as follows:-

Primary fluor - PPO (2,5-diphenyloxazole) - 4 g per litre solvent
Secondary fluor - POPOP (1,4-di 2(5-phenyl-oxazole)-benzene - 0.5 g per litre solvent
Solvent - Toluene (good chemical reagent grade) - dissolve above ingredients in small amount of toluene and make up to 1 litre.

a) Plasma samples - (done in duplicate) Aliquots of 0.1 ml of plasma (measured with a 100-lambda micropipette) were pipetted into glass counting vials. One ml of Nuclear Chicago Solubilizer (NCS) was added. When solution clear, 15 ml of Scintillator counting fluid added.

b) Tissue samples - Weighed samples were placed directly into glass counting vials and 1 ml of NCS added. Vials were then capped and placed in Dubnoff vibrator with water bath set at 36° C until sample completely dissolved and solution appeared absolutely clear against a dark background with cross lighting. Then 15 ml of scintillator solution was added.

Apparatus

All radioactive samples were counted in a Nuclear Chicago Liquid Scintillation System, Mark I with Computer, Model 6860. This is a three channel scintillation counter, geometry optimized, with external standardization which can be used optionally.

The capped, glass vials containing the liquid samples were automatically lowered into a well-type counting chamber (temperature controlled at 4° C). The scintillation solution converts to light the

energy of the primary particles (Beta particles) emitted by the radioactive sample. Two low-noise, 180° horizontally opposed, 13 stage, EMI type multiplier phototubes respond to the light energy by producing a charge pulse which is amplified and counted by a scaling circuit. The circuit responds to a selected range of charge pulses (voltage pulses) and hence to the desired range of energies. In this way the effect of unwanted background counts is minimized.

Statistical accuracy - Because radioactive decay and emission are random events, the longer the counting time, the closer one approaches the actual mean count rate. When n equals the number of counts observed, the standard deviation for each sample, σ , equals \sqrt{n} . The relative standard deviation equals $\sqrt{n} \times \frac{100}{n}$ or $100/\sqrt{n}$. In order to work to 1% standard deviation i.e. for $\frac{100}{\sqrt{n}}$ to equal 1, n must be 10,000.

Therefore, each sample was counted for 20 minutes, so that at least 10,000 counts were recorded to give standard deviation = $\pm 1\%$.

Counting efficiency determined by Channels Ratio Method

A set of quenched standards is necessary for each radioactive isotope used. These may be prepared by adding increasing amounts of acetone to vials containing known amounts of the isotope but the commercially prepared and sealed standards which have been assayed so that exact disintegration per minute (d.p.m.) are known, are more accurate. An unquenched standard is included in each set. This standard is used to establish the balance point (maximum count rate) with

discriminators set at 0 and 9.9 volts, in one of the channels, e.g. channel C. In second channel (channel B), the upper level discriminator is adjusted until the counts in channel B are about $\frac{1}{3}$ of those in channel C. (Fig. 1) Now the ratio of counts in Channel B / ratio of counts in Channel C equals 0.3 for the least quenched standard.

Anything which prevents some of the light flashes from reaching the photomultiplier tube is called a quencher. When quenching occurs the average energy of the pulses is decreased, and the spectrum shifts toward lower energy. If the discriminator settings have been properly chosen, the ratio of the two channel counting rates will change in a way which is a useful measure of the efficiency. Baillie discovered this in 1960.

Channels Ratio Quench Correction Curve (Fig. 2)

From the known d.p.m. for each standard in the quenched series and from the observed counts per minute (c.p.m.) for each standard, the percent efficiency is calculated. This is plotted against the channels ratio for each standard to give a quenching correction curve.

Corrections necessary for Radioactive counting

1) Background counts - A blank sample was counted with each set of samples to determine background counts and to check for contamination. The background counts per minute (usually around 32 c.p.m.) were subtracted from the total c.p.m. for each sample.

2) Radioactive decay (for S³⁵-sulphate samples only)- This correction is negligible for carbon-14 since its half-life is over 5,000

FIGURE 1

RELATIVE CHANNEL SETTING OF ANALYSER
FOR S³⁵-SULPHATE CHANNELS RATIO COUNTING

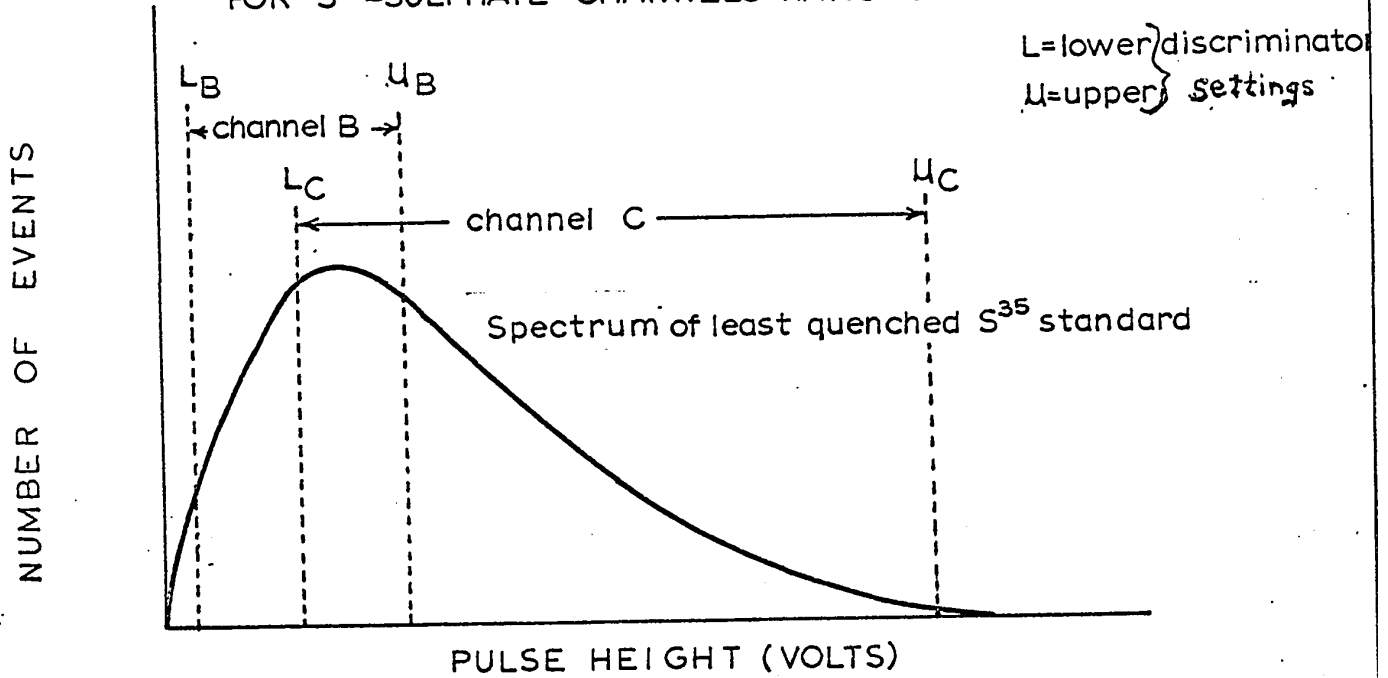
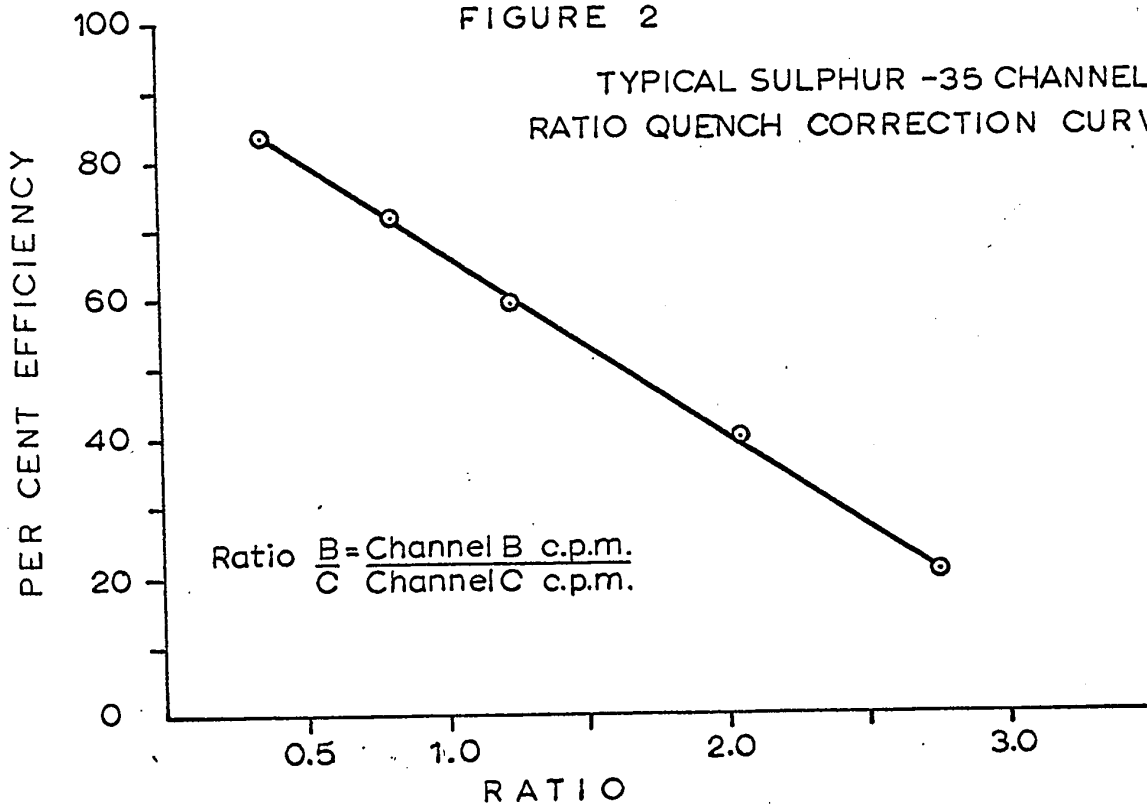


FIGURE 2

TYPICAL SULPHUR -35 CHANNELS
RATIO QUENCH CORRECTION CURVE



years. However, sulphur-35, having a half-life of 87.1 days, it is necessary to correct the observed counts of standards and samples back to the assay date. A prepared chart was used to find the per cent decay for the particular number of elapsed days. e.g. T_{1/2} 6717 on day 50 meant that the tracer had decayed to 67.17% since assayed, so counts were calculated back to T_{1.00} or 100% of d.p.m.

3) Quenching corrections - Substances which cause quenching in the samples, thus preventing all the light flashes from reaching photomultiplier tubes, include any coloured compound, hydrogen ion concentration, water, organic substances, etc. The main cause of quenching in these samples was the presence of hemoglobin e.g. hemolysed serum or muscle samples containing more myoglobin. Therefore, heart muscle always showed more quenching than skeletal muscle. The channels ratio, printed by the computer for each sample, was referred to the quench correction curve (Fig. 2) and the per cent efficiency read for each sample. The efficiency was usually between 70 and 80% and from this 100% of d.p.m. calculated (the absolute activity).

4) Loss due to Dead Time - the pulse pair resolution time for the liquid scintillation counter used was 200 μsec. Therefore, theoretically, % counts lost = $\frac{n^2 T}{60 \times 60} \times 100$ where T = resolution time, n = count rate. Since this works out to only about 1% of 12,000 counts and would be applied to all samples, so that the ratio of $\frac{\text{c.p.m. tissue}}{\text{c.p.m. in serum}}$ would not be significantly altered, therefore,

this correction factor was not included in calculations.

3. Chemical Analyses

a) Total water determination

Weighed tissue samples were placed in pre-weighed, numbered platinum crucibles. Crucibles were covered but a small air space was left between lid and rim. They were placed in drying oven at 95° C for 24 hours, then transferred to a vacuum oven for 15 hours after which they were cooled for 4 hours in a desiccator. Crucibles and dried contents were then weighed, at least twice to make sure they had reached a constant weight. The tissue dry weight was recorded, the per cent dry weight to wet weight calculated; and the total water, by difference, recorded as ml per kg wet weight of tissue.

b) Total Sodium and Potassium determinations

Dry Ashing Method - Crucibles and dried tissue contents were placed in a muffle furnace at 500° C for 24 hours. At the end of this time the furnace was turned off and the samples allowed to cool. Then 2 ml of 5N HCl was added to each crucible to dissolve the ash. Crucibles were placed over a boiling water bath for 30 minutes. Next, the contents of the crucibles were carefully transferred to 10 ml volumetric cylinders, using a Pasteur pipette for washing lid and crucible several times with boiling, deionized, double-distilled water and a special bulbed dropper for the actual sample transfer and washings. When completely cool, the cylinders were made up to volume (last 0.5 ml or less) and sample transferred to labelled polyethylene storage tubes with caps.

Samples were stored in the freezer and aliquots removed as needed for electrolyte determinations.

Alternate methods tried included water extraction methods, with and without sonication, and wet ashing methods using a) concentrated nitric b) hyamine hydroxide and c) Nuclear Chicago Solubilizer.

There is not space to compare results from all the above methods, nor was there time to test fully the merits of each. In general the wet ashing methods had great merit in time saving, but required elaborate preparation of standards because the strong solubilizers necessary to dissolve the tissue interfered with flame photometry. Some of the wet ashing methods affected phosphate determination results, also. Water extraction methods offered too much variation and solutions which appeared quite homogeneous after sonication, tended to block atomiser of the flame photometer. Finally, sodium and potassium recovery studies were performed, using the dry ashing method and this method was used for all electrolyte values reported.

Flame Photometry

Aliquots from the 10 ml samples in storage tubes were diluted and the clear solutions measured by flame emission on a) a Coleman Flame Photometer, Model 21, using oxygen and natural gas as fuel or b) a Jarrell-Ash Flame Photometer, Model 32-600, using hydrogen as fuel and compressed air. When the switch from a) to b) became necessary, 150 control samples were re-measured and the values showed no significant difference between the sodium and potassium measurements taken on either machine.

Factors affecting flame photometry:

1) Hydrogen ion concentration - Standards in distilled water and standards in 0.1 N HCl (as in samples diluted 1:100) were compared. The value for each standard in acid was depressed and the calibration curve shifted toward the right. Therefore, accurate comparison could only be obtained by having the same concentration of hydrogen ion in standards and blank as in samples.

2) Sodium and potassium radiation interference - Comparing potassium standards in distilled water with those containing excess sodium, 25 meq/l (approximately the ratio of Na:K in serum), it was found that excess sodium has an enhancing effect on the per cent transmittance of each standard, thus shifting the calibration curve to the left. Therefore, it was decided to incorporate in the standards, the same proportion of Na to K as is normally found in the samples being measured, e.g. serum 30:1, skeletal muscle 1:4 and heart muscle 1:2.

Preparation of Standards

Since samples, after dry ashing, were dissolved in HCl, and the wide range of sample sizes required different dilutions to fall into measureable range of the flame photometer, therefore, standards containing different concentrations of HCl were prepared. Also, standards containing different proportions of Na to K were prepared for measuring serum, skeletal muscle, and heart muscle. Deionized, metal- and glass-distilled water was used in all standards and samples and many other precautions exercised to prevent contamination by glassware

utensils, etc.

Stock Solution I (for K determination) - KH_2PO_4 was placed in the drying oven for 4 hours and cooled. 68.045g (0.5 M) of KH_2PO_4 were dissolved in glass distilled water and the volume was made up to 1000 ml. This solution contains 500 milliequivalents of potassium per litre.

Stock Solution II (for Na determination) - after drying for 4 hours and cooling, 35.495 g (0.25 M) of Na_2HPO_4 were dissolved in glass distilled water and the volume made up to 1000 ml. This solution contains 500 milliequivalents of sodium per litre.

A concentrated working standard solution was prepared as follows:-

20 ml of stock solution I were placed in a 200 ml volumetric flask and 100 ml distilled water added and mixed. 10 ml of stock solution II were added slowly to the above with constant stirring and finally made up to the mark with water. This working standard contains 50 milliequivalents of K^+ and 25 milliequivalents of Na^+ per litre.

This concentrated working standard was used in the dilute working standards used for measuring heart muscle and one containing 50 milliequivalents K^+ and 12.5 milliequivalents Na^+ per litre was prepared for skeletal muscle standards. Sterox (a commercial detergent for flame photometry) was added to standards and samples to cut down capillary suction in atomiser.

A typical set of dilute working standards was prepared by placing the following amounts of concentrated working standard and

other ingredients into a 500 ml flask and bringing to the mark with distilled water:

TABLE I
Dilute Working Standard Solutions
for Sodium and Potassium determination in Heart Muscle

<u>Volume of the conc. working standard (ml)</u>	<u>Amount of 5N HCl</u>	<u>1% Sterox (ml)</u>	<u>Concentration of Dilute Working Standard</u>	
			<u>K (meq/l)</u>	<u>Na meq/l</u>
1	10 ml	10 ml	0.1	0.05
2	"	"	0.2	0.1
3	"	"	.3	.15
4	"	"	.4	.2
5	"	"	.5	.25
6	"	"	.6	.3
8	"	"	.8	.4
10	"	"	1.0	.5

After diluting to 500 ml each of these dilute working standards was stored in a labelled polyethylene bottle with screw cap.

Calibration curves were prepared with each set of samples. The average percentage error was found to be less than 1% when the concentration of the standard solutions was within range of 0.1 meq ion/litre to 1.0 meq ion/litre for the Coleman flame photometer and 0.01 to 0.1 meq ion/litre for the Jarrell-Ash flame photometer.

Measuring Serum Sodium and Potassium

Serum was diluted with distilled water, therefore, standards in distilled water only were used. Dilutions and standards were done in one of two ways:-

a) Against separate sodium and potassium standards - For potassium measurement, serum was diluted 1:100 and measured on Jarrell-Ash photometer against potassium standards in distilled water, range 0.01 to 0.1 meq/litre. For sodium measurement, serum diluted 1:2500 and measured against sodium standards in distilled water with range of 0.01 to 0.1 meq/litre.

b) Against standards containing sodium and potassium in the proportion of 30 to 1 (as present in normal serum) to overcome interfering radiation.

Sodium and Potassium Recovery Studies

The efficiency of sodium and potassium recovery when using the dry ashing method (page 20) was tested by adding a known amount of each electrolyte, equal to its estimated content, to separate samples of skeletal muscle, approximately 300 mg in size each, from the left thigh muscles of normal rats (8 male, Wistar strain) and comparing with control samples, 600 mg, from the corresponding right thigh.

