

THE UNIVERSITY OF MINNESOTA

GRADUATE SCHOOL

Report

of

Committee on Examination

This is to certify that we the undersigned, as a committee of the Graduate School, have given Charles Edward Shepard final oral examination for the degree of Master of Science . We recommend that the degree of Master of Science be conferred upon the candidate.

Minneapolis, Minnesota

May 15 1922

F. H. Scott
Chairman

Willis Spang

E. J. Lund

E. A. Brown

THE UNIVERSITY OF MINNESOTA

GRADUATE SCHOOL

Report
of
Committee on Thesis

The undersigned, acting as a Committee of the Graduate School, have read the accompanying thesis submitted by Charles Edward Shepard for the degree of Master of Science. They approve it as a thesis meeting the requirements of the Graduate School of the University of Minnesota, and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science.

F. H. Scott.
Chairman

F. C. Mann

E. J. Lund.

Date _____

THE DISTRIBUTION OF CALCIUM
IN THE TISSUES:
WITH SPECIAL REFERENCE TO
SMOOTH AND STRIATED MUSCLE

By

C. E. Shepard.

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MINNESOTA
LIBRARY

A Thesis Submitted to the Faculty
of the Graduate School of the
University of Minnesota
in partial fulfillment of the
degree of
Master of Science
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INTRODUCTION AND PREPARATION.

The work of Sydney Ringer and his predecessors concerning the physiological action of inorganic salts, has been followed by a host of investigations pertaining to the variations in behavior of tissues immersed in solutions of single inorganic salt or in solutions of various combinations of such salts. Changes of irritability have been recorded from many salts, by many investigators, in many ways, and Loeb (1) and others have inquired into changes in cell permeability of tissues immersed in various inorganic solutions. The history of development of our knowledge of this subject is quite recent in origin and rapid in growth - in great contrast to that more fundamental knowledge, the study of the chemical structure of tissues. While the subject of physiological behavior of muscle tissue has been extensively investigated, there remains a most striking paucity of accurate data regarding the chemical constituents of this tissue. The history of this latter subject is far more remote in its origin, and has advanced much less rapidly than that of the former subject. This is mainly due to slow progress in the inauguration of even reasonably accurate methods of quantitative chemical analysis, great variation in results due to calculations of the elements as percentages of the "fresh" whole tissue, the difficulty in obtaining these percentages in terms of "pure" tissue, and lack of regard for "individual variation" in tissues. Slowly we are advancing toward the goal of desired accuracy in this field, and any contribution which leads us toward that end is worthy of consideration.

The history of development of quantitative chemical analysis of the inorganic constituents of the tissues is fascinating from its remoteness and because so much credit is due the earliest workers for

having accomplished so much in the face of the handicap of crude methods of the time as they appear to us today. The history of the subject divides itself voluntarily into three epochs. There has been no one man in this field as Saussure was in the realm of Plant Chemistry, who has devoted his life to the work. Berzelius, working in the first decade of the nineteenth century must be accredited at least with having stimulated an interest in physiological quantitative analysis. His students have developed his ideas. I would compare the work of his students and followers much as Walsh (2) in his history of medicine compares the works of Lavoisier and Berzelius "The first had opportunity to do great things while it was yet possible to do them; to his successor was left only the possibility of developing his thoughts."

Justus von Liebig, pupil of Berzelius developed the work introduced by his teacher and he is the one outstanding figure of the first epoch. Concerning the literature on the subject he states (3) that the only contributors are a small number of chemists whose work is merely suggestive enough to stimulate an interest. Of Berzelius, he says, "Die Untersuchungen welche Berzelius, vor vierzig Jahren begann ----- es hat in dem Geiste dieser Mann kein Nach * ahmung gefunden." The actual quantitative work summarized in this article is brief and of no value, excepting as it serves as a cornerstone of the foundation upon which physiological quantitative analysis is built. The article is interesting in showing a change in the current of scientific philosophy of that time. He emphasized the importance to therapeutic methods, of studying the intimate chemical changes which occur in disease, rather than awaiting "mental inspiration from merely accumulating statistics concerning the symptoms of disease."

which was then in vogue. He continues concerning this latter comment, "Recent times have shown this method of perfecting therapy to be fruitless, but we are now apt to make no less a mistake in the new system (study of chemistry of the diseased body) in that we seek information from others without showing any inclination to work them out for ourselves."

Working under von Liebig, Verdeil (4) has made probably the first systematic study of the inorganic constituents of the blood of different species of animals. Enormous amounts of blood were used. He describes a new method of ashing. Chevreul was the first to use the ashing method described by Berzelius, consisting of evaporating to dryness and subjecting to heat over long periods (weeks and even months), a most time consuming process. Verdeil decreased the time element somewhat by adding ammonium nitrate to the charred residue from evaporation. To prevent spattering, a little water was added and slowly evaporated. In this way the mass was made compact and the crystals of nitrate aided in the ignition of the carbon "without loss of substance." Aliquots of the digested solution were taken for analysis of the various elements. His results are given for whole blood of the ox, sheep, pig, calf, and for man, as percentage of the ash. Calcium was given as the oxide, amounts were very variable even among animals of the same species, and are very much lower than results obtained by more recent investigations.

Franz Keller (5) under direction of von Liebig undertook a chemical analysis of the inorganic elements of tissues. Again, twenty to fifty pounds of tissue were used in a single analysis. Methods are similar to those of Verdeil, but the work is very superficial, not nearly as careful nor as complete as that of Verdeil.

Strecker (6), another student of von Liebig applied the newly introduced method of ashing described by Rose, to correlating inorganic and organic quantitative analysis of blood. Staffel (7) also used this new method of ashing. Stoelzel (8) and Ecchevarria (9) working in von Liebig's laboratories, and others continued in the use of similar methods, with the disastrous consequences to progress of the work which ensued from total disagreement of results. The most variable figures were obtained. In 1850, Weber (10) presented quite a complete analysis of the ash of horseflesh. Unfortunately his methods were those of von Liebig's pupils. His results were given as percentages of ash and vary greatly. But an improvement was made by him in the process of ashing, whereby the volatilization of some of the compounds was hindered. Since there are no volatile salts of calcium, it is unnecessary to utilize this remedy in the present work. Weber's results are subject to far greater error, however, from the unfortunate fact that he perfused the tissues before analysis with distilled water until the perfusing fluid became colorless, in order to wash out the blood, and his discussion makes no mention of the error which was sure to result from departure of some salts from the tissue cells into the perfusing fluid. From this time preference has been given to the analysis of three inorganic elements, potassium, sodium and chlorine. The estimation of calcium was neglected and has never been given special consideration since that time.

There followed a period of two decades during which time very little work was accomplished along this line. Then the second epoch of its history was begun by the publication of work by Bunge (11) who undoubtedly received his stimulus from unsatisfactory

results of previous investigators. He has published several series of inorganic analyses of the ash of blood and muscle tissue (12, 13 14). But his methods were the same as his predecessors and his results varied not only in checks upon his own work, but more widely when compared with the work of others. To him, however, must be given credit for obtaining his analytic figures in percentages of fresh tissue. He has established this desirable precedent. In 1896 the work was taken up by Katz (15). He is the first observer to recognize the importance as contaminating factors, of connective and adipose tissue, blood vessels and nerves, in obtaining good analytical results. Concerning this source of error, he says, "Eine Zerlegung des Fleisches in diese seine Bestandteile wäre natürlich das erste Postulat für eine ganz exakte Analyse seiner chemischen Bestandteile. Leider aber ist diese Bedingung praktisch unerfüllbar ----". To offer a contribution toward the fulfillment of this postulate, which to him appeared practically impossible, has been my guide and the results of my experimental work will indicate to what an extent I have succeeded. Katz considers the importance of the study of chemical structure twofold:- First, in that it gives us a basis for the investigation of contractile substance, and second, since animal tissue is one of the most important food substances for man, it deserves consideration from the standpoint of dietetics. The work of Katz is one of the most important accessions to our knowledge of the subject during this period. It serves as a landmark in the advance of inorganic quantitative analysis. His investigations include a variety of animal species and the analyses were made on all of the important inorganic elements. The results are arranged in commendable tabular form and curves are given to

represent the distribution of potassium and sodium in tissues of various animals. It is by far the most thorough and complete analysis published up to the present time. Considering the method of ashing he employed, the amount of time consumed for the experimental work must have been tremendous. While by far the most emphasis is laid on the analysis of potassium and sodium, estimations on calcium were also made. The element was isolated as the oxide (Aetzkalk) and weighed gravimetrically. Calcium gave by far the most variable results of all elements analyzed ranging from .002% of the fresh tissue in the steer to .04% in the eel and pike. But though he speaks emphatically of the importance of giving figures in terms of percentage of fresh tissue, yet in preparing the tissue for analysis, he has consumed so much time in dissecting out the larger areas of connective tissue and fat and the larger blood vessels, that his tissue ready for weighing can hardly be considered as fresh. From my own experience of weighing fresh tissues, I find that a matter of a few minutes makes considerable difference in weight on account of the rapid evaporation of water from a muscle removed from the warm body. His analyses may scarcely be considered ^{even} of comparative accuracy on account of the varying amount of difficulty encountered in each tissue in removing its contaminating factors.

The origin of history of work concerned with the distribution of the inorganic elements in different tissues of the same animal is of still more recent date. The first reference available to me is given in Kühne's textbook of physiological chemistry (16) in which he states that smooth muscle is richer in sodium than in potassium, without, however, giving reference to any work upon which

the statement is based. Neumeister (17) says that there is little difference in the chemical content of smooth and striated muscle and the context makes it appear that the statement is based on chemical analysis of the ash. Authors of more recent textbooks seem to appreciate this lack of accurate data concerning chemical analysis of tissues. Neumeister comments on the lack of accurate knowledge. Hoppe-Seyler (18) plead for more accurate work. Halliburton (19) quotes Bunge's figures without commenting on their accuracy. Erdmann deprecates the inaccuracy of results of earlier investigators. So little credit has been given to the accuracy of early investigations that Grützner (20) in an excellent resume of work to that date dealing with the physiology of smooth muscle makes this statement, "Über die genaue chemische Zusammensetzung des Glatten Muskels ---- wissen wir äusserst wenig." Concerning the analysis of ash he states that no investigations of even moderate accuracy had been undertaken.