Two 300 mg samples were removed from left thighs and labelled Left A group and Left B group. To Left A group - 0.0075 meq sodium (in 1 ml) was added to each sample. To Left B group - 0.030 meq potassium (in 1 ml) was added to each sample. The added electrolytes were

TABLE II a
 Recovery of sodium added to skeletal muscle
 of male, Wistar rats averaging $286 \pm 8^*$ g in body weight

Rat #	Right Thigh Control Samples	Left Thigh Recovery of sodium chloride added to left thigh samples					Total Na measured μ equiv	Per cent recovered
	Total Na meq/kg or μ eq/g	Left thigh sample wet wt.	Na in sample if left = right μ equiv	Added NaCl μ equiv	Total Na expected μ equiv			
1	16.59	0.324 g	5.375 +	7.500 =	12.875 =	12.60	97.86	
2	16.90	.238	4.022 +	7.500 =	11.522 =	11.35	98.51	
3	17.89	.236	4.222 +	7.500 =	11.722 =	11.65	99.39	
4	18.62	.326	6.070 +	7.500 =	13.570 =	13.50	99.48	
5	18.41	.369	6.793 +	7.500 =	14.293 =	14.15	99.00	
6	19.52	.298	5.817 +	7.500 =	13.317 =	13.45	100.99	
7	17.75	.280	4.969 +	7.500 =	12.469 =	12.60	101.05	
8	17.48	.328	5.733 +	7.500 =	13.233 =	12.97	98.01	
Mean	17.90						99.29	
\pm SEM	± 0.34						± 0.43	

* Standard deviation

TABLE II b

Recovery of potassium added to skeletal muscle
of male, Wistar rats averaging $286 \pm 8^*$ g body weight

Rat #	Right Thigh Control Samples		Left Thigh Recovery of potassium chloride added to the left samples				
	Total K meq/kg or μ eq/g	Left thigh sample wet wt.	K in sample if left = right μ equiv	Added KCl μ equiv	Total K expected μ equiv	Total K measured μ equiv	Per cent recovered
1	121.4	0.324 g	39.3 +	30.0 =	69.3	67.8	97.8
2	120.6	.238	28.7 +	30.0 =	58.7	58.2	99.2
3	120.2	.236	28.3 +	30.0 =	58.3	57.5	98.5
4	120.9	.326	39.4 +	30.0 =	69.4	67.9	97.8
5	120.1	.369	44.3 +	30.0 =	74.3	73.8	99.3
6	118.7	.298	35.4 +	30.0 =	65.4	66.0	100.9
7	121.9	.280	34.1 +	30.0 =	64.1	65.2	101.6
8	120.8	.328	39.6 +	30.0 =	69.6	69.5	99.8
Mean \pm SEM	120.6 \pm 0.34						99.4 \pm 0.49

* Standard deviation

in the form of chloride salts in each case. Samples were then ashed and made up as described under General Methods (page 22). Because of variations in the control samples, eg. sample 6 with highest Na content and lowest K, it was decided to calculate expected sodium and potassium in left thigh samples from the amounts found ⁱⁿ corresponding right thigh samples, rather than from the mean in all controls. Table IIa and IIb show the results of individual sodium and potassium recovery of 8 sets of thigh muscle samples. This study was repeated several times when it was discovered that the ashing temperature had a very significant effect on sodium and potassium chlorides and possibly on other sodium and potassium compounds present in the tissue. It was decided that henceforth the ashing temperature would be kept as low as was feasible for complete ashing; 500° C was the temperature at which all samples were ashed, for all electrolyte results in this study. A temperature closer to 450° C would have been better for complete recovery but resulted in a small residue of insoluble black ash, especially in the case of heart muscle samples.

c) Determination of Total Phosphate

The method used was adapted for muscle determination from "Methods for the Determination of Phosphate, Calcium and Protein in Small portions of mineralized tissues" by J.E. Eastoe.

Reagents used

Ammonium molybdate-sulphuric acid reagent

118 ml of sulphuric acid was mixed with 350 ml of water and allowed to cool.

12.5 g ammonium molybdate $(\text{NH}_4)_6 \text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ was dissolved in this solution and diluted to 500 ml and stored in a polyethylene bottle.

Aminonaphthol sulphonic acid reagent

dissolve 0.10 g of 1-amino-2-naphthol-4-sulphonic acid, 2.5 g sodium metabisulphite and 1 g of crystalline sodium sulphite ($\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$) (or 0.5 g anhydrous Na_2SO_3) in water. Dilute the solution to 50 ml and filter. This reagent must be prepared freshly each week.

Standard Phosphate solution

A stock solution containing 500 parts per million (p.p.m.) of P was prepared by dissolving 2.196 g potassium dihydrogen phosphate ($\text{K H}_2\text{PO}_4$) in water and diluting to 1 litre. This was diluted 10 ml to 1 litre to produce a 5 p.p.m. solution.

Procedure

A portion of a solution containing 1 to 10 μg of phosphate phosphorus was measured into a 10 ml volumetric flask and distilled water added to approximately 9 ml. Then 0.4 ml of ammonium molybdate-sulphuric acid reagent was added and solution mixed; then 0.2 ml of aminonaphthol sulphonic acid reagent was added and water to the mark. Solution was mixed and placed in $1\frac{3}{4}$ inch test tubes (B and L) and immersed in a boiling water bath for 10 minutes, then cooled in water at 15°C for 5 minutes.

The optical density of the blue solution was then measured against water containing the same amount of HCl as the diluted samples, in a Bausch and Lomb Spectrophotometer at 815 $\text{m}\mu$ using a wide range tube and red filter.

Calibration curve

The phosphate content of samples was read from a calibration curve prepared beforehand by carrying out the above procedure on measured volumes of the 5 p.p.m. standard phosphate solution made up in 10 ml flasks according to procedure (above) and containing the same concentration of HCl as the samples. The concentration of P (0.001 to .007 mg P) was plotted on the linear side of semilog paper and the per cent transmittance on the log side, to give a straight line.

It was found that this method of heating to develop the intensity of the blue colour has the great advantage that the standards and samples remain stable. Another method by Gomori, also similar to the Fiske and Subbarow (1925) method was also tried but the optical intensity continued to change, even after 24 hours, and hence the per cent transmittance.

The Eastoe method is well suited for the determination of inorganic phosphate and this is the only phosphate ion present since all of the organic phosphates must have been hydrolysed by the ashing method and 5 N HCl. It is a highly sensitive method and small changes in abnormal tissues can be detected.

4. Statistical Analysis of Data

All results are given as mean value plus or minus the standard error of the mean (SEM). Student's t-test is applied when necessary to determine if differences are significant. The "P" value of significance was considered at the 0.05 level of confidence.

CHAPTER III

Normal Growth and Hypertrophy

Introduction

In the review of the literature, Chapter I, it was noted that many of the characteristics of hypertrophied myocardium, at least up to the critical heart weight (66% increase) were very similar to those of normal heart muscle. In this study factors occurring during normal growth will be taken into consideration in all measured differences.

It has also been shown that some factors change during normal growth. For example, Kobayashi and Yonemura (1967) related inulin space in rat skeletal muscle to the reciprocal of the weight of the muscle (and hence the size of the rat). Changes occurring during normal growth will be examined for any similar changes during hypertrophy.

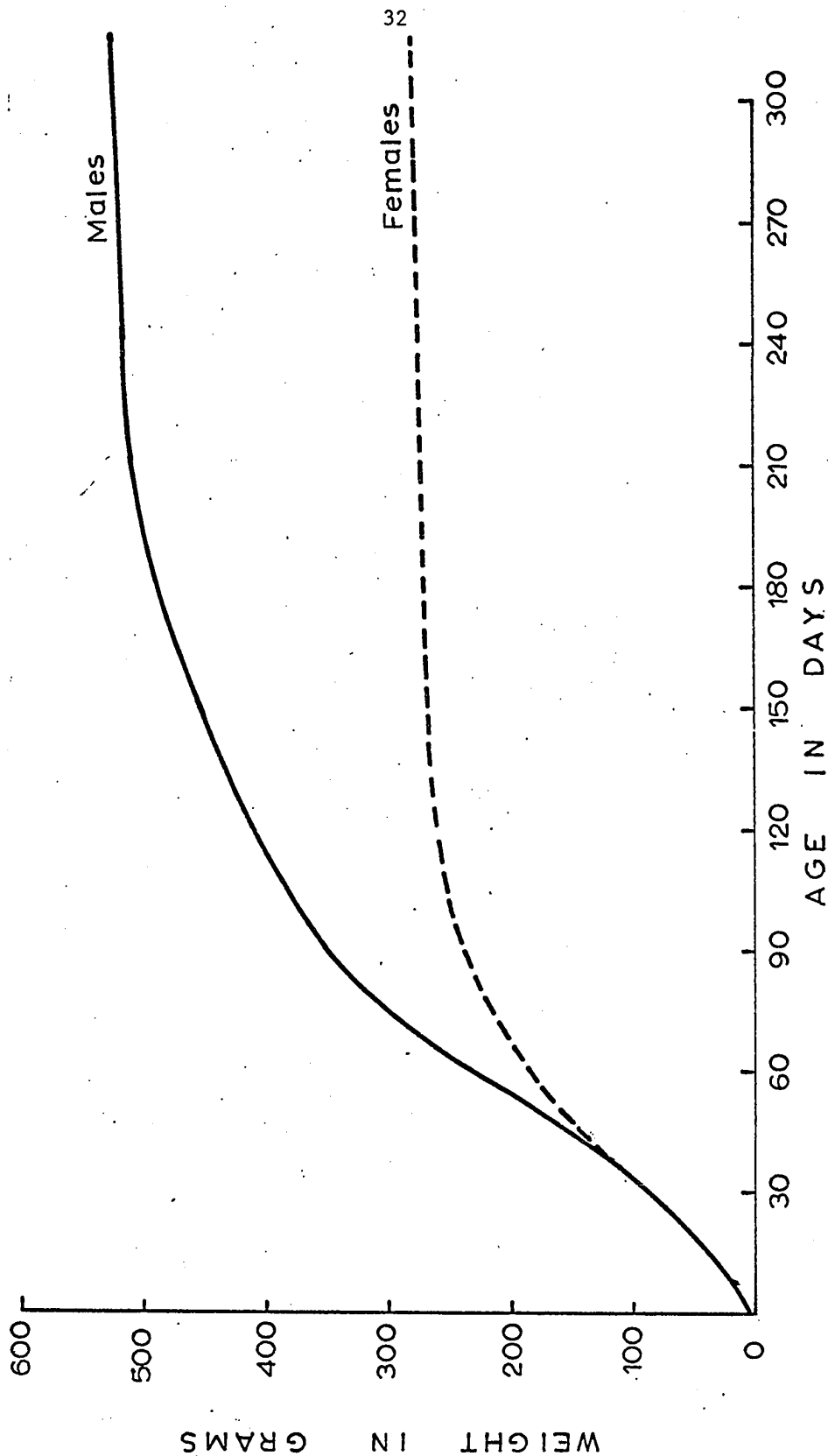
1. Normal growth of the rat

Referring to the growth curve of the rat (Fig. 3) it is apparent that the steepest slope and therefore, the fastest growing period occurs between 200 and 300 grams in the male. This is between 50 and 80 days of age and just before the skeletal muscle of the rat was found to reach chemical maturity at 90 days (Hines and Knowlton, 1939). They also stated that the approach of maturity is associated with a decreased concentration of chloride and water in skeletal muscle and serum.

Many workers have shown that the ratio of the concentration of some ions, e.g. Na, K and Cl in the cell to their concentration in

FIGURE 3

GROWTH CURVE OF THE NORMAL RAT



Ref - Science Service of
Canadian Breeding Laboratories

the extracellular fluid changes in the process of embryonic development and as the organism approaches old age.

2. Normal heart growth

Studies on composition of human hearts at various stages of development before and after birth show that the proportion of water falls with development and the concentration of nitrogenous constituents rises (Widdowson and McCance 1960). The changes are smaller in cardiac muscle than in skeletal muscle.

At birth the heart muscle is believed to have its full quota of muscle fibres and subsequent growth is brought about by "hypertrophy" of existing fibres.

In 1954 Beznak published an important document showing linear regression of heart weight on body weight in rats. In normal rats in the 120 to 240 g body weight group, the expected heart weight in mg can be calculated from the equation $1.88 X + 232$ where X represents body weight in grams. Later the equation $2.14 X + 295$ was published for Wistar strain rats available in this country (1967). These equations were obtained by the method of least squares.

3. Criteria for determining true hypertrophy

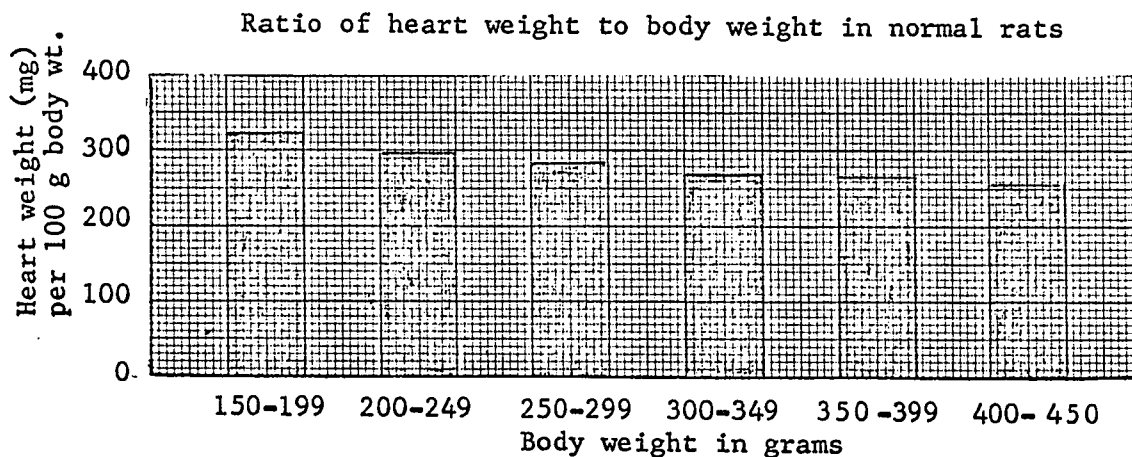
In evaluating changes which occur before or during cardiac hypertrophy, it is important to determine as closely as possible the amount of time which elapses between the application of the stimulus to produce hypertrophy and the response of cardiac enlargement as

distinguished from normal growth. It has been found that the time and degree of hypertrophy vary in different experimental models. The cardiac hypertrophy considered in this section was produced by aortic constriction in rats.

Although other criteria are cited in the literature (e.g. the ratio of dry heart weight:dry body weight), the three main criteria for determining the degree of hypertrophy are as follows:-

a) Ratio of heart weight:body weight or heart weight:100 g body weight - Although this is the criterion most often used, even in recent studies, two factors immediately skew the results. First, this ratio is not constant even in normal animals, it varies with the size of the animal. Fig. 4 A below shows that when the heart weight:body weight ratio of fifty-seven normal rats, grouped according to size, is plotted, the ratio drops as body weight increases.

Figure 4A



Therefore, this ratio in experimental groups can only be compared accurately with controls of the same size groups. Secondly, there is an immediate fall in body weight following aortic constriction (see Table III). The heart weight drops also, in most cases, as one would expect. In 1954, Beznak showed that linear regression exists between heart weight and body weight even in starvation. However, in the ensuing weeks, following the initial body weight loss after aortic constriction, great variation occurs in the rate of body weight recovery and obviously no correlation exists between the degree of hypertrophy and body weight recovery since some rats, with the greatest degree of increase in absolute cardiac mass, remain in the smallest group according to body weight.

b) Observed heart weight vs. expected heart weight, calculated on basis of terminal body weight - From the rat's final body weight, the expected heart weight can be calculated from equations in section 2 and the percentage deviation in the observed heart weight of the experimental animal calculated. This method is very reliable, with great saving in the number of normal controls which would be necessary so long as one is using the exact strain of rat on which the equation was devised.

c) Direct comparison with normal controls of the same age. - Because some of the cardiac enlargement observed after narrowing could reasonably be credited to normal growth and since this variable is being considered in all other parameters dealt with in this investigation, therefore it is considered here as well. As can be seen in

TABLE III
Effect of aortic constriction on heart and body weights

Interval of time since narrowed	Experimental Group					Unoperated Controls				
	No. Rats	Initial wt g	Final wt g	Heart wt mg	Heart wt/100 g b.w.	Calculated Heart wt	No. Rats	Final body wt g	Heart wt mg	Heart wt/100 g b.w.
12 hours	5	185.7 ± 2.3	176.3 ± 0.8	541.5 ± 0.5	306 ± 2.0	567.8 ± 7.9	3	181.3 ± 0.9	604.0 ± 14.0	331.1 ± 8.5
24 hours	13	237.6 ± 9.7	218.4 ± 9.2	616.3 ± 15.2	287.6 ± 12.3	642.7 ± 17.2	5	240.2 ± 2.5	680.2 ± 26.7	283.2 ± 11.0
2 days	3	175.0 ± 2.5	173.7 ± 6.2	**620.7 ± 7.8	358 ± 8.1*	558.3 ± 11.7	3	175.0 ± 2.6	561.3 ± 32.9	320.5 ± 15.9
3 days	10	269.1 ± 4.3	253.9 ± 3.3	703.6 ± 16.6	277.1 ± 5.2*	709.2 ± 6.2	4	262.5 ± 2.2	663.0 ± 13.0	253.4 ± 5.3
4 days	5	178.5 ± 8.8	177.8 ± 2.8	**698.3 [†] ± 30.6	403.3 ± 12.2*	554.0 ± 10.9	11	187.5 ± 5.4	590.8 ± 13.1	317.7 ± 7.2
1 week	17	233.3 ± 8.8	224.5 ± 7.3	**805.4 [†] ± 28.3	366.2 ± 18.6*	654 ± 13.7	6	244.2 ± 11.9	691 ± 22.3	266.2 ± 7.6
10 days	5	185.0 ± 1.2	224.3 ± 3.2	912.3 [†] ± 18.6	407.0 ± 14.2*	653.7 ± 5.8	3	230.6 ± 4.2	750.1 ± 11.1	325.0 ± 4.0
2 weeks	17	232.9 ± 9.5	263.5 ± 12.4	**940.1 [†] ± 34.7	377.5 ± 31.1*	714.8 ± 21.7	4	342.0 ± 8.7	821.5 ± 30.1	240.9 ± 12.0
3 weeks	6	169.7 ± 1.9	257.3 ± 5.6	**1,010.2 [†] ± 58.1	395.2 ± 28.5*	715.7 ± 10.5	6	272.5 ± 1.8	771.7 ± 12.7	283.2 ± 3.5

Continued

TABLE III continued ..

Interval of time since narrowed	Experimental Group					Unoperated Controls				
	No. Rats	Initial wt g	Final wt g	Heart wt mg	Heart wt / 100 g b.w.	Calculated Heart wt	No. Rats	Final body wt g	Heart wt mg	Heart wt / 100 g b.w.
4 weeks	15	203.0 ± 6.9	313.1 ± 5.8	** 1,114.2 ± 37.0	* 358.6 ± 15.2	820.6 ± 10.8	6	317.8 ± 5.6	854.8 ± 15.1	268.9 ± 5.6
6 weeks	6	170.5 ± 4.8	319.0 ± 15.8	** 1,469.3 ± 107.6	* 458.9 ± 37.1	831.7 ± 29.6	3	370.7 ± 9.3	991.5 ± 35.1	268.0 ± 13.6
8 weeks	5	174.2 ± 3.3	370.4 ± 12.8	** 1,522. ± 92.6	* 412.2 ± 27.6	928.3 ± 24.1	6	424.2 ± 8.5	1,096.5 ± 19.9	258.8 ± 3.3

* P < 0.05 significantly greater than heart wt/100 g body of unoperated controls

** P < 0.05 over calculated heart wt

+ P < 0.05 over unoperated controls

Table III, the rats on which aortic constriction has been performed never do catch up to the unoperated controls in body weight, in fact the gap seems to widen. However, this drop in weight appears to be due to undergoing the operation in the first place. Morikin et al (1968) showed that after 5 days the body weight loss of sham-operated rats was slightly greater than that following aortic constriction. Possibly sham-operated normal rats should be used for this comparison.

4. Degree of hypertrophy

It became apparent as the experiments progressed that the absolute amount of cardiac enlargement following aortic constriction varied in different groups of rats. In general it appeared that the size of the rat when the constriction was applied was a factor since the degree of hypertrophy was so much greater in rats in which the aorta was narrowed at 150 - 200 g than in those narrowed when 250 g in weight. Beznak (1954) also noted greater hypertrophies in rats narrowed when smaller.

It was also found that some of the measured parameters varied with the degree of hypertrophy. Therefore, the experimental groups have been classified as Series A or Series B in terms of this factor.

Series A will henceforth denote all groups in which a high percentage increase in cardiac mass occurred.

Series B will denote those groups in which only mild to moderate increases in cardiac mass occurred.

5. Location of hypertrophy

During moderate hypertrophy most of the increase in cardiac mass occurs in the left ventricle and interventricular septum that is continuous with the left ventricle. Beznak (1944) recorded these changes and showed that the atria increased also but contributed little to the total mass. In hearts hypertrophied above 66% level, Linzbach has shown that the right ventricle begins to hypertrophy also.

Method - Throughout the experiments, heart weights and body weights of the control rats and experimental group were recorded. In some cases, the individual heart structures were dissected and weights recorded.