As a brief summary of the literature of the subject up to the first part of the twentieth century, we must admit that no work of true quantitative value, concerning the analysis of the chemical elements of tissues had been accomplished. While various species of animals had been used in the experiments there had been little or no experimental work concerned with the distribution of these elements in different tissues of the same animal. The only contributions of actual quantitative value are from the works of Bischoff (21) for the percentage water content of different organs of the animal body and of Bottazzi and Cappelli (22) who have investigated the water content of muscle tissue.

The modern epoch may be said to have been ushered in by

Grützner's publication. There has been no work equal to that of Katz in thoroughness and completeness, but thanks to the introduction of more accurate methods of quantitative analysis by Neumann (23) and a more rapid process of ashing described by him, the results of more recent workers have been somewhat more accurate, though in no case yet have the results of one observer been confirmed by another. I have been able to learn of only three recent accounts of more or less complete analyses of the ash of tissues. Saiki (24) gives a careful quantitative analysis of the inorganic and of a few organic constituents of smooth muscle. The author seems to have received his stimulus for the work from the statements of Grützner. The quantitative methods and ashing process are those introduced by Neumann. He admits the difficulty in obtaining "pure" specimens of muscle and gives as the chief contaminating factors, connective tissue and residual portions of blood and lymph. He uses the stomach and urinary bladder of the pig "prepared by careful dissection as soon as possible after removal of the organs from the slaughter house and after superficial cleaning." He desires that his results be accepted only for comparative value, and hopes to enhance their absolute quantitative value by using two groups of muscles from different regions and with "distinctly unlike structural environment." Results are given as percentage element of fresh tissue. Again the criticism arises that the tissue was not strictly fresh and that varying amounts of time must have been required for dissecting out connective tissue areas after removal from the body. In addition, no mention was made as to how soon after removal from the body the weighing was made - a most important point in any quantitative calculation. The "superficial cleaning" mentioned must have been the

wiping off of blood stained tissues, an unsatisfactory method to say the least. I have discarded tissues which were contaminated on the surface by blood, since no accuracy can be hoped for by using such muscles. He makes the mistake of attempting in tabular form to compare the results of his analyses on smooth muscle with those of Katz on striated and with Abderhalden's on blood serum, thus reducing greatly his own hopes that his results might be of "comparative accuracy." The values are naturally at great variance with those of Katz; e.g. his figure for calcium oxide for smooth muscle is .044% of fresh tissue as compared with Katzes figure of .011% for striated; and for potassium oxide Saiki's is .081% to Katzes .306%. That there could actually be such an extreme difference is unlikely. Saiki lays considerable emphasis on the large amount of calcium which he found in smooth muscle and suggests (pp.492-493) that this element may have something to do with the well known property of this tissue to remain in tonic contraction for long periods of time. Though he lays no emphasis on the similarity or dissimilarity of the two types of smooth muscle analyzed as to their inorganic content, yet he is the first who has considered the importance of analyzing two types of tissue from the same body.

In 1911, Costantino (25) published a series of quantitative analyses giving the potassium, sodium and chlorine content^{of} striated muscle tissues of various species of animals. Like the work of Katz this is more praiseworthy for its thoroughness and the variety of animal species used, than for its accuracy of method. He has analyzed the ash of striated muscles in the human being, steer, pig, calf, stag, rabbit, dog, cat, chicken, frog, haddock, eel and pike. He made a few analyses on smooth muscle also and it is of interest at

this point to note that in his determinations he used a representative type of smooth muscle, the retractor penis of the dog. The fresh weight of the muscle used was nineteen grams. The fresh weight of the largest muscle used in my experiments which was obtained from a 63 pound collie dog was ten grams, and later histological work demonstrated this muscle to be composed almost one-half of striated fibers! He must have used a good share of sphincter ani externus into which the retractor muscle inserts.

By far the most comprehensive work yet done on this subject was presented in 1911 by Meigs and Ryan (26) in which they undertook an inorganic analysis of smooth and striated muscles of the American bull frog (*Rana Catesbiana*). The stomach muscles of several frogs were used in a single analysis. Care was taken in removal of the mucous coat to prevent contamination by gastric contents. Muscles were invariably still irritable at the time chemical examination was begun. They have attempted to reduce the contaminating factor of residual blood and lymph in the tissue "by pressing several times against hardened filter paper" before weighing. I question the advisability of this method. The quantitative chemical methods employed are accepted accurate methods. The most interesting feature of the work seems to me to be the figures obtained for calcium in smooth as compared with striated muscle. In striated muscles the figures are much higher than those obtained by Katz and earlier workers. In smooth muscle they are one-eighth of Saiki's values. This would indicate that more calcium is contained in striated than in smooth muscle - a finding at utter variance with previous results! Reckoned as percentage of fresh striated muscle, their results are .028 grams of calcium to Katzes figure of .0157

grams and of smooth muscle .0042 grams to Saiki's figure of .032 grams. Such a radical difference should justify further investigations for confirmation or rejection, especially since the series of experiments was a small ~~one~~. The work of Meigs and Ryan is commendable, first, because they have realized the importance in preventing external contamination as from gastric juice, blood, etc., by careful preparation of tissues, secondly because they have at least realized the importance of a strict definition of "fresh tissue" though no attempt was made to limit this definition properly, and thirdly because they have chosen to limit themselves somewhat in the scope of their work and thus tend to augment the accuracy of their results. It is a precedent to be followed by their successors in this field.

PURPOSE AND SCOPE OF THIS INVESTIGATION.

Though the history of inorganic chemical analysis of animal tissues is quite remote in its origin, the history of analyses of true quantitative value has a surprisingly recent origin, and many chapters are still missing. Inaccurate quantitative methods, the tedious process of ashing under older plans, the crude way of calculating the amounts of elements present as percentages of whole tissue and the failure to eliminate the many contaminating factors, have all contributed to our present paucity of accurate data. The attempts of earlier investigators to make their experiments too extensive have also hindered the accuracy which might have been attained by a more intensive study and a narrowed scope. The wide variation in results obtained by older methods, not only in figures on the same elements calculated on different animal species, but more importantly

in the figures offered by different investigators on the same species of animal, shows the utter independability of the work. Peculiarly, up to the modern period no attempts have been made to study the distribution of inorganic elements throughout the different organs and tissues of the same body. Saiki, while still limiting himself to studies on smooth muscle of one animal species has fortunately seen the advantage in using two types of that muscle. But unfortunately he has depended upon work of earlier investigators to obtain figures of a comparable nature! for other types of tissue. Meigs and Ryan, primarily limiting themselves to the study of smooth muscle, commendably, have realized the value of comparing their figures for this tissue with their own figures for the striated variety thus obtaining comparative accuracy, at least. And in limiting themselves to one type of animal they have been able to enhance the thoroughness of their investigations. There is the disadvantage, however, that in so doing they have been unable to arrive at any general statement concerning the distribution of the elements in tissues of other species of animals, by which they might have increased the value of their work both comparatively and phylogenetically. The chief failing in all work to date lies in the fact that no attempt has been made to record a series of determinations on the same tissues of the same species of animal. It has always been the experience of workers in research, be it physiological or medical (though the failing is greater in the latter) that the first few determinations of a series, usually give results according to anticipation, while the progress in the series sometimes brings disagreement with the first and disappointing results to the support of an hypothesis. The goal toward which we should strive,

in the field of experimental research as well as in the field of clinical therapy, is that of preventing the possibility of "individual variation" by accumulating statistics to cover a large series of cases. The living organism is too much subject to individuality in the nature of response to change in environment, to allow the advisability of any other method of studying the responses of living organisms as a class.

In preparing for my experiments I have had these many mistakes of earlier workers to consider and profit by. It is often the errors of our predecessors in research which may add the desired impetus to our own proper advancement. It is my hope to be able to contribute something to the advancement of our knowledge of physiological quantitative analysis which will aid in progress toward the goal of desired accuracy. First it has seemed advisable to limit the scope of the work in the hope of making more thorough that which was accomplished. I have chosen to investigate calcium mainly because of all the elements whose distribution in the tissues has been studied, this element has been given the most variable values by investigators. It has varied in amount in the same animal analyzed by the same investigator, and has varied in different animal species analysed by the same investigator, but has varied the most in the same animal species analysed by different investigators! In modern times it has been shown to vary considerably in different tissues of the same animal; Saiki believing, for instance, that smooth muscle contains far more than striated, which is diametrically opposed to the findings of Meigs and Ryan. Surely the knowledge of no element present in the tissue is more in the "Stage of Disagreement" than that concerning calcium! Other reasons for having chosen

study of this element will appear in the Discussion. I have chosen the mammal as being the species of most interest to us, and because it is easier to obtain larger amounts of tissue in fresh condition from one animal. Realizing the value of drawing conclusions only from a general uniformity of results from a series of experiments performed under the same conditions, in order to avoid the error of individual variation, I have obtained results from a series of thirty dogs. The dog was chosen mainly because of the thickness of the sheet of smooth muscle which lines the alimentary tract in the pyloric region of the stomach, which facilitates removal; and also because of the size and ease in decapsulation without injury and external contamination of the retractor penis muscle, which was to be used as a second type of smooth muscle; and also because I wish to obtain normal calcium values on the normal dog to serve as a check in some future experimentation. However, though the largest series has been run on the tissues of this animal, the cat as a second type of carnivorous animal was also used, and as type of herbivorous animal the rabbit was used.

I have already stated how little inquiry has been made into the distribution of the elements in different tissues of the same animal. Saiki has attempted to compare his figures for smooth muscle with those of Katz for striated, in itself an impossible comparison, but valuable in that it has led to further investigation of the matter. Meigs and Ryan were the first to use different tissues from the same type of animal, in their experiments on the frog. But the tissues from several frogs were subjected to a single analysis. The important feature of individual variation has never been taken into account, and this is of considerable quantitative

importance. I have used for comparison in every case the tissues from the same animal. There has never been a comparison between the inorganic constituents of muscular tissue and the elements in blood and blood plasma. I have compared these two, also in the same animal. Quantitative figures for the normal ratio of tissue calcium might aid somewhat in the solution of the many problems concerned with the selective permeability of cell membranes. And it might also serve as an additional fact which would point toward the conclusion that smooth muscle fibers are not surrounded by semi-permeable membranes. Normal figures for this ratio would also give us a basis of inquiry into the changes in that ratio which occur from increased or decreased calcium intake; and more especially, might give light upon the cause of symptoms produced in diseases where blood calcium is diminished, as in infantile tetany, spasmophilia and rickets.

METHODS EMPLOYED TO ELIMINATE SEVERAL IMPORTANT SOURCES OF ERROR.