The data are collected and analysed to show:

- a) heart and body weight changes, in over 100 rats (Series A and Series B combined) following aortic constriction at intervals of 12 hours to 8 weeks.
- b) Expected heart weight calculated on the final body weight.
- c) Body and heart weights for normal unoperated controls at the same intervals are collected.
- d) Changes in size of the atria, left ventricle, right ventricle and septum of a Series A group is recorded.
- e) Changes in size of atria, right ventricle, left ventricle and septum of a Series B group are presented and compared with changes in unoperated controls at same interval.

Observations and Conclusions - Actual changes in the heart and body weights of over 100 rats following aortic constriction are shown in Table III. Expected heart weight calculated on the final body weight is shown in the centre column and body weight, heart weight and ratio of H.W./B.W. are given for unoperated controls on left-hand side of Table III. Thus, the onset of hypertrophy can be judged by the three methods described above:-

Method 1. The ratio of heart weight per 100 g body weight is shown in Table III for unoperated controls and experimental group. Changes after aortic constriction are also shown in Fig. 4B. It can be seen that the ratio drops by the first 12 hours following constriction but then rises rapidly compared with normals so that by the end of 2 days, it is significantly higher ($P < .05$) than unoperated controls.

Method 2. When the actual heart weights of the experimental group are compared to the calculated heart weight (based on final body weight), the cardiac mass is shown to increase significantly ($P < .05$) by 2 days after aortic constriction also.

Method 3. When the experimental group are compared with unoperated controls, the heart weight is not significantly larger until the 4th day following constriction.

The differences in Method 2 and Method 3 are plotted graphically in Figure 5 which shows that the difference between hypertrophied hearts and unoperated controls is usually smaller than between the hypertrophied hearts and the heart weights calculated on final body weight.

FIGURE 4.B

CHANGES IN RATIO OF HEART WT/BODY WEIGHT FOLLOWING AORTIC CONSTRICTION

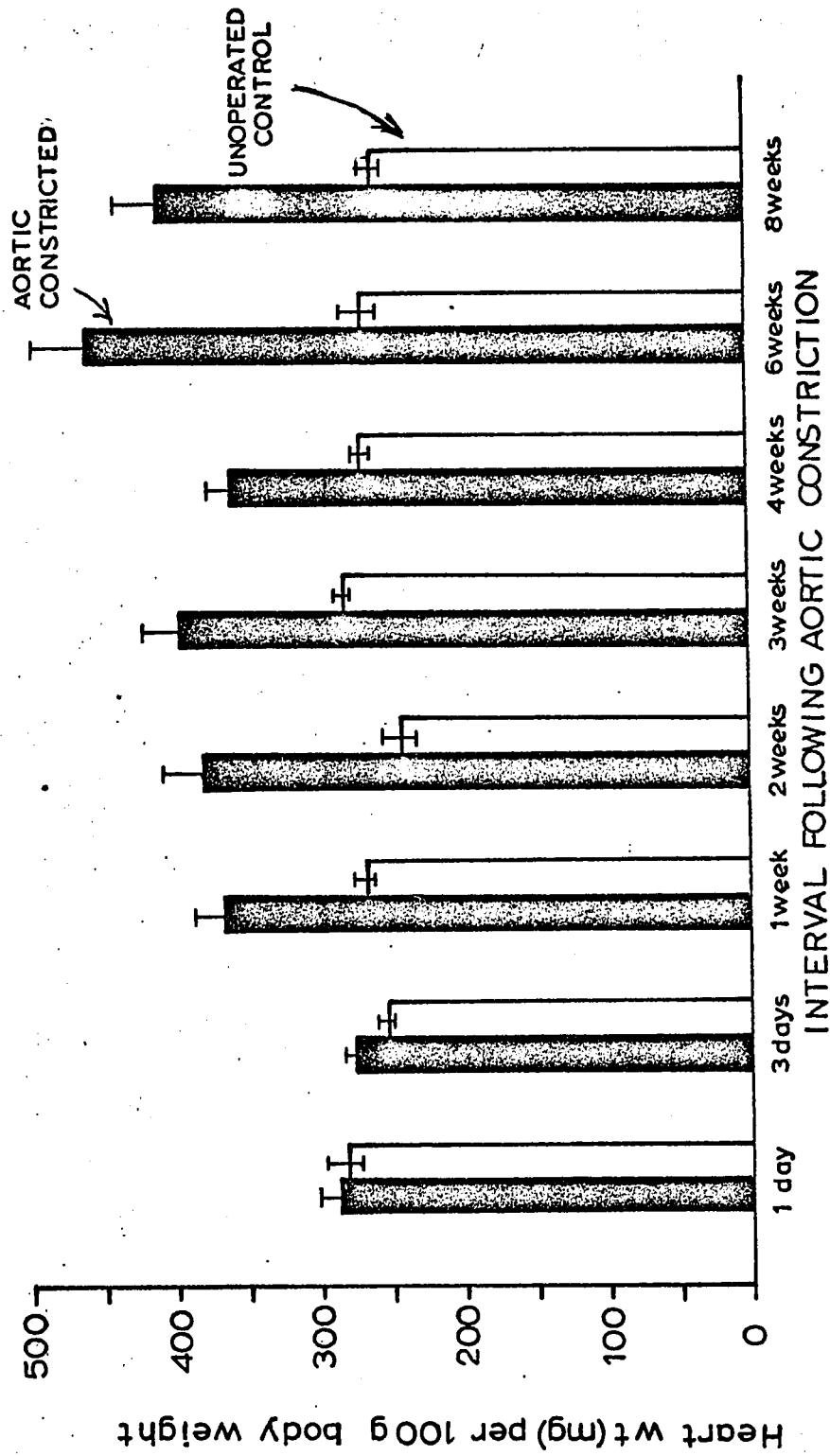
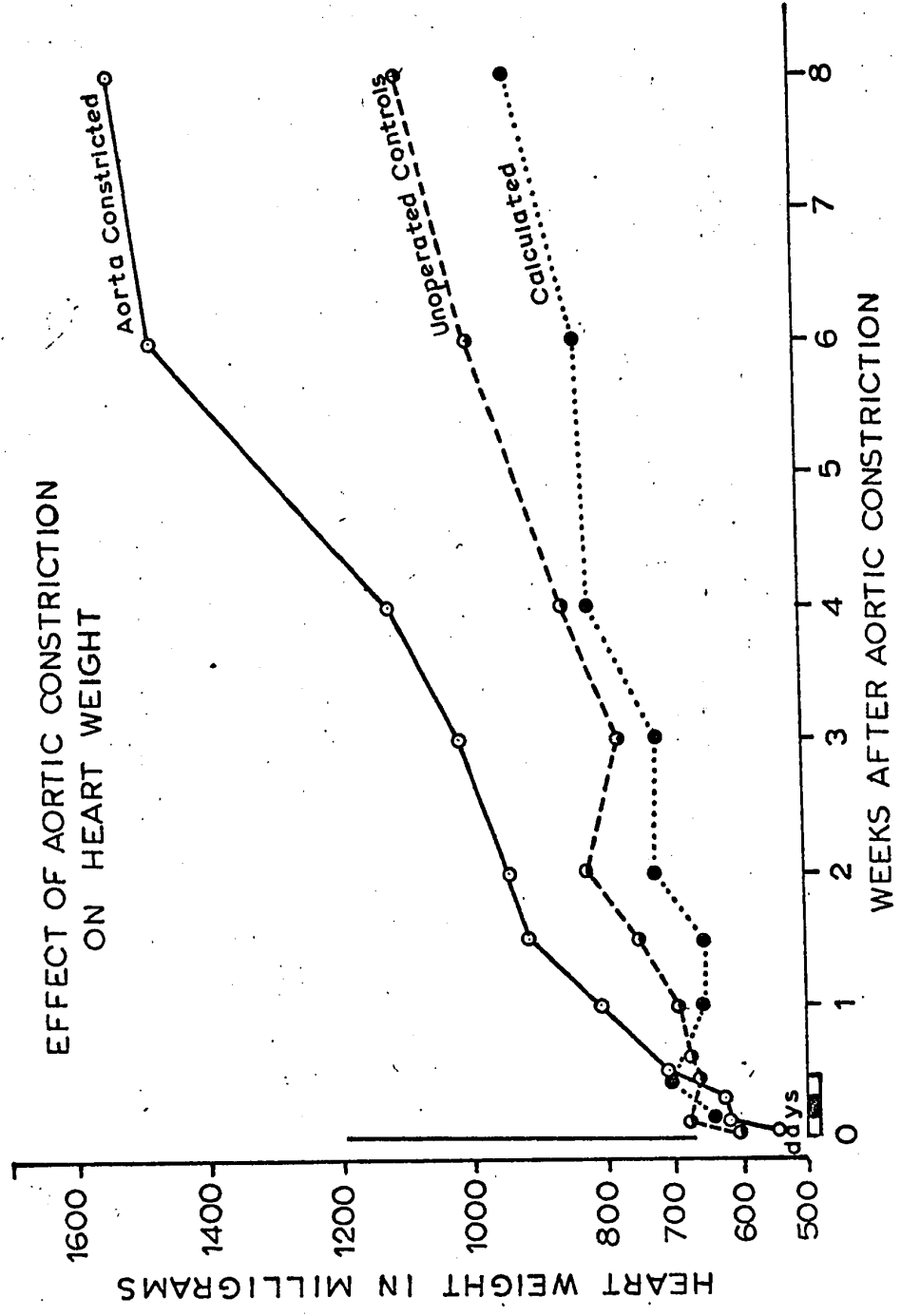


FIGURE 5
EFFECT OF AORTIC CONSTRICTION
ON HEART WEIGHT



Degree of hypertrophy

Table IV shows the changes in weight of heart structures in Series A-4, a small group of rats with cardiac enlargement up to 70% over the control group. A larger group with smaller degree of hypertrophy, Series B-4, also shown in Table IV. If Method 2 were used to judge the degree of hypertrophy this group would be about 30% hypertrophied. Over normal growing controls, the degree of enlargement is only about 15%.

It is apparent that in Series B-1 the entire change in cardiac mass is due to changes in the left ventricle whereas in Series A-4 changes in the right ventricle and atrium are apparent at one week after constriction when the increase in left ventricle and septum has exceeded 70%.

TABLE IV

Changes in weight of heart structures during moderate and severe hypertrophy

Series A-4	No. Rats	Body wt	Atrium		Right Ventricle		Left Ventricle		Septum		Total Heart	
			Controls	Exptl	Controls	Exptl	Controls	Exptl	Controls	Exptl	Controls	Exptl
Controls	4	226 g	32.0 ± 2.3		108.5 ± 4.8		283.8 ± 28		183.8 ± 9.7		608.1 ± 29.8	
1 wk narrowed	4	214.5	44.5 ± 4.9		137.0 ± 10.8		410.5 ± 19.3		319.8 ± 14.1		911.8 ± 34.1	
2 wk narrowed	4	227.0	50.8 ± 8.9		148.3 ± 16.0		418.3 ± 28.3		386.0 ± 38.6		1003.3 ± 88.7	
Series B-1 Interval after aorta constricted	No. Rats		Atrium		R V		L V and Septum		Total Heart			
			Controls	Exptl	Controls	Exptl	Controls	Exptl	Controls	Exptl		
24 hours	10		34.0 ± 2.0	34.5 ± 2.5	130.0 ± 6.0	115.5 ± 3.9	458.5 ± 23.5	476.3 ± 14.8	622.5 ± 27.5	626.3 ± 18.4		
3 days	10		39.0 ± 2.1	41.1 ± 1.9	122.3 ± 5.2	125.2 ± 5.9	503.8 ± 8.1	538.2 ± 10.6	665.0 ± 13.0	704.5 ± 16.4		
1 week	10		40.5 ± 2.5	38.9 ± 2.1	144.5 ± 8.5	140.9 ± 7.4	546.0 ± 7.0	600.4 ± 31.4	731.0 ± 18.0	779.5 ± 39.0		
2 weeks	10		44.5 ± 4.5	48.3 ± 2.7	144.0 ± 12.0	148.2 ± 7.6	595.0 ± 15.0	678.2 ± 24.0	783.5 ± 31.5	874.7 ± 29.4		
4 ¹ / ₂ weeks	9		60.0 ± 4.3	66.9 ± 4.3	170.2 ± 7.3	172.8 ± 5.4	739.2 ± 10.2	841.4 ± 50.2	969.4 ± 21.0	1081.1 ± 57.1		

CHAPTER IV

Fluid Shifts in Normal and Hypertrophied Hearts

1. The extracellular fluid volume in heart and skeletal muscle

Introduction

In 1859 Claude Bernard in Liquides de l'organisme (Paris: Bailliere) defined interstitial fluid as a special liquid in each tissue, which does not circulate. He included it in the classification "Anatomical Extracellular Fluids" along with a) the circulating fluids (blood and lymph), b) cerebrospinal fluid and other stationary fluids and c) secretions. Nearly a century elapsed before Bernard's extracellular fluid (ECF) compartments were rearranged slightly on the basis of ion distribution. Manery (1954) defined the physiological extracellular water as, "Water of the plasma and of fluids into which ions and small molecules diffuse freely from the plasma i.e. liquids which resemble a plasma ultrafiltrate".

Investigation of ECF volume was closely tied in with electrolyte research and both were studied in skeletal muscle sooner, and to a greater extent, than in heart muscle. Histological measurements of interstitial spaces were reported by Hermann in 1888. Electrolyte content of tissues was of great interest around the turn of the century and by 1908 Urano associated sodium chloride with connective tissue and interstitial fluids in a concentration similar to that in plasma. During this first decade many investigations were made concerning the mechanism which prevented the diffusion of sodium chloride from the tissue spaces into the fibres and of potassium

phosphate from the fibres into the spaces. The importance of tissue spaces was well established by 1912 when Meigs estimated the volume of interstitial fluid in both striated and smooth muscle to be between 15 and 25%.

An important era in extracellular space measurement began in 1934 when Fenn reported that all the chloride in frog muscle diffused freely into the surrounding medium. Thus all the chloride was assumed to remain extracellular and from the total chloride content of the muscle and the serum, its "chloride space" could be calculated. First the assumption was made that the concentration of chloride in the interstitial fluid is that of an ultrafiltrate of the plasma concentration, so two factors had to be taken into account:-

a) Water content of the plasma - Because of the presence of the plasma proteins (7.5 g/100 ml in human plasma) the water content of the plasma must be calculated as follows:-

$$[H_2O]_p = 984 - (7.18 \times \text{protein conc. in g/100 ml plasma})$$

This equation was published by Eisenman et al (1936) as tested for protein concentrations from 3 to 8g/100 ml plasma.

b) Gibbs-Donnan equilibrium - Because the plasma proteins contribute to the total anions in the plasma and are separated from the interstitial fluid by a semipermeable membrane (the capillary wall), hence the permeable ions distribute so that the product of diffusible anions and cations on each side of the membrane is equal, causing an excess of diffusible cations in the plasma and an excess of diffusible

anions in the interstitial fluid (Gibbs-Donnan equilibrium). The ratio of an ion's concentration in the serum to that in the plasma is called its Donnan factor. In the case of cations, the plasma water correction tends to cancel the Donnan factor but for anions, the Donnan and plasma water correction act in the same direction. The theoretical Donnan factors for different ions were calculated by Van Slyke et al in 1923 and experimental results were in good agreement, especially for univalent ions, but since pH, ratio of albumen to globulin in different species, CO₂ tension, etc. were all influencing factors, Manery (1954), from in vitro studies, suggested that the factor r equals 0.95 should be used for all negative ions.

Thus the "chloride space" could be calculated as follows:

$$[Cl]_e = \frac{[Cl]_p \times 1000}{r \times (H_2O)_p}$$

where $[Cl]_p$ is the chloride concentration of the plasma meq/l

$(H_2O)_p$ equals the ml water per litre of plasma

r = the Donnan factor.

Also it became apparent that this extracellular space was not a homogeneous compartment but contained connective tissue proteins which form a network of thin, collagenous fibres which anchor the capillaries in place. Solids of the blood and lymph capillary walls and nerve fibres are also part of this extracellular phase. Manery (1954) showed that the connective tissue of muscle, like tendon, contains much collagen. She also showed that the water of the connective tissue cells contributes to the extracellular fluids. By measuring

the amount of collagen nitrogen in the tissue, correction could be made for the connective tissue present.

Autoradiographs made with $S^{35}O_4$ (D.K. Hill 1965) show that space between the muscle fibres is distributed in an irregular way; its volume is made up of pockets and occasional wide channels as well as very narrow inter spaces approximately 0.1μ in width.

Corrections were also introduced to give a blood-free, fat-free, as well as connective tissue-free "chloride space". An elaborate present-day study of this nature is that of Moulder et al (1966).

At the same time, other ions, presumed to be located extracellularly, were used to determine extracellular "space" e.g. sodium. However, in 1940, Yannet and Darrow warned that the prediction of intracellular fluid concentration from consideration of extracellular sodium or potassium concentration may be deceptive. They had found sodium in excess of the chloride space in dog muscle (Harrison et al 1936) and had reported data which strongly suggested that in some tissues a considerable fraction of the chloride was not extracellular.

Thus the concept of a cell membrane impermeable to extracellular electrolytes gave way to a new theory of slight permeability of the muscle membrane to chloride ions (Boyle and Conway 1941). Their "sieve" or "pore" theory included the following:-

- i) that diffusible ions distributed across the cell membrane according to the Gibbs-Donnan laws.

ii) that electroneutrality existed on both sides of the cell membrane.

iii) that solutions on either side of the cell membrane are isosmotic.

Now that electrolytes found in the extracellular fluid could no longer be considered to remain extracellular, other small electrolytes were tried and new "ion spaces" measured, e.g. thiocyanate, thiosulphate, iodide, bromide, etc. Also small non-electrolytes were used, such as mannitol, sucrose, raffinose and inulin. The main criteria for any substance used as an extracellular volume indicator were as follows:- a) that it cross the capillary membranes with ease, but be excluded by the cell membrane, b) that it must not be metabolized or become adsorbed on any extracellular site. It is not surprising, therefore, that no ideal molecule or ion has been discovered for the purpose. It has become more practical to speak of the "substance space" because of lack of well-defined boundaries, the rapid rate of water exchange across cell walls and lack of knowledge about cell permeability to each specific substance.

Properties of the molecules or ions themselves, their size, hydration, solubility, charge, etc. have been shown to affect their distribution in the extracellular fluid and hence their calculated "space". In 1965, Barr found that the volume of "space" occupied by each molecule or ion varies inversely with its molecular size (or hydrated radius in the case of ions). Thus urea equilibrated with the

entire volume of tissue water while arabinose and mannitol equilibrate with about 70% of tissue water; sucrose, raffinose and inulin with smaller proportions and radioiodinated serum albumin (RISA) with the smallest proportion.

Numerous comparative measurements of extracellular fluid volume based on the distribution of a variety of substances were reported. For example, in 1956 Robertson and Peyser compared sucrose space with that of chloride and sodium in myocardium. The Conway-Boyle hypothesis was applied to calculations of chloride and sodium "spaces", and the intracellular chloride was assumed to be equal to the extracellular potassium. Even then the sucrose space was consistently smaller than the other "spaces". However they concluded that, "Any method for determining extracellular volumes which takes cognizance of intracellular chloride or sodium would give better results than chloride or sodium space".

With the advent of radioactive isotopes for use in biological tracer studies came more sensitive methods for measuring volumes of distribution. No longer need such large quantities of test substances be used as to disturb the balance between compartments osmotically. Radioactive tracers brought a new approach to the problem of how far sodium or chloride could be assumed to be extracellular and the volumes of distribution of these ions could be compared with the best estimate of the volume of extracellular fluid made with substances foreign to the body. For example, in 1954, Cotlove compared inulin and sucrose

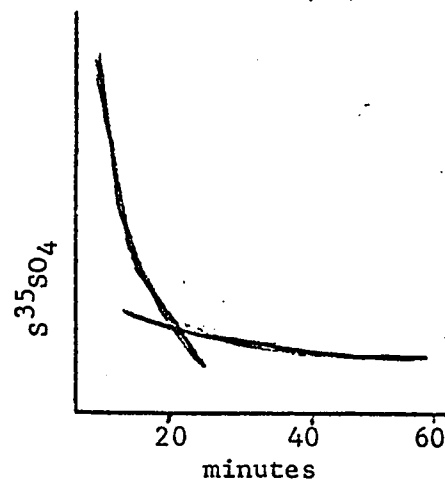
spaces with chloride space in rat skeletal muscle. The chloride space was found to be 114 ml/kg wet weight. Cotlove showed that the inulin and sucrose rapidly equilibrated with 80% of this value but over a period of several hours, their distribution approached that of chloride space. Walser et al (1954) did simultaneous determinations of chloride and sulphate space on muscle of nephrectomized dogs. They found that the sulphate rapidly equilibrated with about 75% of the chloride space and unlike inulin and sucrose did not appear to penetrate further unless very slowly over long distribution intervals.