Sources of error in the subject fall into two groups, chemical and histological. Those of the latter group may be considered as the most formidable, but nevertheless a careful study of the former should be undertaken.

A. Chemical.

In the effort to select for this work the most satisfactory quantitative chemical method for the determination of calcium a survey of the literature has been undertaken in order to study the various types of processes used and to select that method which has proven most satisfactory. After a critical review of the methods,

the ashing-volumetric method was adopted as the most satisfactory and after it had been used in more than one-half of the experimental work, an article appearing in December, 1931, by Clark (27) confirmed its adoption. This author after some two thousand calcium analyses by various methods, adopts the ashing-volumetric method as the standard of accuracy.

A critical summary of the various ashing and analytic methods is here given in order to justify the adoption of those used in the present work. Classified according to the manner of obtaining solutions from which calcium may be precipitated, methods fall into three groups. 1. Direct Precipitation in the Presence of Proteins. This method has been described recently by Kramer and Tisdall (28). It is applicable only to blood and serum. It serves satisfactorily as a very rapid method for small amounts of specimen but is not at all accurate. 2. Precipitation of Proteins by Chemical Precipitants. Picric, tungstic, and trichloroacetic acids are most commonly employed. Aliquots of the filtrate are used. Halverson and Bergeim (29) have used this method and it is also used by Lyman (30); McCrudden (31) has a modification and Clark (32) further modifies the latter's technique. It is used only on organic solutions containing calcium, such as blood, plasma, serum, milk, etc. 3. Destruction of Organic Matter by Ashing or by Digestion with an Oxidizing Agent. This is followed by a solution of the ash in hydrochloric acid. The method involves the choice of one of two ashing methods, either direct ashing, in platinum or silica dishes, the heat applied over long periods of time. This has previously been a slow and tedious procedure but more recently has taken on quite different proportions from the adoption of the use of the electric furnace, or

the "Säuregemisch-Veraschung", introduced by Neumann. It was desired to compare the accuracy of these two methods. Two samples of material, each containing the same amounts of calcium were ashed, one by Neumann's method, the other placed in an electric furnace. Calcium analyses of the ash gave almost identical results. Had an electric oven not been available the oxidation method would have been used, but after considering in this method the difficulty of regulating heat to avoid splattering, the regulation of inflow of the oxidizing substance and the time consuming element, and after considering the favorable comparison of the two methods as to accuracy, the direct ashing method has been adopted. The criticisms concerning the use of the oxidation and direct ashing methods are here summarized:-

I. Digestion with nitric and sulphuric acid as suggested by Neumann and used by Marriott and Howland and others is objectionable:-

1. Because it cannot be adopted so readily to the ashing of solid tissues.
2. Because it is difficult to prevent mechanical loss during oxidation (as by foaming of blood).
3. Because accuracy in digestion of small amounts, even in the best of glassware is questionable.
4. Because of the length of time required to complete oxidation and to evaporate the excess of acid.

II. Direct ashing by means of the electric oven may be criticized:-

1. Because there may be an expulsion of small particles during early stages of incineration by rapidly evolving gas.
2. Because there is danger of loss of material by splatter-

ing unless the first heating is carefully regulated.

3. Because it is difficult to completely dissolve the fused residue in hydrochloric acid.
4. Because of the length of time required.

The first and second criticisms have been minimized by placing tissues to be ashed in a cool oven and allowing the temperature to rise slowly. The third is a real criticism as I have found by running blank determinations with calcium free reagents, using the ashing dishes emptied and rinsed of contents (the digestion process supposedly having been completed). It was discovered that such dishes whose contents had been subjected to the action of hot hydrochloric acid after which digestion was allowed to continue three hours and then expelled for analysis, gave a blank test for calcium of from .5 to 1.5 milligrams. This error has been over-come by subjecting the digestion material to heat for an hour with frequent stirring and then allowing the digestion to continue ten or twelve hours longer, after which blank tests on the emptied and rinsed dish were negative. The minimization of the first three sources of error leaves as the main criticism to this as to the oxidation method, the amount of time required. This factor has been so much diminished by the use of the electric furnace that much less time is required for it than for the ashing by Neumann's method.

After digestion of the ash with hydrochloric acid, the calcium (present as CaCl_2) is precipitated. Almost all methods utilize for this purpose the oxalate ion. Axon (34) uses Neumann's method for ashing and precipitates the calcium as sulphate, claiming greater accuracy thereby. This may be satisfactory for large amounts of calcium where gravimetric methods are used, but for small amounts

where volumetric methods are the most accurate it is hardly to be considered. His figures of comparison between the Neumann - calcium sulphate method and the direct ashing - calcium oxalate method vary within a few tenths milligrams - not enough to warrant an adoption of his more complicated procedure. Considerable variation is observed in methods of different workers in obtaining the optimum H ion concentration at which calcium oxalate is best precipitated. And this indeed is an important question, as has been observed from the present series of experiments. The size of the particles of precipitate, and consequently the error in loss of calcium in filtration depends largely on the slightest changes of the pH of the solution. Treadwell (35) has used methyl orange as an indicator in the neutralization of the excess acid. Other authors (Marriott and Howland) have used phenolsulphonphthalein. Jones and Nye and McCrudden (36) have used alizarin. In order to obtain proper H ion concentration in the present series of experiments bromphenol blue has been used, as introduced by McClendon and described in an article on experimental rickets (37). At a pH of 4 which is the turning point of bromphenol blue from yellow to lavender, the calcium oxalate will be practically insoluble, and yet no calcium will precipitate as the phosphate.