A new type of study emerged with the use of radio isotopes, - the kinetics of isotope distribution from the blood into and out of individual tissues. It was established that each tissue exchanges water with the plasma at an independent and characteristic rate, that there are "fast" and "slow" compartments and that, in general, the rates of exchange are faster for visceral tissues than for bone and muscle.

By plotting the concentration in the plasma against time, the existence of more or less distinct fluid compartments could be determined. A single homogeneous phase should yield a simple experimental function. If a more complex curve was obtained, it could be analysed to yield a series of exponential functions with different time constants characterizing compartments or masses which equilibrate with the plasma at different rates.

For example, Johnson (1955) published "The kinetics of release of sodium-24, sulphate-35 and carbon-14 sucrose from frog sartorius muscle".

After equilibrating muscles with each of these isotopes, the rate at which each isotope diffuses from the tissue was measured (a washout study) and plotted, as at right. The change in slope of the curve indicates that the substance is diffusing from a "fast" and a "slow" compartment in the tissue. The diffusion coefficients (as



per cent of diffusion values in free solution for the three isotopes were 67% for sodium-24, 63% for carbon-14 sucrose and 93% for sulphate-35 showing that, of the three, radioactive sulphate has the least tendency to enter the cell or to become metabolized.

In 1962, Page used the "osmotic gradient" method, an intracellular microelectrode technique for determining whether an uncharged water-soluble molecule enters the cell or remains extracellular. By substituting sulphate for the permeable chloride in the medium and observing potassium concentration in the cell (K_i), cell volume changes and changes in voltage across the membrane, he showed that arabinose, with a hydrated radius of 3.5 \AA entered the cell whereas mannitol (hydrated radius 4.0 \AA) remained extracellular. Inulin (diameter around 30 \AA) was shown to equilibrate with 24% of the total water within 1 hour and then to slowly increase its volume. Both Cl^{36} and Br^{82}

were found to have higher cell concentrations than could be accounted for from passive distribution alone. S^{35} -sulphate was found to remain impermeant for 5 hours, in cat papillary muscle. Maltose and sucrose spaces became very large after long periods of distribution indicating that they may penetrate the cell slowly e.g. by solution in the membrane, active transport, chemical or metabolic processes, etc.

Danielson (1964) measured extracellular fluid volumes in different areas of frog heart and found that the C^{14} -sucrose spaces in the sinus venosus, atrium and ventricles did indeed continue to increase even after 40 hours whereas a mean value of 148 ml/kg was obtained in skeletal muscle with no change in value after a steady state was reached. Thus a substance which is satisfactory for measuring extracellular fluid volume in one type of muscle may be unsatisfactory in another type of muscle.

Danielson also showed that a substance may equilibrate differently in different regions of the heart. C^{14} -mannitol reached a steady state in the sinus venosus and skeletal muscle in 5 and 15 hours respectively, whereas in atrium and ventricle the C^{14} activity continued to rise over a very long period. Thus the choice of substance used to measure extracellular fluid volume is very important; many factors must be taken into account. There are many reasons for considering S^{35} -sulphate as the best choice for use in heart muscle. These reasons can be summarized as follows:-

(a) S^{35} -sulphate has been shown to equilibrate rapidly, within 4 minutes in rat ventricle (Page and Page 1968).

(b) Mammalian heart muscle cells were found to be impermeable to sulphate ions (Page 1962) possibly because the negatively charged heart cell mem-

brane may repel divalent anions such as sulphate (Teorell 1953).

(c) The sulphate space in toad muscle is found to be greater than that of mannitol, sucrose or inulin, none of which are considered to penetrate the connective tissue (Selkurt 1963); but sulphate space is shown to be less than that of sodium or chloride, both of which have been shown to enter the intracellular phase.

(d) Since the amount of S^{35} -sulphate necessary is negligible in weight, the serum concentration is not altered; hence the osmotic pressure of the ECF is unaffected.

(e) S^{35} -sulphate has a convenient half-life of 87.1 days.

Like every other material used for measuring ECF volume, it has a few disadvantages. It is rapidly excreted by the kidney, however this factor should not affect the serum to tissue ratio as long as it diffuses freely from the tissue and is not bound or metabolized to any extent.

Hitherto it has been considered to remain impermeant for 5 hours (Page 1962). Page and Page(1968) now show it to penetrate a "slow" compartment during the 4 to 70 minutes of distribution time. Since this study was performed "in vitro" and the volumes found are very large compared to those of other investigators (Table XVII) it is possible that permeability factors change in tissues isolated in artificial media, thus affecting measured ion "spaces". Barclay (1959) observed that the sodium content increased and the potassium content decreased in isolated, perfused rat heart.

Johnson (1955) showed the coefficient of diffusion of S^{35} -sulphate from heart muscle to be 93%. It is possible that the remaining 7% becomes
(p.56)
part of the chondroitin sulphate B₁ contained in tendon such as is present

in heart valves. Sulphate is not used in the biosynthesis of proteins as the S-containing amino acid, methionine, is essential to the diet.

Eventually some sulphate may be incorporated into S-containing proteins in the intestine and reabsorbed (Dziewatkowski 1954) but if the distribution time is kept short the amount of protein synthesis which could occur in the heart would be relatively small. Furthermore, sulphate ions have usually formed ethereal conjugates with indole, phenol and -17 ketosteroids by the time they enter the intestine.

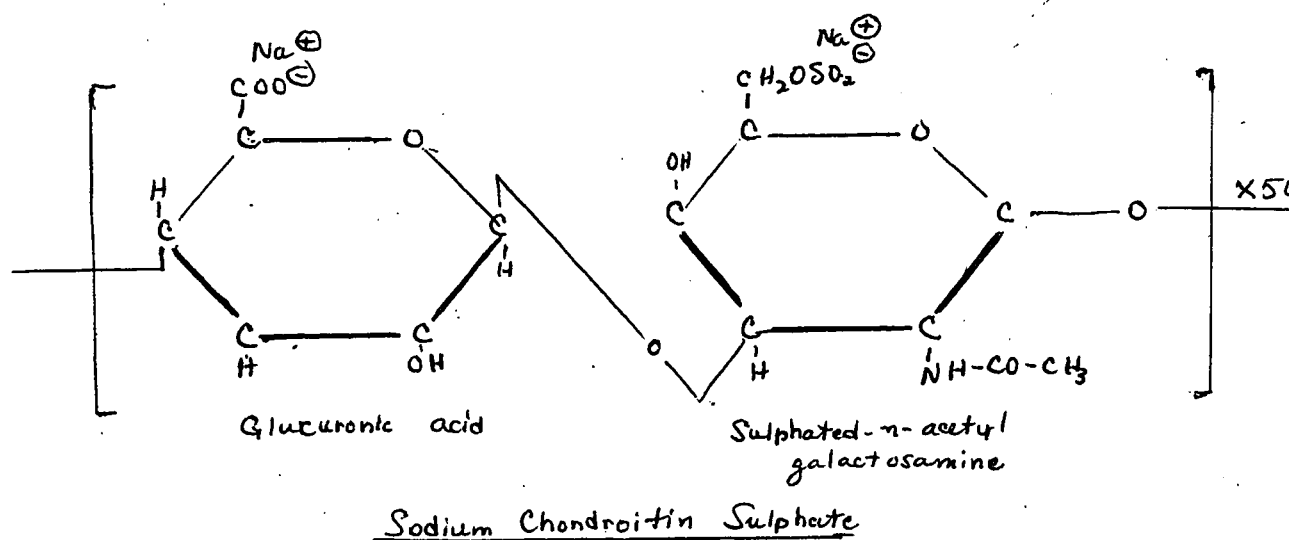
Concerning the uptake of S^{35} -sulphate in rat heart "in vitro", Page and Page (1968) show that 36 - 41% of the total muscle water has equilibrated within 4 minutes after the start of perfusion with $S^{35}O_4$. A second fraction of water equilibrates slowly in the interval between 4 and 70 minutes. This slowly equilibrating fraction accounts for 20% of the total water. For the next 20 minutes i.e. 70 to 90 minutes after start of infusion, the sulphate space remains constant and thus about 40% of the total water content of the muscle fails to equilibrate with sulphate ions. The conclusion is thus drawn that there are three distinct water compartments in mammalian left ventricle.

There have been shown to be extracellular structures which might account for the "slow" compartment of sulphate distribution. Electron microscope studies by Simpson and Oertelis (1962) revealed invaginations of the sarcolemma of sheep ventricular muscle, indicating that interstitial spaces are continuous with an extensive tubular system which appears to run transversely into the cell interior at the longitudinal level of the Z-bands but are blocked from the cell itself by the thick-walled membrane. Thus

the "third" compartment might be located between the cell and the "fast" extracellular compartment.

Since Page and Page (1968) have also shown that this "slow" compartment contains little chloride and sodium and is slowly permeable to sulphate and sucrose, they suggest that it is an extracellular compartment but that its ionic composition is that of a cellular subcompartment. However, the possibility that it is a cellular compartment such as the intra-mitochondrial water, the water within the tubules of the sarcoplasmic reticulum, or the water content of a cell type different from the myocardial cells, is not eliminated. It has even been suggested that the cardiac mitochondria may be divided into subcompartments (O'Brien and Brierly 1965).

Thus the previously held concepts regarding the extracellular and intracellular fluids are changing and the questions now asked are, - "What is the relationship of the compartments to one another? What is their ionic composition and permeability to ions? What form their boundaries? And finally, what is their physiological significance?"



Methods

1. C^{14} -inulin space in heart and skeletal muscle

Normal rats weighing approximately 200 g. were anaesthetized with 0.2 ml of 2% nembutal solution per 100 g body weight, injected intraperitoneally. A lateral incision was made on each side of the back and the renal pedicles tied off with thread, taking care not to include the blood supply to the adrenal glands. C^{14} -inulin solution containing 5 microcuries per 0.1 ml was injected into the jugular vein in amount to give 5 microcuries per 100 g body weight. The radioisotope was allowed to distribute for one and one-half hours. At the end of the distribution period, the rats were anaesthetized with ether, and samples of blood, atrium, right and left ventricles and skeletal muscle from the thigh region were collected and prepared for analysis of their radioactive content; as described in Chapter II.

2. S^{35} -sulphate space in heart and skeletal muscle

From the assayed supply of carrier-free S^{35} -sulphate, a standard solution was prepared in saline containing 10 microcuries per 0.1 ml. The dose was determined, as outlined below, to give a constant specific dose of 5 microcuries per 100 g body weight. The rats were anaesthetized with 2% nembutal (0.2 ml per 100 g injected intraperitoneally). The dose, as determined below, injected into jugular vein and the labelled solute was allowed to distribute for 1 hour unless otherwise stated. At the end of this period, the rats were anaesthetized with ether. Blood samples and tissue samples of atrium, left ventricle, right ventricle and skeletal muscle from the thigh region were collected and prepared for radioactive analysis as described under General Methods, Chapter II.

The above procedure to determine sulphate space in heart and skeletal muscle was carried out on the following groups of rats,-

(a) A group of 25 normal rats weighing around 200 g in which the labelled tracer was allowed to distribute for different lengths of time, from 20 minutes to 24 hours. The results were used to determine the distribution time for the groups which followed.

(b) Three groups of rats, between 50 and 60 rats in each group, on which aortic constriction had been performed. The first two groups averaged 180 ± 10 g and the last group were all around 250 g when narrowed. The sulphate space was determined at intervals of 1 hour, 3 hours, 7 hours, 12 hours and 24 hours after narrowing; then at periods of 3 days, 1 week, 2, 3, 4, 6 and 8 weeks post-operatively.

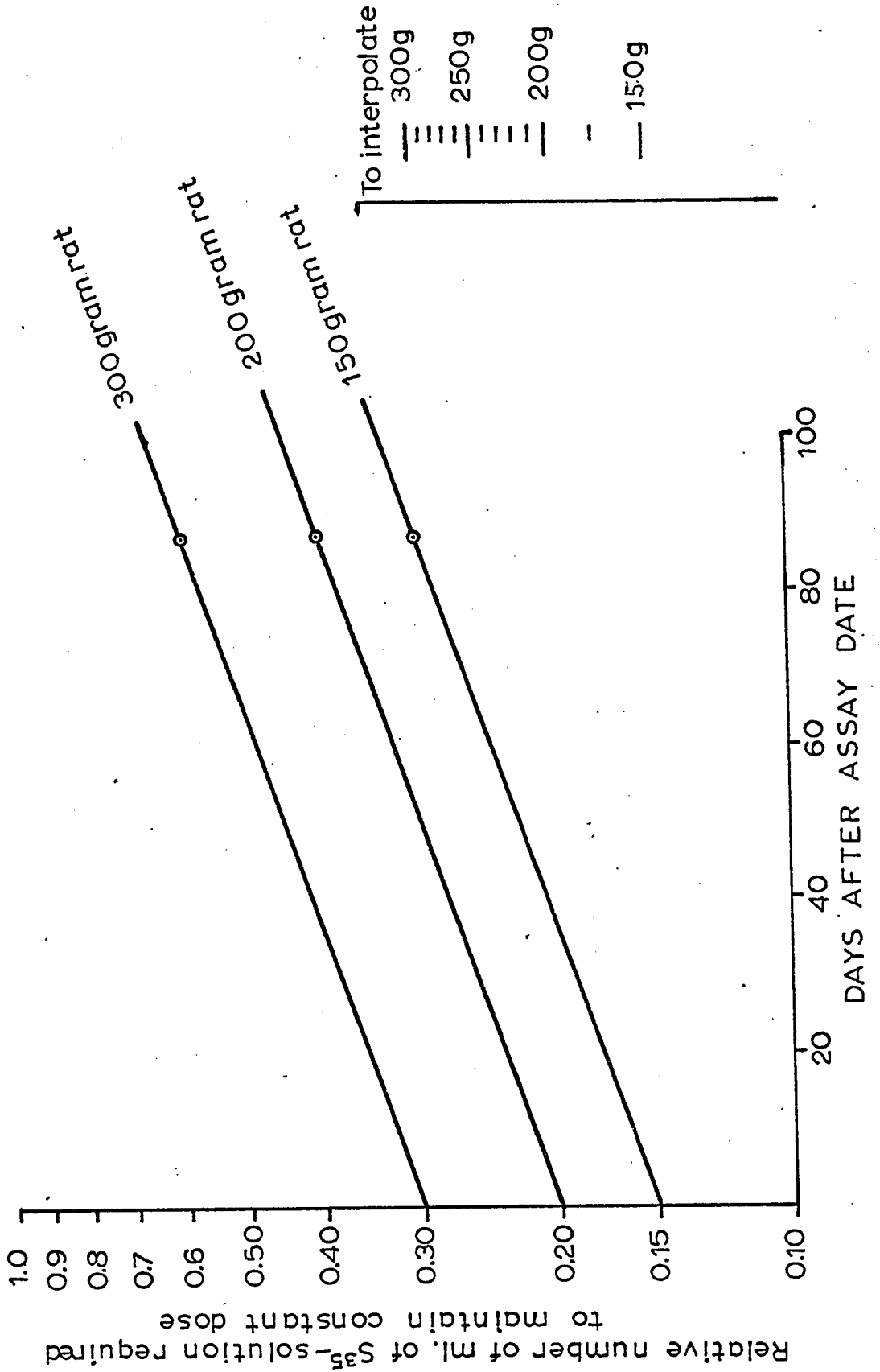
(c) Normal controls - A few of the groups included unoperated normals of the same original size which were used as controls. In addition, another group of 40 normal rats was included to cover all sizes of rat from 150 to 450 grams.

Inulin space was determined in heart and skeletal muscle of 15 rats as described in section 1 above.

Dose determination for S^{35} -sulphate experiments (Figure 6)

Because of the radioactive decay factor, a constant dose of 5 micro-curies per 100 g body weight for various sizes of rats on different days involved lengthy calculations until a method was discovered whereby the dose could be ascertained immediately from a graph plotted on semilog paper. Since decay rate follows an exponential curve, the days elapsing after the assay date were plotted on the linear side and the dose, in ml

FIGURE 6
TO DETERMINE CONSTANT SPECIFIC DOSE
OF S^{35} -SULPHATE



on the log side. This is shown in figure 6. Since the required dose doubles in 87 days, dose determination lines are easily placed for the main body weights, e.g. 200 g, 250 g. etc. For each day, starting from the assay date, the dose for intermediate weights can be interpolated.

Calculations

After samples were counted in the Liquid Scintillation Counter, the corrections were applied for background, radioactive decay, quenching, etc. as described in Chapter II. The extracellular fluid volume based on the sulphate space was then calculated as follows:

(1) The plasma protein concentration of rat blood is 3.5 g/100 ml plasma (Rauen 1956). Therefore the correction for solute volume in the plasma becomes, - $[H_2O]_p = 984 - (7.18 \times 3.5) = 959$ ml water/litre plasma.

$$(2) \quad [S^{35}O_4]_E = \frac{[S^{35}O_4]_p \times 1000}{r \times [H_2O]_p}$$

where $[S^{35}O_4]_E$ equals the S^{35} -sulphate concentration in the extracellular water in counts per minute per litre;

$[S^{35}O_4]_p$ equals the S^{35} -sulphate concentration found in the plasma in counts per minute per litre;

and r equals the Donnan factor, which is 0.95 for sulphate (Manery 1954).

$$(3) \quad \text{The sulphate space is thus } \frac{[S^{35}O_4]_m \times 1000}{[S^{35}O_4]_E}$$

Where $[S^{35}O_4]_m$ equals the counts per minute $S^{35}O_4$ per 100 g wet tissue (after corrections in Chapter II)

and $[S^{35}O_4]_E$ is the $S^{35}O_4$ concentration in the extracellular water calculated above.

Observations

Distribution time for S^{35} -sulphate

Sulphate space was determined in atria, left ventricle, right ventricle and skeletal muscle in normal rats after different distribution times (20 minutes, 1 hour, 3 hours, 5 hours, 18 hours and 24 hours. Table V lists the results for all distribution times up to 5 hours and the mean \pm S.E.M. for all 31 rats. It is apparent from the table that S^{35} -sulphate in the tissues equilibrated rapidly with the injected $S^{35}O_4$. The values reached in 20 minutes are not significantly different from the values after 5 hours although the sulphate was rapidly being excreted, as shown by the drop in counts per minute/ml serum and by measuring the activity in 0.1 ml of urine. Because the sulphate space calculations are based on the ratio of $\frac{\text{c.p.m. tissue}}{\text{c.p.m. serum}}$ which is shown to remain constant for distribution times up to 5 hours, therefore there is no evidence that sulphate ions are being adsorbed, metabolized or entering another fluid compartment in these "in vivo" experiments. In fact linear regression of c.p.m. in the tissue on c.p.m. in the serum was plotted (Figure 7) and was found to be linear in all parts of the heart and in skeletal muscle, but only up to 5 hours.

For distribution times of 18 and 24 hours the ratio of $\frac{\text{c.p.m. tissue}}{\text{c.p.m. serum}}$ was found to increase greatly indicating that the sulphate ions were becoming bound or metabolized in some way or crossing a membrane into another fluid compartment from which diffusion is slowed.

Because the mean values after 1 hour of distribution time were so similar to the mean values for all distribution times, up to 5 hours, it was decided to allow 1 hour for distribution time in all the experiments

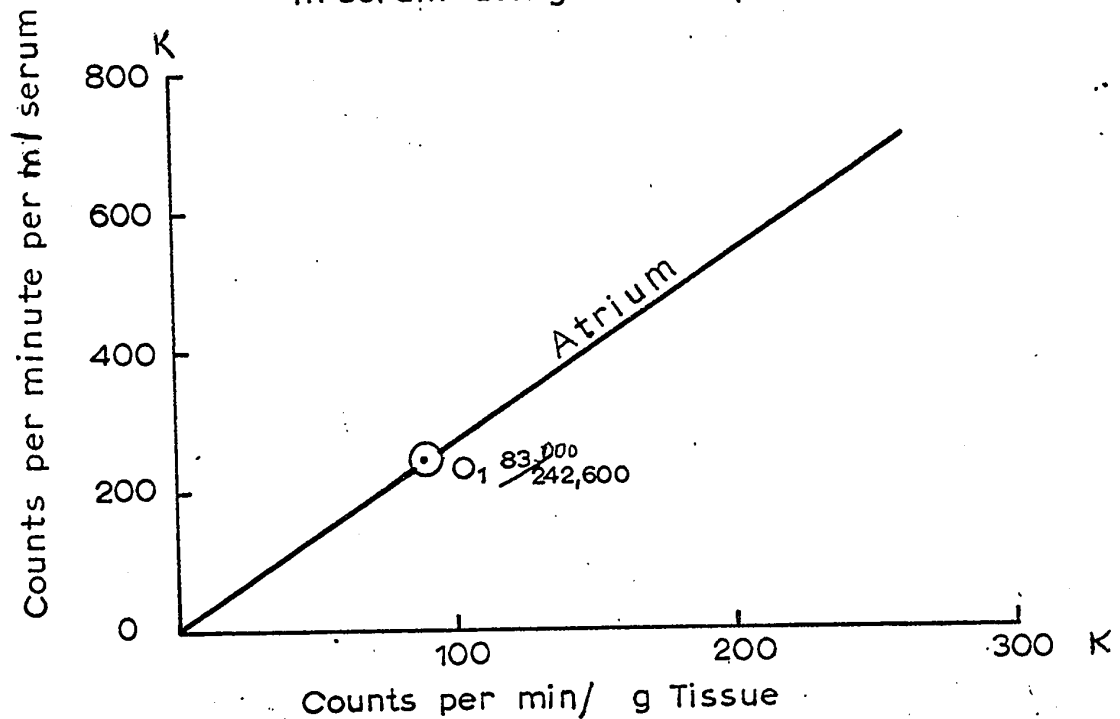
TABLE V

The S^{35} -sulphate space in different regions of the heart and in skeletal muscle after different periods of equilibration

Distribution period	No. rats	Atria	Left ventricle	Right ventricle	Skeletal muscle
20 - 30 mins	4	342.0	199.8	210.6	90.0
1 hour	13	295.2	188.1	196.2	106.8
3 hours	10	310.5	192.6	190.8	83.7
5 hours	4	338.4	199.8	208.8	101.9
Mean \pm S.E.M. for total	31	307.8 \pm 7.9	189.9 \pm 4.1	196.2 \pm 6.3	101.4 \pm 3.9

FIGURE 7

Regression of c.p.m. in tissue on c.p.m. in serum using S^{35} -sulphate



which followed.

C¹⁴-inulin and S³⁵-sulphate spaces compared

From Table VI it is apparent that C¹⁴-inulin space is smaller than S³⁵-sulphate space in all regions of the heart in which they were determined and in skeletal muscle. Inulin space remains close to 70% of sulphate space in all tissues in which it was measured. Both sets of measurements compare well with those of other workers. For example, Ledingham (1956) found inulin space in rat skeletal muscle to be 75 ml/kg. Assuming sulphate space to be about 80% of chloride space as stated by Walser et al (1954) the sulphate space measurements on cardiac muscle seemed quite reasonable for cardiac muscle but came closer to 90% of chloride space measurements in skeletal muscle. Since inulin space was proven to increase its distribution with time and required extra operative procedures (see General Methods) it was decided to use S³⁵-sulphate only as extracellular fluid volume estimator in experiments which follow.

Sulphate space changes in muscle during normal growth

The S³⁵-sulphate space measurements found in atria, right and left ventricles and thigh muscle of 56 normal rats, grouped according to size, are listed in Table VII. A gradual increase in sulphate space occurs in all parts of the heart and in skeletal muscle as the rat grows from 150 to 300 g. The relative amounts and changes in sulphate space in heart and skeletal muscle as the rat grows from 150 to 450 g are shown in Figure 8. It can be seen that atrium, with the largest sulphate space shows the least amount of change with growth (only 6%) whereas skeletal muscle with the smallest sulphate space changes the most during normal growth (27%). Left ventricle increases by 17% and right ventricle by 12%. Sulphate space reaches a maximum in all muscle

TABLE VI

C^{14} -inulin space and S^{35} -sulphate space in different regions of heart muscle and in skeletal muscle of normal, male, Wistar strain rats
(Body weights 160 ± 10 g)

Tissue	C^{14} -inulin space ml/kg wet wt.	No. rats	S^{35} -sulphate space ml/kg wet wt.	No. rats	Ratio $\frac{C^{14}\text{-inulin}}{S^{35}\text{-sulphate}}$
Atria	214.3 ± 9.5	15	308.3 ± 7.9	18	0.69
Right ventricle	139.2 ± 6.1	15	196.2 ± 6.3	18	0.70
Left ventricle	141.7 ± 5.2	15	189.9 ± 4.1	18	0.72
Thigh muscle	71.5 ± 5.8	15	100.5 ± 4.0	18	0.71

TABLE VII

S^{35} -sulphate space in different regions of the heart and in skeletal muscle of normal, male, Wistar rats grouped according to size

Body weight g	No. rats	S^{35} -sulphate space ml/kg wet wt			
		Atrium	Right ventricle	Left ventricle	Thigh muscle
150-199	18	312.0 ± 7.0	210.2 ± 3.7	199.0 ± 4.0	124.2 ± 5.0
200-249	16	316.9 ± 7.5	223.4 ± 6.9	207.7 ± 4.1	137.6 ± 8.7
250-299	7	330.4 ± 14.0	235.8 ± 10.4	233.8 ± 14.2	157.8 ± 13.4
300-349	6	319.0 ± 6.0	212.4 ± 8.5	212.6 ± 10.0	141.0 ± 7.7
350-399	3	289.2 ± 22.4	209.4 ± 1.5	196.8 ± 11.1	118.8 ± 8.1
400-450	6	295.6 ± 6.7	200.1 ± 3.1	190.1 ± 6.0	111.3 ± 7.7

FIGURE 8

S^{35} - SULPHATE SPACE IN RAT HEART AND SKELETAL MUSCLE DURING NORMAL GROWTH

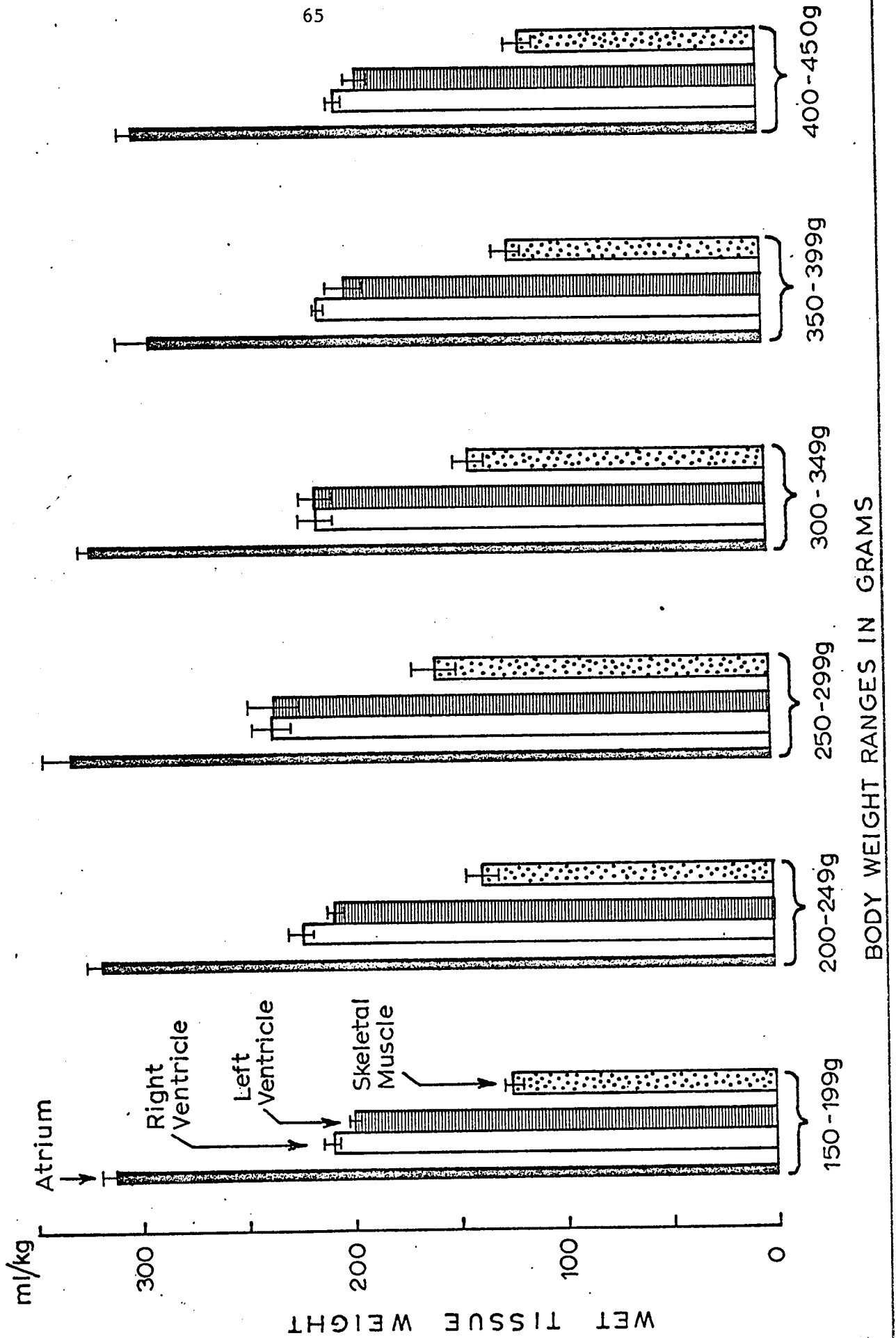


FIGURE 9

EFFECT OF NORMAL GROWTH ON SULPHATE SPACE OF RAT HEART AND SKELETAL MUSCLE

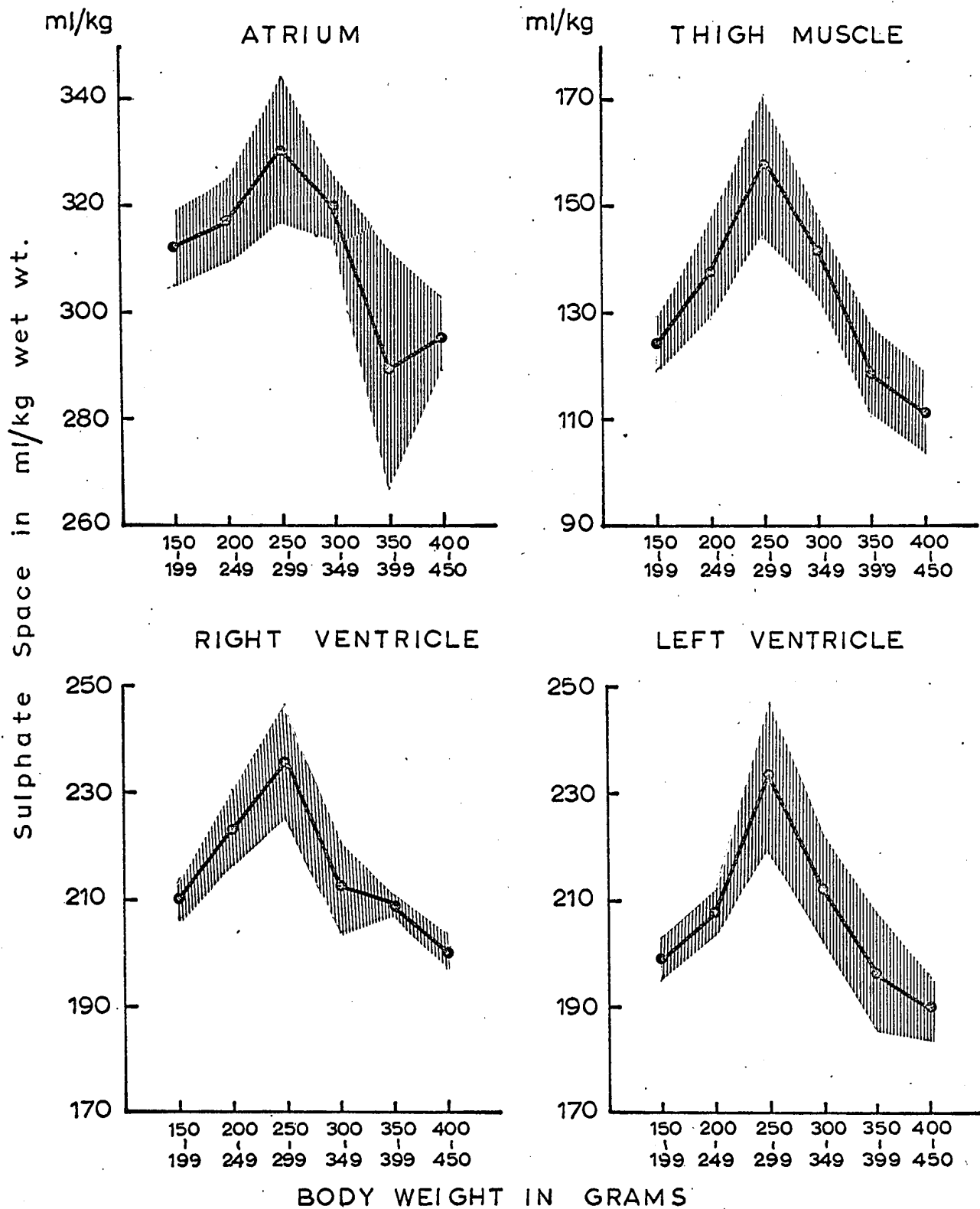


TABLE VIII

S^{35} -sulphate space in different regions of the heart and in skeletal muscle of rats, male, Wistar strain, after aortic constriction

Interval after coarctation	Rats No.	Body wt. g	Atrium	S^{35} -sulphate space in ml/kg wet wt.			Thigh muscle
				Right ventricle	Left ventricle		
Controls	7	185 ± 6	305.0 ± 7.4	211.4 ± 3.5	192.1 ± 4.1	126.7 ± 6.6	
1 hour	6	183 ± 2	301.1 ± 8.4	201.4 ± 7.7	186.7 ± 4.9	133.2 ± 10.3	
3 hours	6	178 ± 2	315.2 ± 11.0	* 233.6 ± 10.7	194.9 ± 8.9	122.6 ± 9.1	
7 hours	10	200 ± 6	* 339.5 ± 8.8	* 242.6 ± 10.1	204.3 ± 7.6	134.8 ± 5.9	
12 hours	11	181 ± 3	* 346.3 ± 5.8	* 241.9 ± 7.2	199.2 ± 4.9	123.2 ± 14.4	
24 hours	10	172 ± 4	* 351.0 ± 19.7	* 241.3 ± 17.0	* 215.5 ± 8.6	114.4 ± 7.1	
2 days	10	183 ± 3	* 344.0 ± 15.1	* 262.6 ± 14.9	* 211.4 ± 3.9	129.3 ± 8.7	
4 days	10	178 ± 3	* 315.6 ± 4.4	** 292.0 ± 15.2	209.4 ± 9.9	132.7 ± 9.3	
1 week	6	189 ± 4	* 335.2 ± 12.9	** 291.4 ± 16.2	* 223.9 ± 8.9	123.1 ± 6.7	
10 days	13	184 ± 2	** 342.2 ± 16.5	** 288.8 ± 13.4	* 227.6 ± 5.9	122.5 ± 6.0	
2 weeks	3	197 ± 8	** 342.3 ± 10.2	* 287.6 ± 2.0	** 231.6 ± 9.8	137.7 ± 9.2	
3 weeks	6	257 ± 6	304.0 ± 15.7	* 255.3 ± 15.8	201.4 ± 4.8	120.6 ± 1.7	
4 weeks	6	309 ± 6	304.8 ± 4.9	230.4 ± 4.0	212.6 ± 3.6	111.9 ± 8.1	
6 weeks	6	319 ± 16	313.4 ± 20.2	224.8 ± 2.8	204.0 ± 8.1	109.6 ± 8.9	
8 weeks	5	370 ± 13	311.4 ± 17.5	* 244.1 ± 14.4	205.0 ± 6.7	120.4 ± 9.9	
Controls	6	370 ± 5	296.5 ± 6.8	203.8 ± 4.0	193.0 ± 5.7	121.9 ± 7.0	

* P < 0.05
 ** P < 0.001

during the 250 to 299 g period when the rat is growing at the fastest rate (Fig. 3). In the larger size groups the sulphate space declines. In Figure 9 it is apparent that sulphate space in the 400 to 450 g group is lower than in the 150 to 199 g group.

Sulphate space in hypertrophied hearts

From Table VIII it can be seen that within 3 hours after constriction of the aorta there is a rise in sulphate space. This increase is significant ($P < 0.05$) by the end of 3 hours in the right ventricle, by 7 hours in the atria and by 24 hours in the left ventricle. This increase in interstitial fluid volume amounts to 16% in the atrium, 12.5% in the left ventricle and 17% in the right ventricle by the end of 24 hours. The increase in sulphate space becomes even more significant by the end of 4 days in the right ventricle ($P < 0.001$) and at 2 weeks after constriction in the left ventricle ($P < 0.001$). Between 2 and 3 weeks after aortic constriction the sulphate space rapidly decreases back to close to normal values in unoperated controls of the same size.

No similar trend in sulphate space is observed in skeletal muscle during the same intervals, although great variation occurs in these values which may be due in part to the sizes of the rats (Fig. 8) or to inadvertent analysis of different thigh muscles (see Discussion, Chap. VI). Following preliminary tests, it was suggested that the S^{35} -sulphate dose be doubled to 10 microcuries per 100 g body weight. This had no effect on sulphate space measurements in heart muscle, but resulted in a significant increase in sulphate space of thigh muscle (Table VI and Table VII).

2. The Total water content of normal and hypertrophied hearts

Method

(a) Water content of different regions of normal hearts; samples of atrium, left and right ventricles, interventricular septum and skeletal muscle were examined for differences according to the method described on page 20.

(b) Water content of right and left ventricles only of 35 rats, during normal growth, were tested for water content

(c) Water content of different regions of hypertrophied hearts; - samples of atrium, right and left ventricles, interventricular septum and skeletal muscle from Series A-4. (8 rats with extreme cardiac hypertrophy) were examined 1 and 2 weeks after aortic constriction for water content.

(d) Water content of right and left ventricles of 57 hearts, series B-1, was determined at periods of 24 hours, 3 days, 1, 2 and 4 weeks after aortic constriction.

Observations and discussion

From Table IX showing water content in different regions of normal hearts and skeletal muscle, it is apparent the water content of all regions of the heart is higher than that of skeletal muscle. The atrium has the highest water content. It is significantly higher ($P < 0.01$) than the ventricles and septum, between which no significant differences occur in normal hearts. Other investigators, e.g. Danielson (1964), have observed the water content of the heart to be highest in the pacemaker region and conducting tissues.