After obtaining the washed calcium oxalate the following procedures have been used for the actual estimation of calcium.

I. Gravimetric. By conversion to calcium oxide or sulphate (Avon) and weighing. This method was used entirely in work previous to the time of Neumann and Treadwell and by some German authors even since the introduction of volumetric methods (38). The method may be satisfactory for large quantities of calcium but is not accurate

enough for minute amounts.

II. Nephelometric. Suggested by Lyman (30) as a rapid means to be used in blood and milk. He precipitates proteins with trichloroacetic acid, the calcium as oxalate, then converts it into the calcium soap (stearate). As a rapid and rough method it is satisfactory. Had the author not emphasized its accuracy (claims 1% error) the work would not have received so much criticism. Clark's comment on this method is enlightening as to its real accuracy, "In beginning experimental work on calcium, Lyman's nephelometric method was selected because of the apparent rapidity with which determinations could be made. A large number of determinations made on solutions of known calcium concentration ----- gave such discordant results that but few determinations were used on blood samples." Jones and Nye (39) have used the method in a series of thirty four cases, and support its accuracy above the permanganate method. But they have now completely abandoned it for the latter method. Halverson, Mohler and Bergeim (40), and more recently Kramer and Howland (41) have shown that the limits of error by this method are so great as to make it valueless as an accurate quantitative procedure. The change in size of colloidal particles with the slightest variation in the H ion concentration of the solution seems to be the chief criticism.

III. Color^{imetric}. From the fact that ferric thiocyanate is decolorized by oxalates. This method is used by Marriott and Howland. They describe the use of a suction filter in the calcium oxalate filtration and in the process ^{several} filtrations are required. This brings in considerable error. On account of the size of oxalate crystals obtained in my experiments it was necessary to observe the greatest care in filtration to prevent loss through the pores of the filter,

(Whatman No. 40 double washed filter paper was used), and I question the accuracy of several filtrations, especially where suction is employed.

IV. Iodometric. This method is offered as a supplement to Jansen's (42) review of the accuracy of direct ashing - oxalate methods. It consists in reducing calcium oxalate to the oxide, dissolving it in excess of n/100 hydrochloric acid, adding a known amount of standard solution of potassium iodide, and determining the amount of excess acid by estimating the amount of iodine liberated by titration with thiosulphate solution. This method was offered only as a remedy for the inaccuracy of the very dilute permanganate solution used by Krüger and Lamers (43).

V. Volumetric.

1. By solution in excess acid and titration of excess acid, as used by Jansen.
2. By titration with potassium permanganate.

This latter has proven the most satisfactory method, indicated at least by the number of workers who have adopted it in their investigations. It is advocated by Neumann, Treadwell, Lamers, Kramer and Howland, Halverson and Bergeim, McCrudden (in his recent work), McClendon, Clark, and others. Four precautions must be observed in the use of this method in order to prevent criticism.

1. The permanganate solution must be neither too dilute (Lamers) nor too strong.
2. The standard permanganate solution must be frequently checked by titration with a standard oxalate solution.
3. The end point of titration, namely the first definite pink color which persists for thirty seconds, must not be

passed.

By a critical review of the literature on the subject it has been hoped that the best method might be adopted which would eliminate as far as possible in the present series of experiments the sources of error in quantitative chemical analysis. The next and by far the most difficult problem has arisen in the attempt to eliminate as far as possible the sources of error of an anatomical and histological nature.

B. Histological.

No matter how accurate chemical methods may be, the smallest variations in the original weight of a "fresh" tissue will make large differences in the calculation of per cent element to total tissue. These variations fall into two groups.

1. Those caused by loss in weight of the tissue from the time of removal from the body until weighing takes place.
2. Those caused by the variability in the amount of connective and adipose tissue, residual blood and lymph, which are included with the weight of the muscular element itself.

The first appears to be the primary causal factor for such great variation in results of previous investigators. Either we must define strictly the meaning of "fresh tissue", or hereafter give our results in terms of dried tissue. A fresh tissue is ordinarily thought of as being one which will still respond to a stimulus. This seems to have been accepted as satisfactory by Meigs and Ryan. But a muscle will remain irritable for some time after it has begun to dry. The drying process begins from the moment the tissue in situ is exposed by the incision. The most variable results have been obtained in the present series of experiments, in calculating

per cent calcium to fresh tissue, simply by varying the time between removal of tissue from the body and weighing. The warm tissue just removed from the body naturally loses water vapor quite rapidly, and consequently loses rapidly in weight. This has necessitated the establishment of a quick technique for removal as described in the Procedure. The tissue was placed directly upon removal into a glass stoppered weighing bottle, small enough that it was almost completely filled by the tissue. This prevents evaporation before and during weighing. Thus, a "fresh" tissue in the present work is defined as a tissue whose weight gives very nearly the weight of that same volume of tissue in situ, and the element calculated as per cent should give very nearly the percentage of that element in the tissue as it exists in the body. Thus a grave ambiguity has been overcome and an important source of error in previous investigations has been removed.