Water content of the heart during normal growth - Table X(A) gives the results of total water determinations of left and right ventricles of 35 normal rats

TABLE IX

Total water content of different regions of normal and hypertrophied hearts and of skeletal muscle in male, Wistar strain rats (- Series A-4)

Preparation	Rats No.	Body wt. g	Total water in ml/kg wet wt.				Skeletal muscle
			Left ventricle	Septum	Right ventricle	Atrium	
Unoperated controls	4	226 ± 3	771.1 ± 1.8	768.9 ± 2.3	773.2 ± 1.1	817.2 ± 2.4	757.5 ± 2.7
1 week after aortic constriction	4	215 ± 7	778.0 ± 2.6	774.5 ± 4.6	* 787.6 ± 4.3	812.4 ± 4.4	759.1 ± 4.7
2 weeks after aortic constriction	4	227 ± 8	780.3 ± 2.8	775.7 ± 2.2	* 787.1 ± 3.5	806.1 ± 0.7	758.3 ± 3.2

* P < 0.05

+ P < 0.02

TABLE X a)

The water content of left and right ventricles of
35 normal rats grouped according to body weight

	Body weight g	No. of rats	Left ventricle ml per kg wet wt.	Right ventricle ml per kg wet wt.
1)	200 - 249	6	770.9 ± 1.2	773.6 ± 1.2
2)	250 - 299	12	* 773.0 ± 1.7	* 775.0 ± 1.8
3)	300 - 349	5	767.3 ± 3.9	769.5 ± 4.1
4)	350 - 399	5	758.2 ± 3.2	764.8 ± 1.5
5)	400 - 450	7	759.0 ± 4.1	763.3 ± 2.2

* P < 0.05 (significantly higher than groups 4) and 5)

TABLE X b)

The water content of left and right ventricles of
57 rat hearts hypertrophied by aortic constriction

Interval after coarctation	Rats		Left ventricle ml per kg wet wt.	Right ventricle ml per kg wet wt.
	No.	Body wt. g		
24 hours	10	235 ± 3	766.5 ± 2.3	775.4 ± 2.9
3 days	10	254 ± 3	768.5 ± 0.2	775.9 ± 2.8
1 week	10	234 ± 7	772.5 ± 1.9	* 784.5 ± 2.7
2 weeks	10	278 ± 12	771.7 ± 2.1	773.9 ± 2.8
4 weeks	9	316 ± 9	768.9 ± 1.8	776.6 ± 2.2

* P < 0.05

grouped according to size. A very definite increase followed by a decrease is observed in both ventricles during normal growth of the rat from 200 to 450 g. The increase reaches a peak in both ventricles in the 250 to 299 g group of rats. At this time the total water content of both ventricles is significantly elevated ($P < 0.05$) above both groups with body weights over 350 g.

Water content of hypertrophied hearts - Tables IX and X b) show the total water content of both ventricles of Series A-4 and Series B-1 rats following aortic constriction. No significant changes in total water occur in the left ventricle no matter what degree of hypertrophy occurs. Considering that during the intervals listed the cardiac mass has increased up to 70% (Series A-4, Table IV) the total water in the left ventricle has remained remarkably constant. In the right ventricle very little change occurs compared with normals of the same size except at 1 week following aortic constriction when the total water is elevated ($P < 0.05$) in Series B-1 rats. In Series A-4, this increase remains significant at 2 weeks after constriction also. Thus it appears that total water of the right ventricle varies with the degree of hypertrophy but only during the first 2 weeks after aortic constriction as the sulphate space soon returns to normal whereas the hypertrophy continues to increase.

Discussion

Sulphate space and total water in normal hearts

In normal growing hearts it has been shown that both total water and sulphate space reach a maximum in rats weighing 250 to 299 grams, the period of fastest growth rate in both heart and body of the rat. In both ventricles the total water and sulphate space were found to be significantly greater than total water and sulphate measurements in all rats over 350 g in weight.

However the increase in total water is much less than the increase in sulphate space. The per cent of the total water in the sulphate space increases from 25 to 30 % (Table 18). Therefore a shift of fluid must occur between compartments within the tissue. Sulphate space must have expanded either at the expense of another extracellular compartment or a compartment within the cell. Possible locations for this compartment were discussed in the introduction to this chapter.

Sulphate space and total water in hypertrophied hearts

Similarly, the large increases in sulphate space following aortic constriction are not accompanied by similar increases in total water. In the left ventricle where all the increase in cardiac mass occurs in mild hypertrophy, the total water remains amazingly constant. Although the actual hypertrophy is not measurable for 2 to 4 days following constriction, depending on the criterion used, the sulphate space is significantly elevated by the end of 24 hours ($P < .05$) and the increase is even more significant ($P < .001$), compared with controls, at the end of 2 weeks. During this time the increase in per cent of total water occupied by the sulphate space is only from 27.5 to 30%, similar to the per cent of total water occupied by sulphate space in normal growing hearts during their fastest growing period.

The same pattern of changes occur in the atria and right ventricle. The increases in sulphate space, significant by the end of 3 and 7 hours after constriction in these areas of the heart, may be partly the effect of the increased work load on thin-walled structures. Hort(1951) observed that right ventricle muscle fibres become thinner and the interstitial spaces wider (p.2). The per cent of the total water in the sulphate space increases to 37% of the

total water by the end of one week. A physical decrease in the cross-sectional area of each muscle cell could account for the apparent decrease in intracellular water.

3. Intracellular water volume

When total water has been determined and extracellular fluid volume measured, it is very simple to calculate the intracellular fluid volume, by difference. Obviously the validity of this datum will depend entirely on the validity of the estimator used to measure the extracellular fluid volume. The proper value will be obtained only if its distribution is limited to the extracellular spaces, no more, no less.

Intracellular water volumes in ml/kg wet wt. are included in Table XVIII for normal and hypertrophied left ventricle and in Table XIX for normal and hypertrophied right ventricle.

It can be seen that because the total water changes so little, the intracellular fluid volume is lowest when the sulphate space is highest, i.e. at 250 - 300 g in normals and at 2 weeks after aortic constriction.

CHAPTER V

Electrolyte Content of Normal and Hypertrophied Hearts

Introduction - Theories regarding ion accumulation in the cell.

Once upon a time^{*}, a tiny, organized cell drifted in a great pre-cambrian sea. Its cell wall enclosed its intracellular fluid which reflected the high potassium and phosphate composition of a still more ancient primordial sea, before even cell membranes existed. All fluid outside of the cell wall was "extra" cellular. Multi-celled, complex bodies evolved and left the sea, each cell bathed in a fluid medium resembling the pre-cambrian sea.

To this day the cell has retained its high concentration of potassium despite the fact that the predominating cation in the extracellular fluid is sodium. This ability of the living cell to accumulate potassium has caused redistribution of the potassium in the earth's crust. E. J. Conway (1943) has shown that the concentration of potassium in the oceans has dropped as the evolution of life progressed whereas the concentration of sodium in the oceans continues to rise.

Less than a century has elapsed since it was first recognized that muscle ash is rich in potassium and poor in sodium, while blood contains much sodium and little potassium. G. von Bunge (1873) was one of the first to calculate salt concentrations on the basis of fresh weight. By the turn of the century information regarding the electrolyte content of muscle from many species had been collected.

Interest next centred on the mechanism which prevents the diffusion of sodium chloride from the tissue spaces into the fibres and of potassium phosphate from the fibres into the spaces.

* Between 1 and 1.5 billion years ago.

Two schools of thought developed to explain this phenomenon. They centred around the following two theories;-

- a) The Ostwald-Bernstein "Membrane Theory" in which a semipermeable membrane separates intra- and extracellular compartments and is permeable to the smaller cations, hydrogen and the hydrated potassium ion, but impermeable to anions and to the larger hydrated sodium ion.
- b) The "Phase" or "Sorptions Theory" which assumes the cell to be an organized structure of molecules like a polyelectrolyte resin, the concentration of various ions resulting from selective adsorption by the resin.

The concept of the cell as surrounded by a porous semipermeable membrane was presented by Ostwald in 1890. Bernstein (1902) extended the theory by suggesting that the membrane potential was produced by the diffusion of potassium ions through the pores of this membrane. The theoretical relationship between the magnitude of this diffusion potential and the potassium concentrations inside and outside the cell, was formalized in an equation derived by Nernst (1908),-

$$E = \frac{R T}{F} \log_n \frac{[K]_i}{[K]_o}$$

where R is the Gas constant,
F " " Faraday constant
T " " absolute temperature

$[K]_i$ is the concentration of potassium inside the cell,

$[K]_o$ is the concentration of potassium outside the cell,

and E is the potential across the membrane in millivolts (inside negative with respect to outside).

Taking the Donnan relationship for resting muscle into consideration-

$$[K]_o \times [Cl]_o = [K]_i \times [Cl]_i$$

the equation becomes $E = 61.5 \log \frac{[K]_i}{[K]_o} = 61.5 \log \frac{[Cl]_o}{[Cl]_i}$ at 37°C.

The applicability of this equation was tested and confirmed by Boyle and Conway in 1941. They increased the product of $[K]_o \times [Cl]_o$ by addition of KCl to Ringer's fluid bathing frog sartorius muscle (at 20°C). This caused KCl to diffuse into the muscle fibres to restore the equilibrium. The new values of $[K]_i$ and $[Cl]_i$ were confirmed by chemical analysis.

Boyle and Conway concluded,- "Potassium in muscle (and it would seem generally in cells) may be considered as held by the electrostatic attraction of the indiffusible ions. An osmotic balance occurs also and the cell maintains a definite equilibrium volume. The indiffusible anions which hold the electrical balance with potassium are mostly esters of organic acids and phosphoric acid. Inorganic phosphate ions diffuse into the cell and are then changed to indiffusible phosphate complex anions. An equivalent amount of potassium enters with the phosphate and is held when the latter become indiffusible." Negatively charged amino acids were also believed to cross the membrane where they became indiffusible negatively charged protein.

With this view, potassium would automatically accumulate with the natural growth of the cell; no special mechanism being required apart from the membrane and its characteristic permeability.

In 1946 Graham and Gerard introduced the microelectrode technique whereby the membrane potential between the cell interior and the external bathing solution could be measured directly. The technique was improved by Ling and Gerard three years later (1949) and agreement between measured and calculated potentials improved. For example, Hoffman and Suckling (1952) found a resting membrane potential of 85 mv in dog and cat ventricle, for

which the calculated value is 84 mv.

The introduction of isotopes into biology instigated a reconsideration of older theories about ion accumulation based on assumptions of impermeability. It was discovered that true impermeability of the membrane is rare and reinterpretation of older findings became necessary. To explain the movement of ions against their electrochemical gradient, the concept of active transport of ions became important. In 1941, Dean suggested that muscles keep their sodium concentration down by means of the outwardly directed "sodium pump". The distribution of potassium and sodium could be accounted for by the active transport of only one of them, the other then being in electrochemical equilibrium across the membrane.

Meanwhile arguments raged regarding the role of membrane transport systems vs. fixed cytoplasmic charged sites to account for the familiar distribution of ions in the ECF and ICF. Criticism of the membrane theory included the following,-

- a) Innumerable different kinds of specific ion pumps would be required for regulating the passage and distribution of, not only individual electrolytes and organic ions, but also non-electrolytes as well.
- b) According to the Boyle-Conway Theory, hydrogen ions, like potassium ions should be distributed between the cell and its medium in the same proportions but this has not been fully proven.
- c) Some anions, such as certain proteins, organic phosphate compounds, etc. alleged by the Conway-Boyle theory and sodium pump hypothesis to "not penetrate" the cell membrane, had in fact been shown to easily pass through.
- d) Finally, the "Membrane Theory" group were accused of assigning to the

membrane properties of the protoplasm itself.

The Sorptional (or Phase) Theory starts with the proposition that the dispersion medium of the protoplasm (water) is in a special state and behaves like a non-aqueous phase. The solubility of different substances is thus significantly different from their solubility in the water of the surrounding medium. Protoplasm is believed to have a coacervate structure. Coacervates are liquid drops, rich in colloids and containing from 50 to 85% water. They are immiscible with the surrounding medium. Much research has been done to prove that protoplasm and coacervates exhibit the same physicochemical properties. (Troshin 1966) Living cells, with their high content of protein macromolecules, water, nucleic acids, lipids, etc. are believed to be a system of multiple coacervates.

The difference in adsorption of different substances (i.e. their permeability) by the cells from the medium is basically due to their differing capacity to be bound by the protoplasm colloids. Thus a substance entering the cell may be distributed as follows;- a) in firmly bound state (unionised) b) less firmly bound with the cell colloids, by adsorptional forces (ionised) or c) dissolved in the mobile cation and anion fraction. Thus there can be a high concentration of a substance in the adsorbed or bound state and yet the substance can still enter the cell along its concentration gradient, if there is a lower concentration in the dissolved fraction.

The dependence of the concentration of a substance in the cell (C_c) on its concentration in the medium can be expressed by the formula

$$C_c = C_s K \left(1 + \frac{A_{\infty}}{C_s K + a} \right)$$

where K is a constant showing how many times the solubility of the substance in one phase (the cell) differs from its solubility in the other phase (the medium),

A_{∞} and a are constants characterizing the limit and the steepness of rise of the isotherm of adsorption or chemical binding. It follows from this formula that the greater C_s (concentration in the medium), A_{∞} and K , the greater the value of C_c ; and the smaller the constant a , the smaller will be the concentration in the medium at which binding by the cell will reach its limit.

For non-electrolytes the rate of enzymatic reactions is another factor which can lower its concentration in the cell in comparison with its concentration in the medium. Although the solubility of non-electrolytes in the protoplasm is 2-3 times less than in the aqueous surroundings, mono- and disaccharides as well as urea, creatinine and other non-electrolytes do enter most cells. The capacity of the cell colloids for chemical binding or adsorption differs for non-electrolytes. Some are believed to be adsorbed on the micelle proteins where they function to protect the proteins against coagulation.

Both membrane and sorptional theory adherents had difficulty explaining the selective accumulation of potassium in the cell.

According to the phase theory a large proportion of the potassium in the cell is in the "bound state" and potassium ions have greater affinity for the macromolecules of the protoplasm than sodium. To explain the difference in "affinity" it was thought by many investigators that the more hydrated the ion, the more weakly it adsorbed. In view of this, the

weakly hydrated potassium ions will compete successfully with the sodium ions which have a powerful hydrate shell. Ling (1952) suggested the hypothesis of fixed charges. The irreversible binding of K^+ , as an explanation of preferential K^+ retention by the cell, cannot be seriously considered since it fails to account for these rapid changes in permeability. Realizing this, Ling has extended his original fixed charge theory to account for the reversibility of specific K^+ binding. In his new hypothesis, the "fixed charge-induction hypothesis" account was taken of all the known physical forces (Coulomb, van der Waal's etc) that determine the interaction of ions and water molecules, in calculating the energy of adsorption of various cations.

The most recent revision of the Sorption Theory was suggested by Liberman (1961). It is the Phase-Membrane Hypothesis. The differences between this theory and the previous phase theories are that a considerable amount of potassium and sodium are believed to be in the free (ionised) state and also the surface layer of the protoplasm is assumed to have a high resistance.

An important factor in the maintenance of potassium concentration in the cell, from the point of view of both the membrane and fixed-charge theories, is the stability of the anionic macromolecules in the cells. In mammalian tissue the maintenance of cell potassium seems to be dependent upon respiration. Mullins and Noda(1963) have shown phosphate loss from frog muscle to be accompanied by a loss of cell potassium. In mammalian muscle where the dependence on respiration is greater, the loss of phosphate from the cell could cause greater loss of potassium.

In conclusion it would appear that Troshin's comment on membrane theories applies to all theories to date;- "Each is capable of explaining one group of facts but cannot correctly explain the totality of facts relating to the phenomena." However, the weight of evidence at present favours the view that the physical properties of the cell membrane, and in particular the pore size, determine that it is the potassium ion and not the sodium ion which enters the cell to balance the internal negative charge.

Methods

Samples of left ventricle, right ventricle, atrium, septum, skeletal muscle and blood were collected and treated according to the methods described in Chapter II under Chemical Analyses. Aliquots of the prepared samples were then used to measure the sodium and potassium content by flame photometry and to measure phosphate content by the Eastoe method, described on p. 28.

The following groups of rats were analysed as described above:

- a) Sodium and potassium content of left ventricle, right ventricle, septum, atrium and skeletal muscle of 8 normal rats.
- b) Sodium and potassium content of left and right ventricles of 22 normal rats grouped according to body weight.
- c) Serum sodium and potassium of 29 normal rats.
- d) Sodium and potassium content of left and right ventricles of a group of 57 rats at intervals of 1 day, 3 days, 1 week, 2 weeks and 4 weeks after aortic constriction. Another 15 rats were used as controls and samples taken at the same intervals for comparison with experimental groups.
- e) Total phosphate was determined in different regions of normal hearts and in skeletal muscle of approximately 25 normal rats.
- f) Total phosphate was determined in different regions of hypertrophied hearts from Series A, with a high degree of hypertrophy.
- g) Total phosphate was determined in left and right ventricles of 57 rats from Series B, with mild to moderate degree of hypertrophy.

Observations

Sodium and potassium content of normal hearts; comparing the different

regions of the heart with skeletal muscle (Table XI) it can be seen that the sodium content of all parts of the heart is much higher than the sodium content of skeletal muscle whereas the potassium content is much higher in skeletal muscle. This finding is well documented (see Tables XVI and XVII for sodium and potassium values found by other investigators in cardiac and skeletal muscle). Within the heart the sodium content of the atrium > right ventricle > septum > left ventricle. On the other hand the atrium has the lowest potassium content of heart structures and left ventricle has the highest. Although there is no significant difference between potassium content of left and right ventricles, that of the left ventricle was found to be higher in every individual case, except one, of 73 rats. Because low sodium and high potassium values are found in some tissues whereas high sodium values are related to low potassium values, the sodium and potassium content of muscle appears always to vary inversely.

Table XII gives the results of grouping all normals according to size to determine if any changes in sodium and potassium content of the ventricles result from normal growth and aging. There is a definite increase in sodium content of the left ventricle with aging; that of the 400 to 450 g group is significantly higher than that of the 200 to 250 g group. No changes in sodium content of the right ventricle occur with aging but the potassium content of the right ventricle drops significantly in the 400 to 450 g group. No significant change occurs in left ventricle potassium although potassium content of both ventricles is highest in the 250 to 300 g group.

TABLE XI

Sodium and potassium content of heart structures,
skeletal muscle and serum of normal rats

Preparation	No. of rats	Sodium meq/l or kg	Potassium meq/l or kg
Serum	29	146.6 ± 1.4	4.50 ± 0.07
Left ventricle	10	30.6 ± 0.5	84.7 ± 0.9
Septum	"	34.6 ± 0.7	78.8 ± 0.9
Right ventricle	"	38.8 ± 1.2	82.5 ± 1.4
Atria	"	43.8 ± 0.5	70.3 ± 1.2
Thigh muscle	8	17.9 ± 0.3	121.4 ± 0.5

TABLE XII

Sodium and potassium content of left and right
ventricles of normal rats grouped according to size

Body weight g	No. of rats	Sodium meq/kg wet weight		Potassium meq/kg wet weight	
		Left ventricle	Right ventricle	Left ventricle	Right ventricle
200 - 249	6	30.05 ± 0.45	38.03 ± 0.93	84.78 ± 0.98	82.54 ± 2.61
250 - 299	6	31.96 ± 0.53	38.36 ± 1.34	85.73 ± 0.59	84.58 ± 0.85
300 - 399	4	31.38 ± 0.34	37.34 ± 0.69	84.93 ± 0.51	83.19 ± 0.55
400 - 450	6	33.25* ± 0.61	38.10 ± 0.59	84.95 ± 0.37	* 80.30 ± 0.69

* P < 0.05

Sodium and potassium content of hypertrophied hearts

Tables XIII and XIV show the relative sodium and potassium contents of rat myocardium at intervals of 1 day, 3 days, 1 week, 2 weeks and 4 weeks after aortic constriction. It is apparent from Table XIII that the sodium content of both ventricles increases after aortic constriction. Within the first 24 hours there is an 11% rise in left ventricle sodium which is significant at the 95% confidence level. By the end of 1 week sodium content has increased very significantly ($P < 0.001$) in both ventricles. Between 2 and 4 weeks the sodium content decreases until it is not significantly greater than unoperated controls of the same size (Table XII).

No significant changes occur in potassium content of the left ventricle after aortic constriction but an immediate decrease occurs in right ventricle potassium following aortic constriction. This decrease in potassium was even more significant in a small group of series A rats in which the potassium dropped to 77.2 meq/kg wet wt. by the end of 2 weeks after narrowing.

Figures 10 and 11 compare sodium content and sulphate space in left and right ventricles before and during cardiac hypertrophy. Since changes in sulphate space and sodium content were both significant at 24 hours after aortic constriction in the left ventricle, correlation between the two was checked and found to be very high ($r = 0.915$). Correlation between these factors was high in both ventricles in hypertrophied hearts, but only in the right ventricle of normal hearts.

Total phosphate in normal hearts

Table XV shows the total phosphate content of heart structures and

TABLE XIII

The sodium content of left and right ventricles of
57 rat hearts hypertrophied by aortic constriction

Interval after coarctation	Rats		Left ventricle meq/kg wet wt.	Right ventricle meq/kg wet wt.
	No.	Body wt. g		
Controls	6	244 ± 4	30.50 ± 0.46	38.13 ± 1.39
24 hours	10	235 ± 3	*33.51 ± 0.62	40.48 ± 1.19
3 days	10	253 ± 3	*33.72 ± 0.56	*41.75 ± 0.86
1 week	14	239 ± 8	**34.98 ± 0.53	**44.10 ± 1.12
2 weeks	14	298 ± 10	**34.92 ± 0.62	40.46 ± 1.30
4 weeks	9	316 ± 9	33.40 ± 0.89	38.08 ± 1.68

* P < 0.05
** P < 0.001

TABLE XIV

The potassium content of left and right ventricles
of 57 rat hearts hypertrophied by aortic constriction

Interval after coarctation	Rats		Left ventricle meq/kg wet wt.	Right ventricle meq/kg wet wt.
	No.	Body wt. g		
Controls	6	244 ± 4	84.65 ± 0.74	82.01 ± 0.54
24 hours	10	235 ± 3	85.53 ± 0.76	** 78.23 ± 1.03
3 days	10	253 ± 3	84.36 ± 0.92	** 79.89 ± 1.14
1 week	14	239 ± 8	84.69 ± 1.20	** 79.11 ± 0.63
2 weeks	14	298 ± 10	85.34 ± 0.73	** 80.92 ± 0.87
4 weeks	9	316 ± 9	83.04 ± 0.99	* 80.59 ± 1.04

*P < 0.05 **P < 0.02 (significantly lower than normal of same size)

FIGURE 10
LEFT VENTRICLE

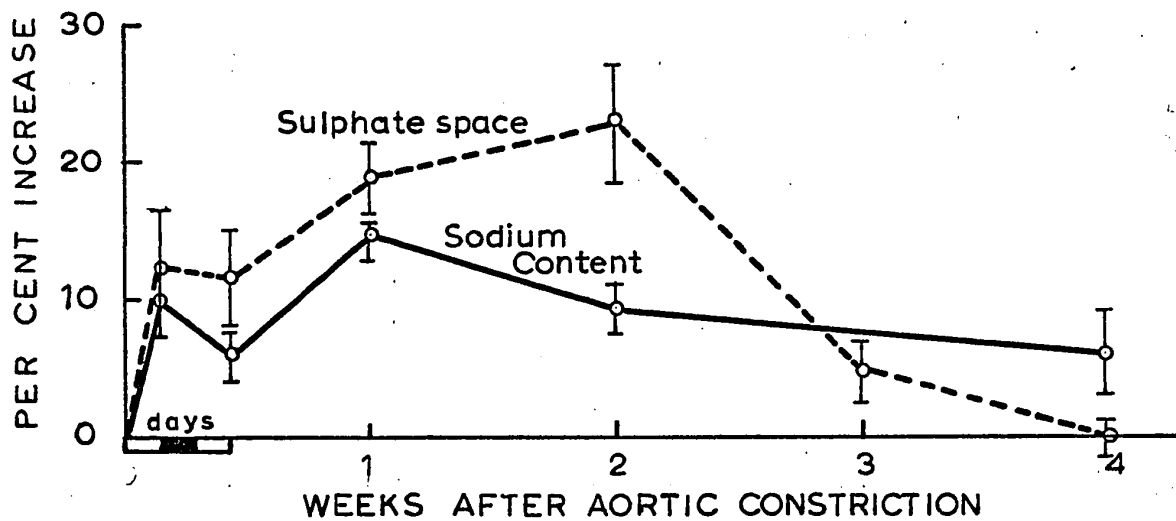


FIGURE 11

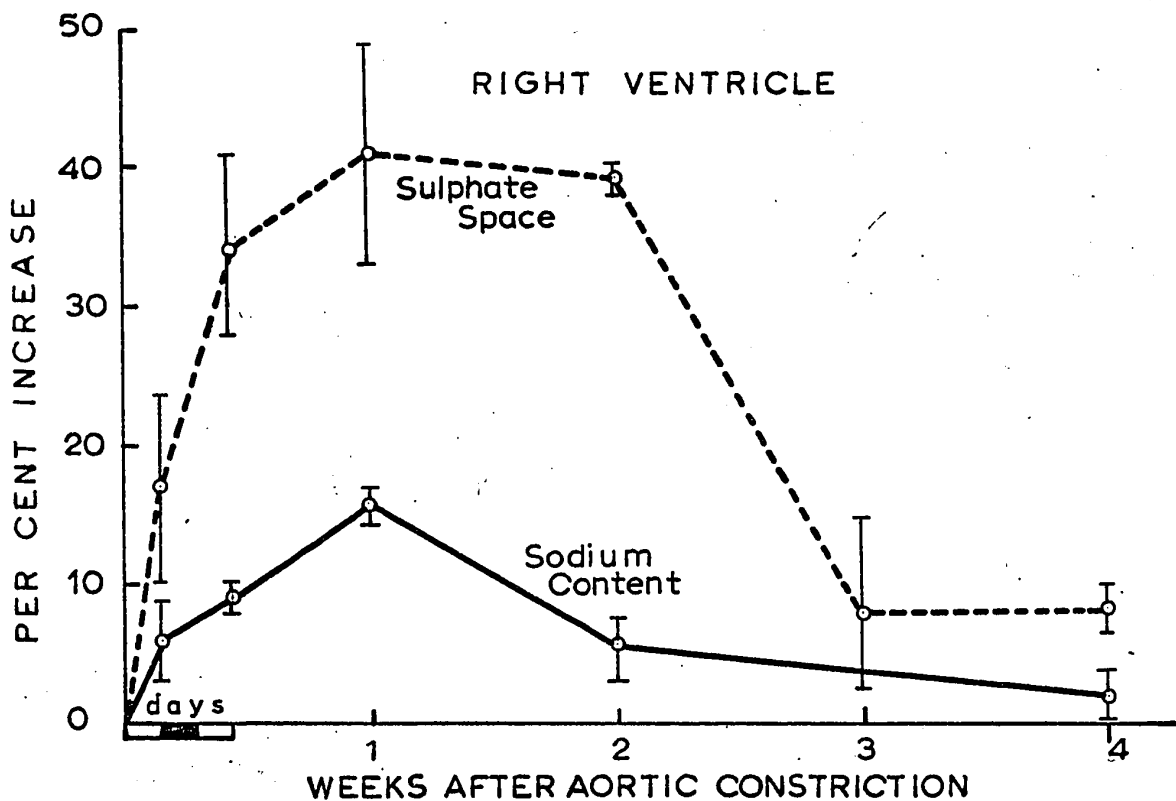


TABLE XV (a)

Total phosphate content of heart structures and skeletal muscle of normal rats and rats after aortic constriction

Tissue	Total phosphate in mg/100 g wet wt		
	Unoperated controls	1 week narrowed Series A-4 (8 rats)	2 weeks narrowed
Left ventricle	260.8 ± 4.2	223.1 ± 5.8	216.2 ± 5.3
Septum	246.9 ± 4.9	245.8 ± 6.1	228.1 ± 8.4
Right ventricle	241.3 ± 3.8	214.4 ± 6.6	209.8 ± 6.4
Atrium	213.0 ± 3.6	200.0 ± 3.9	187.0 ± 5.5
Thigh muscle	259.0 ± 4.8	256.8 ± 3.5	255.4 ± 6.3

TABLE XV (b)

The phosphate content of left and right ventricles of 49 rat hearts hypertrophied by aortic constriction Series B-1

Interval after coarctation	Rats		Left ventricle mg/100 g wet wt	Right ventricle mg /100 g wet wt.
	No.	Body wt. g		
Controls	6	244 ± 4	263.3 ± 4.1	246.7 ± 5.2
24 hours	10	235 ± 3	268.2 ± 2.6	250.9 ± 5.4
3 days	10	253 ± 3	265.3 ± 3.3	233.7 ± 3.7
1 week	10	239 ± 8	*249.9 ± 3.5	
2 weeks	10	298 ± 10	261.8 ± 4.0	
4 weeks	9	316 ± 8	260.3 ± 3.1	
Controls	4	320 ± 5	265.1 ± 1.2	

P < 0.05

skeletal muscle in normal controls. Phosphate of left ventricle > septum > right ventricle > atrium in normal hearts, similar to potassium distribution.

Total phosphate in hypertrophied hearts

Table XV (a) shows the phosphate content of hypertrophied hearts in Series A-4 rats, with severe hypertrophies. It is apparent that there is a significant decrease in total phosphate in all parts of the heart by the end of 1 week after aortic constriction. A further significant decrease occurs by the end of 2 weeks in these greatly hypertrophied hearts, but no decrease is noted in skeletal muscle from the same animals. Table XV (b) gives the results of total phosphate determinations in a Series B group of 49 rats with mild hypertrophies. Although there is a significant drop in left ventricle phosphate at 1 week after constriction, no other changes occur in left ventricle phosphate at any time after narrowing. Thus the decrease in total phosphate in hypertrophied hearts appears to be greater in hearts with a greater degree of hypertrophy as was found with potassium decreases in the right ventricle.

CHAPTER VI

DISCUSSION

C¹⁴-inulin space vs. S³⁵-sulphate space

The differences in extracellular space fractions as estimated by radioactive inulin and sulphate were so markedly different (Table VI) that further discussion is warranted. In the Introduction to Chapter IV many reasons were set forth for choosing S³⁵-sulphate to measure extracellular space in cardiac muscle. These reasons were based on the work of others. The following reason is based on observations included in this study.

To compare the intracellular sodium concentrations based on the extracellular space measured by each estimator, one must first apply the corrections for water content of the plasma and Donnan factor to plasma sodium value (Na)_p (Table XI), which was found to be 146.6 ± 1.2 meq/l. The sodium concentration of the extracellular fluid (Na)_E becomes:

$$\begin{aligned}(\text{Na})_E & \text{ equals } (\text{Na})_p \times \frac{0.95}{0.959} \\ & = 146.6 \times \frac{0.95}{0.959} = 145.1 \text{ meq/l}\end{aligned}$$

From this, the concentration of sodium in the cell water (Na)_{Cell} is calculated according to the following formula:

$$(\text{Na})_{\text{Cell}} \text{ equals } \frac{(1000 \times [\text{Na}]_m) - (\text{ECV} \cdot (\text{Na})_E)}{[\text{H}_2\text{O}]_m - \text{ECV}}$$

where $[\text{Na}]_m$ represents the total sodium content of the muscle,
ECV equals the measured extracellular fluid volume in ml/kg wet wt.
(Na)_E is the sodium concentration of the ECF as calculated above

and $[H_2O]_m$ equals the total water content of the muscle in ml/kg wet wt. The intracellular sodium, in milliequivalents can also be calculated as the difference between the total sodium $[Na]_m$ (meq/kg wet wt) and the $[Na]_E$ sodium contained in the extracellular fluid in milliequivalents.

Thus the intracellular sodium amounts and concentrations based on the two "space" indicators used are as follows:

	<u>C¹⁴-inulin</u>		<u>S³⁵-sulphate</u>	
	RV	LV	RV	LV
Space measured ml/kg wet wt	139	141	196	190
Total sodium meq/kg wet wt	37.3	31.4	37.3	31.4
$[Na]_E$ meq	20.2	20.4	28.5	27.6
$[Na]_i$ meq (by difference)	17.1	11.0	8.8	3.8
Total water ml/kg wet wt.	773.6	770.9	773.6	770.9
Intracellular water ml (by difference)	634.6	629.9	555.6	559.9
(Na) _{Cell} meq/l cell water	27	17.5	15.8	7.0

From these derived data for sodium distribution it is apparent that intracellular sodium calculated by C¹⁴-inulin space is much higher than that calculated from S³⁵-sulphate space. According to inulin measurements, from 35 to 45% of the total sodium in the tissue would be located inside the cell. It is generally accepted that the concentration of sodium in the intracellular water is quite similar to the concentration of potassium in the extracellular fluid. Robertson and

Dunihue (1954) give 6.5 meq/litre of cell water as the intracellular concentration of sodium in cat heart muscle. Experiments on the kinetics of sodium diffusion out of muscle have shown that about 95% of the sodium in heart muscle is freely exchangeable within 10 - 20 minutes (Holland 1964). This leaves only 5% of the total sodium to be bound, adsorbed or hindered by the membrane of a cellular compartment. Heart muscle has been shown to have a higher ECF sodium concentration and a lower ICF sodium concentration than skeletal muscle. In fact, ICF sodium concentration in heart muscle is believed to be similar to ECF potassium concentration (Holland 1964). Thus data derived by using S^{35} -sulphate to measure ECF volume are closer to accepted values than those derived by using C^{14} -inulin to measure ECF volume.

Page and Solomon (1960) used inulin as the ECF volume indicator in cat heart muscle. They came to the conclusion that intracellular concentrations were too large in heart muscle when inulin was used, or else theories regarding transmembrane potentials were incorrect.

In addition, if we examine the chances of sulphate ions crossing the cell membrane in the light of the transmembrane hypothesis as applied to anions;-

$$\text{from } E = \frac{RT}{nF} \cdot \log_n \frac{A_o}{A_i}, \text{ we obtain } \frac{A_o}{A_i} = e^{\frac{nFE}{RT}}$$

where A_o is the concentration of the anion outside the membrane,

A_i is the concentration of the anion inside the membrane,
 n equals the valence,

and e represents the base of the Napierian or natural logarithm.

Then at 80 mv, the generally accepted potential across striated muscle membrane, the ratio of $\frac{A_o}{A_i}$ for monovalent ions is $\frac{24}{1}$ but for divalent anions, $\frac{A_o}{A_i}$ becomes $\frac{570}{1}$. Therefore there is only a small chance that a divalent anion, such as sulphate will cross the charged membrane. However, if the membrane potential drops to 20 mv for any reason, then a different

situation ensues. For monovalent anions $\frac{A_o}{A_i}$ becomes $\frac{2.2}{1}$ and for divalent anions the ratio becomes $\frac{4.8}{1}$ so that theoretically, at least, there is a much greater chance for divalent anions to cross the cell membrane, but only if the membrane potential is altered or the concentration of the anion outside the membrane becomes very large. Because the transmembrane potential across heart cells has been shown to be 88 mv when measured by microelectrodes (Robertson and Dunihue 1954) there is less chance of divalent anions crossing into heart muscle cells than into striated skeletal muscle cells in which the transmembrane potential is around 80 mv.

Frater et al (1959) have shown that skeletal muscle is permeable to sulphate in practice, but according to this group, sulphate on penetrating muscle fibre, is distributed in less water than sodium. Thus according to the Sorption Theory, sulphate is not included in those anions bound or adsorbed by the cell but may be in the "free" phase.

D. K. Hill (1965) limits the space within striated muscle which is accessible to sulphate to a small fraction. From autoradiographs made with $S^{35}O_4$, he estimated that only from 2.5 to 11% at the most, of the space within the muscle fibre was occupied by the S^{35} -sulphate.

In Tables 18 and 19 showing calculated data for intracellular concentrations in hypertrophied hearts a few very small negative intracellular sodium concentrations appear. This can mean one of two things;-

- a) sulphate space is overestimating the ECF volume very slightly, or
- b) sodium measurements are slightly low.

Page and Page (1968) have specifically shown, by raising the

concentration of sulphate in the medium that in rat ventricle muscle at least, sulphate is not being adsorbed at saturable surface sites or incorporated to a significant degree into cellular compartments. However a few sulphate molecules may find their way into the endoplasmic reticulum, a series of invaginations and tubes through the cytoplasm which open on the cell surface. These channels could be penetrated by many molecules since their radii are of the order of 1,000 angstrom units.

Another possibility is that the sulphate ion forms CaSO_4 when it enters the transverse tubular system because the T-system is known to be the site of much calcium adsorption and CaSO_4 has a low dissociation constant.

It was shown in Chapter IV that S^{35} -sulphate equilibrated rapidly with about 26% of the total water and remained at this level for 5 hours. Thus it is shown that "in vivo" sulphate space in rat ventricle is much lower than sulphate space "in vitro". Page and Page (1968) using isolated, perfused rat ventricle found that S^{35} -sulphate equilibrated with 39% of the total water within 4 minutes and then increased to 60% of the total water. At no time did the sulphate space exceed 37% in the present study. It seems abundantly clear that ion "space" measurements "in vivo" and "in vitro" differ, possibly due to changing permeability, lower transmembrane potential, causing higher Na^+ and hence higher "space" measurements (Intro.)

Thus most evidence in the literature, plus our own findings, all point to the fact that S^{35} -sulphate is a very good estimator of ECF volume, even if it overestimates by 1 or 2%. When one considers the alternatives e.g. Low estimates of C^{14} -inulin which would put nearly 40% of the sodium

and chloride intracellularly when a distribution period of 1 hour is used. If longer distribution time is used, inulin continues to increase and in addition a constant infusion technique is necessary or an operation to tie off the renal pedicles. All things considered, S^{35} -sulphate appears to come as close to the ideal substance for measuring extracellular fluid volume as any substance suggested in the literature.

Normal values for water and electrolytes in rat heart and skeletal muscle

a) Skeletal muscle

Values collected from the literature for normal rat skeletal muscle are listed in Table XVI. At first glance it appears that the only value all are agreed on is that of total water content of the tissue. The work of Sreter and Woo (1963) clarifies this somewhat. They show that enormous variation in total sodium, potassium and inulin space occurs depending on which skeletal muscle is analysed. Their results range from 16.5 meq/kg wet wt. up to 32.1 meq/kg. They equate low sodium values with a high proportion of white muscle fibres and high sodium values with the proportion of red muscle fibres. Sreter and Woo also show that inulin space varies directly with sodium values. This could explain the variation found in sulphate space measurements in thigh muscle in Table VIII. Various thigh muscles were used, some with red fibres and some without. Later, in the electrolyte recovery studies, white muscle from the thigh was used, which could explain the slightly low sodium content of 17.8 meq/kg and high potassium value of 121.7 meq/kg. Compared with other species, rat skeletal muscle is low in sodium. Human, cat and dog all have higher sodium content and lower potassium content.

TABLE XVI

Collected values for water and electrolyte content of normal rat skeletal muscle

Tissue	"space" measured	ECF vol... (space size) ml/kg wet wt.	Total H ₂ O ml/kg	Total sodium meq/kg wet wt.	Total potassium meq/kg wet wt.	Author
Skeletal muscle Sprague-Dawley	Chloride	104.2 ± 0.2	773.1	20.1	98.9	Woodbury (1956)
Rat skeletal muscle	chloride	114 ± 3.1				Cotlove (1954)
	inulin (1 hr)	95 ± 2.7				
	inulin 15 "	106 ± 3.9				
	sucrose (2 hrs)	98 ± 5.1				
	sucrose 45 ")	110 ± 5.0				
Sherman strain rat skel. m.	chloride ion	100	7620	20.8	106	Cotlove (1951)
Wistar strain rats - 184 g	chloride ion	124	763		113.3	Lowry et al (1942B)
Yale-Cornell strain - 311 g	Chloride	125	762		110.6	" " "
Wistar strain	inulin	75	742	18.8	116.1	Ledingham (1956)
gastrocnemius muscle	chloride Cl ₃₈	124 130	761			Manery & Haege (1941) " " "
Rat skeletal muscle			770	23.1	108	Widdowson & Dickerson (1964)
Rat skeletal			763.5	17.43	112.5	Kalant (1966)
Rat "			770.4	18.79	122.0	Petschek & Timeras (1963)
Wistar king -soleus " " -EDL	inulin inulin	111 97				Kobayashi & Yonemura (1967)
Rat gastrocnemius	inulin	76.2	759	19.2	112.6	Sreter & Woo (1963)

b) Cardiac muscle

Values collected from the literature for water and electrolyte content of normal rat cardiac muscle are listed in Table XVII. There is not really much basis for comparison unless the region of the heart analysed is known since there is a difference in electrolyte content even between the ventricles. Whole heart values are presumed to have higher sodium and lower potassium values because they include the atria and right ventricle. Also the size of the animal should be known to compare values, since sodium content of the left ventricle has been shown to increase in older rats. As was the case with skeletal muscle, rat cardiac muscle is low in sodium compared to other species. Human, ox, dog and rabbit cardiac muscle all contain more sodium and less potassium than rat cardiac muscle. (Widdowson & Dickerson 1964).