The second type of variation has proven a much more difficult factor to control. Earlier investigators have shunned it completely. More recent workers have attempted to solve the problem by various methods, all of which are objectionable. The method of pressing out the residual blood and lymph used by Meigs and Ryan is not advisable; because the pressure must be uniformly applied and invariably a considerable one in order to express all of the residual lymph and blood and when such a pressure is applied, one cannot avoid injury to the tissues which may change the cell permeability and at least will result in the expression of some fluid from within the cells. The attempt to dissect out larger areas of connective tissue as done by Saiki, Katz and others has proven the greatest source of error in their results. And probably the most unphysiological means employed to rid the tissue of residual blood was made by Weber when he perfused

the vessels with distilled water until the perfusing fluid became colorless! This brief review shows how unsatisfactory the attempts to solve this problem have been. There are two avenues of approach toward its solution. Can it best be undertaken by chemical methods or by morphological methods? The former was soon abandoned. A hypothetical plan was considered, namely, that a sample of each tissue be subjected to an analysis in such a way that the chief chemical constituent of each might be isolated and a proportion worked out for each, thus giving comparative values; as for instance from such a basis as :- that nucleoprotein is the chief organic constituent of smooth muscle, mucoid is the chief protein in connective tissue, and that a quantitative estimation of iron in the sample might indicate roughly the amount of blood present. The method is absolutely impracticable. Attention was then turned toward morphologic methods as the only possible means of solving this important problem.

By histological definition, a tissue is a group of cells similar in origin and structure and possessing a common function. This would limit quantitative chemical analysis on muscle tissue, strictly to the chemical contents of the muscle fibers themselves. If this were practically possible, many of our ideas concerning the chemical nature of intracellular processes which are now only theories might be developed into proven facts and many of the chemical secrets of the living cell would be disclosed. We cannot hope with our present knowledge of physiological quantitative analysis and morphologic methods to shed much light upon this matter but any contribution toward that end would be most valuable. Contaminating factors of muscle tissue are connective, adipose, nervous, vascular tissue, blood and lymph. The more completely these various factors can be

removed the more absolute will be the quantitative results. At present it seems an impossibility to remove all; nor can all of any one factor be eliminated. But at least most of the main contaminating factors have been removed by the method used in these experiments. Solution of the problem lies in determining by as accurate morphological means as possible the proportions of the various contaminating elements present in the given volume of muscle. Next it is necessary to know how much calcium is contained in the whole muscle and in each amount of contaminating tissue present. By deducting from the calcium content of the whole tissue, the sum of the amounts of calcium contained in the various amounts of contaminating tissue, the amount of calcium contained by the muscle fibers themselves can be obtained. The three chief contaminating factors are blood, lymph and connective tissue. It was sought at first to estimate the proportions of each of the first two in a very rough way, by sectioning pieces of muscle with a Vallentine knife immediately after removal from the animal, and immersing this section in a drop of Ringers solution on a glass slide. Irritability was tested by weak induction shocks. Then under a microscope the proportions of space occupied by lymph spaces and blood vessels were roughly estimated. The method has been discarded as too inaccurate, though it has been used later as the only practical means (though it is rough) of estimating the proportion of volume occupied by lymph spaces. I have finally adopted a volumetric method used by Hammar (44) in determining the proportions of parenchyma and stroma, and the size of Hassel's corpuscles in the thymus, the method having been modified and extended by Jackson (45) in studies on the hypophysis and suprarenal bodies. The method will be described in the procedure.

It cannot be hoped, even by this means that nearly all of the contamination will be removed. Neither has any one of the factors been wholly removed. Adipose tissue scarcely acts as a contamination of the muscle tissue used in these experiments. It is easy to remove the fat in the stomach muscle by quickly stripping off the mesenteric attachments before the muscle is excised. Nervous tissue forms such a very minute part of the whole muscle that it has been disregarded as a contamination factor. Vascular areas were calculated quite accurately by this method and found to form a very small proportion of the total, especially in smooth muscle. Lymph spaces cannot be accurately calculated by this method because they usually do not remain patent even with the most careful preparation and embedding and because of the shrinkage of the tissue in fixation. Stoelzner (46) has calculated the shrinkage in organs fixed by different methods and suggests a fixing agent (of very nearly the same osmotic pressure as Ringers fluid) in which least shrinkage occurs. But chromic salts are best for fixation of tissues when specific connective tissue stains are to be used and they produce shrinkage. Work by Rasmussen has shown that the various tissue elements tend to undergo a uniform shrinkage in fixation with Zenker's fluid. Connective tissue which forms by far the most important contaminating factor, has proved by this method the best subject for almost complete elimination. By using Mallory connective tissue stain (47) the larger masses are very easily distinguished and eliminated. Only the fine interlacing fibrils which form the interstitial network between the individual muscle fibers cannot be eliminated. This does not constitute a very great error in either type of muscle tissue although the amount of connective tissue present in this form is some-

what greater in smooth than in striated muscle.

In the foregoing discussion review has been made of previous methods employed in determining the inorganic constituents of body tissues, with criticism when it was due and in the methods to be described in the Procedure an attempt has been made to improve the accuracy of our present state of knowledge on the subject. If the methods serve as a contribution toward the attainment of greater accuracy in physiological quantitative methods, the purpose of the work has been fulfilled.

PROCEDURE.