The effect of normal growth on water and electrolyte balance in cardiac and skeletal muscle of the rat

By collecting all the data on water content, sulphate space, sodium and potassium content for normal rat hearts throughout this investigation, many interesting and unusual findings have come to light. The fact that all of these factors, except sodium content of the left ventricle reach a maximum in the 250 to 300 g group is of particular interest. It is also shown in Tables XVIII and XIX that intracellular sodium reaches a low and intracellular potassium reaches a high at this stage of the rat's growth. Since it was shown in Chapter III that 250 to 300 g stage is synchronous with the steepest part of the growth curve (Fig. 3) in Wistar strain rats therefore the increases noted in sulphate space, total water, intracellular potassium concentration, and the decrease in intracellular sodium may all

TABLE XVII

Collected values for water and electrolyte content of normal rat cardiac muscle

Tissue	"space" measured	ECF vol. (space size) ml/kg wet wt	Total H ₂ O ml/kg	Total sodium meq/kg wet wt.	Total potassium meq/kg wet wt.	Author
Sprague Dawley			768	33.46	69.11	Woodbury (1956)
Wistar strain	inulin (3 hrs)	128	741	30.8	93.1	Ledingham (1953)
Rat - whole heart			776	32.2	82	* Widdowson & Dickerson (1964)
Sprague-Dawley			768.6	41.07	80.95	Kalant (1956)
Wistar strain					82.5	Prioreschi (1963)
Rat - cardiac chloride		234				Manery & Haege (1941) 3
Rat "	Cl ³⁸	220				" "
Rat - "					84.7	Noonan et al (1941)
Rat - R.atrium	inulin sucrose	248 329				Barclay et al (1959)
L. atrium	inulin sucrose	213 258				" "
R.ventricle	inulin sucrose	151 224				" "
L.ventricle	inulin sucrose	91 195				" "
Rat, Wistar strain						
L.ventricle	S ³⁵ -sulphate	192	771	30.5	84.7	Horwood (1968)
R.ventricle	"	211	774	38.1	82.0	" "

* averaged from 7 authors

be factors pertaining to rapid or accelerated muscle fibre growth. The fact that thigh muscle and left ventricle with the lowest sulphate space show the greatest increases (Figures 8 and 9) may also be significant

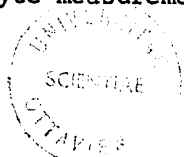
It is of particular interest that Kobayashi and Yonemura (1967) have recently discovered a relationship between size of muscle (or rat) and inulin space. They conclude that inulin space in rat skeletal muscle (soleus and extensor digitorum longus) is related to the reciprocal of the weight of the muscle (and hence the size of the rat). The regression equation of the space on the reciprocal is worked out and assumed to apply to all weights of rats between 70 and 395 g.

Lowry et al (1942) concluded that water and chloride decrease in rat skeletal muscle during growth and rise again in old age (approx. 668 days). However their extracellular measurements were based entirely on chloride space (calculated) and according to the report, measurements were only made at the ages of 71, 153, 449 and 668 days so changes occurring during the most rapid growth period may have been missed.

The increase in total sodium in cardiac muscle of older rats is not surprising. A decrease in potassium content was also noted by Lowry et al (1942).

Intracellular electrolyte concentrations

Now that we have established that our sulphate space measurements cannot be more than slightly higher than the true extracellular fluid volume and our electrolyte measurements compare favourably with



those of other investigators, we are now in a position to calculate intracellular concentrations of sodium and potassium,

Although it has been shown that mammalian heart muscle has at least three fluid compartments, the third compartment is really a subcompartment of either the extracellular or intracellular phase and as mentioned earlier there are many possible locations for subcompartments. Basically there is still the ECF where sodium ion predominates and the ICF where potassium ion predominates. Therefore we can still assume that there are two main compartments.

The method for calculating intracellular sodium was shown on page 90. The calculations for intracellular potassium are similar;-

First the corrections for water content of the plasma and Donnan factor are applied to the plasma potassium value $(K)_p$ which was found to be 4.50 meq/l (Table XI). The potassium concentration of the extracellular fluid then becomes:

$$(K)_E \text{ equals } (K)_p \times \frac{0.95}{0.959} \quad \text{From this, the concentra-}$$

tion of potassium in the cell water $(K)_{Cell}$ is calculated as follows:

$$(K)_{Cell} \text{ equals } \frac{(1000 \times [K]_m) - (ECV \cdot (K)_E)}{[H_2O]_m - ECV}$$

where $[K]_m$ represents the total potassium of the muscle,

ECV equals the sulphate space in ml/kg wet tissue,

$(K)_E$ is the potassium content of the ECF as calculated above,

and $[H_2O]_m$ is the total water content of the muscle in ml/kg.

Table XVIII shows the electrolyte distribution in left

TABLE XVIII

Estimated electrolyte distribution in normal and hypertrophied left ventricle of rat, male, Wistar strain

	Original data			Derived data		Total cations			
	Total water ml/kg wet tissue wt.	Sulphate space	Cell water	Total sodium meq/kg	Total K	$\text{Na}^+ + \text{K}^+$			
				$[\text{Na}]_{\text{cell}}$ meq	$(\text{Na})_{\text{cell}}$ meq/1 cell water	$(\text{K})_{\text{cell}}$			
<u>NORMALS</u>									
150 - 199 g	768.4	192.1	576.3	29.83	84.0	27.9	3.3	144.2	147.5
200 - 249	770.9	207.7	563.2	30.05	84.8	30.1	0	149.0	149.0
250 - 299	773.0	233.8	539.2	31.96	85.7	33.9	-1.9	156.8	154.9
300 - 349	767.0	212.6	554.4	31.38	84.9	30.9	0.9	151.5	152.4
350 - 399	758.2	196.8	561.4	31.40	84.0	28.6	5.0	149.7	154.7
400 - 450	759.0	190.1	568.9	33.25	84.9	27.6	10.0	147.7	157.7
Mean					84.7			149.8	152.7
\pm S.E.M.					± 0.3			± 1.7	± 1.6
<u>HYPERTROPHIED</u>									
24 hrs. 172 g	766.5	215.5	551.0	33.5	85.5	31.3	4.0	150.3	154.3
2 days 180	768.5	211.4	557.1	33.7	84.4	30.7	5.4	150.0	155.4
1 week 189	774.1	223.9	550.2	35.0	84.7	32.5	4.3	151.9	156.2
2 weeks 197	771.7	231.6	540.1	34.9	85.3	33.6	2.4	155.7	158.1
4 weeks 309	768.9	212.6	556.3	33.4	83.0	30.9	4.5	147.2	151.7
Mean \pm S.E.M.					84.6			151.0	155.1
					± 0.4			± 1.4	± 1.1

TABLE XIX

Estimated electrolyte distribution in normal and hypertrophied right ventricle of rat, male, Wistar strain

	Original data			Derived data		
	Total water	Sulphate space ml/kg wet tissue wt.	Cell water wt.	Total sodium meq/kg	Total K meq/kg	Total cations Na ⁺ + K ⁺
<u>NORMALS</u>						
150 - 199 g.	771.2	211.4	559.8	38.0	82.0	157.7
200 - 249	773.6	223.4	550.2	38.03	82.5	158.3
250 - 299	775.0	235.8	539.2	38.36	84.6	162.5
300 - 349	769.5	212.4	557.1	37.34	83.2	159.3
350 - 399	764.8	209.4	555.4	37.34	83.2	160.4
400 - 450	763.3	200.1	563.2	38.10	80.3	156.8
Mean					82.2	159.2
± S.E.M.					±0.7	± 0.8
<u>HYPERTROPHIED</u>						
24 hrs. 172 g	775.4	241.3	534.1	40.5	77.0	152.4
2 days 180	775.9	262.6	513.3	41.8	78.6	158.6
1 week 189	785.5	291.4	494.1	44.1	77.6	160.0
2 weeks 197	773.9	287.6	486.3	40.5	79.5	160.8
4 weeks 309	776.6	230.4	546.2	38.1	79.4	151.8
Mean					78.4	156.7
± S.E.M.					±0.5	± 1.9

ventricle of normal and hypertrophied hearts and Table XIX shows the electrolyte distribution in right ventricle of normal and hypertrophied hearts. At first glance it is apparent that intracellular potassium concentration remains very constant in spite of changes in intracellular fluid volume. The total cation ($\text{Na}^+ + \text{K}^+$) concentration of the cell remains quite constant also when values for normal and hypertrophied ventricle are compared. The intracellular potassium values compare well with intracellular concentrations found for other mammalian heart muscle. For example, Page and Solomon (1960) reported 155 ± 5 meq potassium per litre of cell water in cat papillary muscle.

The two cases of very small negative values (approx. -1.5 meq/l) were discussed previously. They are close enough to 0 to be within the realm of experimental error. They are included for their relative value only. The importance of including this derived data (negative or positive) is to compare the changes which occur, not the absolute values. Therefore it is the relative changes in intracellular sodium concentration which are important, the fact that the lowest intracellular concentrations occur during the 250 to 299 g group in the normal hearts in both ventricles and at the interval of 2 weeks after aortic constriction in the hypertrophied hearts.

Hypertrophied hearts - water and electrolyte shifts

The most important changes occurring in water and electrolyte balance following aortic constriction are the changes in sulphate space and in sodium content. The per cent increases are shown graphically in Figure 10 for left ventricle and Figure 11 for right ventricle (page 88). The increases

in both sulphate space and sodium content were shown to be significant ($P < 0.05$) within 24 hours after aortic constriction, whereas measurable increases in cardiac mass were not significant until 2 to 4 days after aortic constriction (Chapter 3). Thus there is the possibility that increased extracellular fluid and increased sodium content of the tissue play some role in the mechanism which stimulates cardiac hypertrophy.

Because these two factors increased concurrently for the first two weeks following aortic constriction and then decreased concurrently the following week, the data were tested for correlation. It was found to be very high between sulphate space and sodium content of hypertrophied hearts ($r = 0.915$). Since sodium is the main cation in the extracellular fluid, this relationship is not unexpected, but which factor is dependent on the other, or if both factors are dependent on a third factor, is unknown.

If we assume that the increase in extracellular fluid volume is the first change to occur then the question is, "Does the fluid which expands the extracellular phase come from the plasma or from the cell?". In other words, does it cross the capillary membrane or the cell membrane? This question is answered by the fact that the total water remains fairly constant in spite of the changes in extracellular fluid volume. Even in the right ventricle where a significant rise in total water was observed, the increase in sulphate space greatly exceeds the increase in total water. Therefore, the fluid does not come from the plasma and edema in the usual sense does not occur. The only logical explanation, based on present knowledge, is that the extracellular fluid expands at the expense of another compartment. This may be a subcompartment of the ECF or the ICF, the

possible locations were discussed earlier. The important feature is that it appears to be a sodium-free fluid which swells the ECF, making it hypotonic. Thus sodium ions would immediately begin to diffuse into the ECF from the plasma in order to maintain its tonicity since it is an ultrafiltrate of the plasma. In this way, the total sodium content of the tissue would increase, as observed, while the total water remains constant.

What useful purpose would it serve a heart subjected to a chronic work load to have its ECF volume suddenly increase? There are two remote possibilities which should be mentioned. a) It has been shown in liver growth and regeneration that dilution of the extracellular amino acids acts as a stimulus to protein synthesis (Glinos 1958). and b) The increase in interstitial spaces in all regions of the heart may simply be due to changes in the physical dimensions of the fibres caused by stretching. In the atrium and right ventricle the increase in sulphate space is significant long before the increase in sodium content. Hort (1951) has shown that the number of layers of muscle and cross sectional area decreased when stretching occurred in thin-walled structures of the heart. Assuming that water enters to fill the void, this could explain the decrease in intracellular volume and the significant drop in intracellular constituents, potassium and phosphate, when, at the same time, the intracellular potassium concentration is calculated to be close to normal value for rats of the same size (Table XIX).

These changes in physical dimensions of the muscle fibres of the atrium and right ventricle may result from the immediate drop in cardiac output which has been shown to follow aortic constriction (Beznak 1954), since a drop in cardiac output could cause increased venous pressure and

hence increased atrial and right ventricular pressure. However, although the cardiac output returns to normal within a day or two, the sulphate space and sodium content continue to rise significantly. Therefore, no correlation appears to exist between cardiac output and increased sulphate space. Nor is there correlation with other hemodynamic factors, such as hypertension. Blood pressure has been shown to increase markedly after aortic constriction (Beznak 1954) and to continue increasing long after the sulphate space has returned to normal range.

What seems more likely is the assumption that sodium enters the tissue first. What reason brings it there? One possibility is its relation to protein synthesis. Allfrey et al (1957) have shown that the transport of amino acids into the nucleus is strongly sodium-dependent. Since the rate of protein synthesis has been shown to increase in the first stage after aortic constriction (Meerson 1962), thus it is quite feasible that amino acids are being transported into the cell soon after aortic constriction and sodium ions with them, because of their dependence on sodium. As the amino acids are utilized, the excess sodium ions would be actively transported out of the cell, and water with them, since the principle process for the shift of water out of the cell is believed to be the net transfer of ions, passively or actively.

This theory could also explain the observed drop in potassium and total phosphate which appear to vary with the degree of hypertrophy. Allfrey et al (1957) have also shown that potassium has no effect on the incorporation of labelled amino acids, in fact the rapid concentration of amino acids causes loss of potassium at the same time that accumulation of

of sodium is observed. Thus metabolic, as well as physical and hemodynamic factors might explain the observed changes.

The decreases in potassium and total phosphate which vary with the degree of hypertrophy may both be associated with excess energy requirements of the cell during hypertrophy, e.g. for protein synthesis, active transport, etc. If the phosphate complex anions reach the metabolic stage where they are being broken down faster than they are being replaced, then potassium ions, normally held in the cell by the negatively charged macromolecules (Chap.V, Introduction), would leave the cell also.

Throughout the experiments the assumption has been made that radioactive sulphate space represents the extracellular fluid volume both in the normal as well as in the hypertrophied myocardium. Alterations in cell permeability of tissues normally impermeable to sulphate would result in apparently higher values but there is no evidence to indicate that such changes occur. Thus it is more likely that all of the sodium remains extracellularly located. Manery (1954) stated that since radioactive sodium-24 equilibrates with the whole sodium space in heart muscle within 8 minutes, there is the possibility that "excess" sodium may occupy extracellular sites. For instance, it may become associated with an anionic polyelectrolyte, an osmotically inactive anion in connective tissue ground substance. If we examine the structure of chondroitin sulphate^(p.56), we see that each of its 50 repeating disaccharide units contains two anionic binding sites.

Mineralocorticoids, growth hormone and thyroxine, all of which have been found instrumental in producing hypertrophy experimentally (Chap. I, Introduction), have also been shown to have a direct effect on the

synthesis of connective tissue, stimulating collagen fibre synthesis and synthesis of ground substances such as chondroitin sulphate and hyaluronic acid. Thus an increase in connective tissue ground substance could be one of the first changes to occur prior to cardiac hypertrophy. This could cause retention of sodium, bound to chondroitin sulphate and could also cause retention of water in the protein-polysaccharide gel between cells and collagen fibres. The Gibbs-Donnan effect of this changing protein-polysaccharide system would greatly affect water and electrolyte content of the extracellular phase.

Furthermore, it has been shown that deoxycorticosterone acetate (DOCA) causes precisely the same changes in water and electrolyte balance that were observed to follow aortic constriction in the present study. Gaudino and Levitt (1949) showed that when 30 mg of DOCA per day were administered to dogs, the extracellular space expanded and reached a peak in 9 to 10 days, then returned to normal. At the same time, intracellular fluid volume decreased and sodium and intracellular potassium were found to be elevated.

Thus, although very few studies exist with which one can compare the observed changes in water and electrolyte values in hypertrophied hearts, similar shifts have been observed in studies involving experimental hypertension and hormonal changes.

Ledingham (1956) noted that inulin space rose in the whole body as well as in skeletal and cardiac muscle during experimental renal hypertension produced in rats. He also noted a significant increase in the total sodium content of the heart and decreased potassium and intracellular sodium concentration. He later (1964) observed that the level of circulating renin increased

for approximately the same length of time that the extracellular fluid volume was found to increase after renal constriction.

Another study showing shifts in water and electrolyte values is that of Willard (1968). During hypertension induced by L-triiodothyronine and salt he observed significant increases to occur in the water, sodium, potassium and chloride content of the aorta, but small increases are shown for the heart as well.

Thus the shift of water to the extracellular phase, the retention of sodium and the decreases in total potassium and phosphate may be related to one or several of the factors discussed. Hemodynamic factors do not appear to play a part and physical factors may play a questionable if incidental role. However there seems little doubt that metabolic and hormonal factors are very important.

CHAPTER VII

Summary and Conclusions

1. S^{35} -sulphate was used to measure the extracellular fluid volume in rat heart and skeletal muscle. It proved to give a constant ratio of counts per minute in tissue for up to 5 hours, unlike "in vitro" counts per minute in serum reports of sulphate space in rat heart muscle.
2. Cardiac hypertrophy was produced in rats by aortic constriction. Following aortic constriction, sulphate space increases were found to be significant ($P < 0.05$) by the 3rd hour in the right ventricle, by the 7th hour in the atrium and by 24 hours in the left ventricle. The increases in sulphate space became even more significant ($P < 0.001$) at 1 and 2 weeks after aortic constriction and then decreased to within 5% of normal values by the end of the 3rd week.
3. A similar increase in sulphate space was shown to occur in normal, growing rat heart and skeletal muscle at the period when the growth curve of the rat is steepest. When normal rats with body weights between 150 and 450 g were grouped according to size, it was found that the sulphate space in atrium, right and left ventricles and skeletal muscle increased to a maximum in the 250 to 300 g group in each case and then dropped significantly in the 350 to 400 g group and further in the 400 to 450 g group.
4. An increase in sodium content of the left ventricle was also found to be significant by the end of 24 hours after constriction ($P < 0.05$).

Correlation was found to be very high ($r = 0.915$) between the increases in sulphate space and the sodium content of both ventricles.

5. Because the sulphate space expands while the total water remains constant, the ECF must increase at the expense of another fluid compartment, possibly a subcompartment of the cell, since it is deduced to be a sodium-free fluid which swells the ECF.

6. In normal hearts, total water content, potassium (intracellular) and sodium (of the right ventricle only) all reached maximal values in the 250 to 300 gram rats. The values reached during this period of rapid growth were quite similar to those of hypertrophied hearts at two weeks after aortic constriction. A relationship is thus established between normal growth and hypertrophy, not previously reported.

7. The decreases in total phosphate and potassium appear to be related to the degree of hypertrophy. They may be associated with excessive energy demands of the cell, leading to breakdown of phosphate complex anions and hence fewer negatively-charged macromolecules to balance the cations, so potassium content of the cell decreases as well.

8. The observed shifts in water and electrolytes may be associated with hormonal changes since similar shifts have been observed by others when deoxycorticosterone was administered and also during experimental renal hypertension when the amount of circulating renin was elevated. Deoxycorticosterone has also been shown to stimulate connective tissue substances such as chondroitin sulphate which has many anionic binding sites and may thus account for sodium retention.

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