I. PREPARATION OF TISSUE.

Normal adult male animals were always used. Voorhoeve (48) has showed in experiments concerned with calcium equilibrium in the body, i.e. the ratio of ingestion, that after the third day of starvation the amount of calcium becomes quite constant and is quite low in amount. In the fasting dog, I have found that blood calcium varies considerably in the first two days of starvation but thereafter becomes quite constant. In order that the present results be not affected by ingestion of foods the animals were fasted four days before death. Dogs VI, X and XVI (Table I) show the effect on blood and tissue calcium of only one day of starvation. One other precaution in this regard was observed. Judging from results of investigations on the variability in blood chlorides after ingestion of water, it has been deemed advisable to restrict water from the animals several hours before death. Samples of blood were taken for analysis directly after killing (usually ashed and analyzed in duplicate). No difference was found in the calcium content of blood removed from the right auricle and that removed from the left. As

soon as circulation had ceased work was begun on the removal of the tissues.

The abdominal cavity was exposed by a transverse incision in the right upper quadrant. With a sharp scalpel the pyloric end of the stomach was freed of mesentery. Two circular incisions were then made around the stomach with a razor just to and not through the mucosa, one just above the pyloric sphincter, the other farther up on the wall of the stomach where the muscle sheet was still quite thick. A longitudinal incision joined the two and the muscular layer was quickly freed from the underlying mucosa as is described in Rammstedt's method of operation for pyloric stenosis. This author lays stress on the speed with which the technique is accomplished. Practice aided in attaining desired speed in technique and accuracy in separation of the muscle coat from both mucosa and submucosa. From the excised sheet of muscle several pieces were cut and dropped into Zenkers fluid (see III) and the remainder placed in a weighing bottle as previously described. From the moment the stomach was exposed by the incision until the weighing bottle was stoppered did not exceed three minutes, and more often was ^{less} ~~two~~ - the same amount of time required to excise the other muscles used. The retractor penis muscle was first used for chemical analysis by Katz and has later been used by Bayliss and Starling in experiments on tone of smooth muscle. Its anatomy may be found in Ellenberger and Baum "Anatomie des Hundes" (49). It is well encapsulated and therefore can be removed quite easily and without external contamination. It arises as a tendon attached to the prepuce and extending posteriorly into a muscular belly is inserted by interdigitations with the sphincter ani externus. The belly has been thought previously to be composed almost purely of

smooth muscle fibers, but study of microscopic sections in this work shows that it is ^{often} more than half striated. The analytical results as shown in Table I for this muscle are quite confusing and it was not until histological corrections had been made that the reason became obvious. Cardiac muscle was taken from the wall of the left ventricle near the apex without cutting into the chamber. The sartorius was taken as an example of striated muscle because it is so often the subject of physiological experiments and also because it can be quickly cleaned of surrounding fascia and removed. The blood was removed into tubes, tightly corked and weighed, then removed to ashing dishes and the emptied tubes recorked and weighed. The tissues after weighing were placed in silicate dishes and ashed in an electric furnace.

II. CHEMICAL.

The ash was digested in hydrochloric acid with the precautions described in the preceding section and analyzed in the following manner:

1. After digestion, filter through ash free filter paper into 200 cc. Pyrex flask, rinsing ashing dish once with hydrochloric acid and several times with distilled water.
2. Add three drops of .04% solution of bromphenol blue and 20 cc. 2½% oxalic acid.
3. Neutralize with 20% sodium carbonate from a burette, slowly with rapid shaking to just the point where color changes from yellow to colorless or faint lavender.
4. Boil for some time to remove carbon dioxide and to increase the size of crystals, and allow to stand overnight.
5. Filter very carefully through ash free filter paper so as to

minimize loss of crystals through filter, and rinse flask several times.

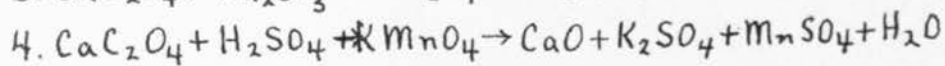
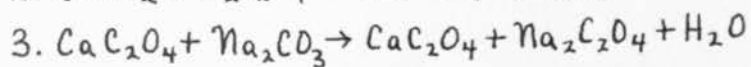
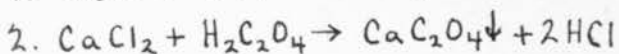
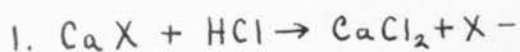
6. Return funnel into Pyrex flask from which the crystals of calcium oxalate have been removed and puncture filter with a fine glass point.

7. Wash down crystals into flask, remove but save filter paper and rinse glass point and the funnel several times with distilled water.

8. Add 2 cc. concentrated sulphuric acid and heat to 75° .

9. Titrate solution in flask and filter paper with n/10 potassium permanganate solution shaking rapidly between each addition until the first pink appears which will persist thirty seconds.

The equations and calculations are as follows:-



$$\text{Ca}^{++} = 40 \quad \text{Ca} = 20 = \frac{1}{10} \text{Ca} = .002 \text{ qms. Ca.}$$

$$\text{Mwt. KMnO}_4 = 157.93 \quad \text{Valence of K} = 5$$

$$\frac{1}{5} \text{ of } 157.93 = 31.586 \quad \frac{1}{10} \text{ KMnO}_4 = 3.1586$$

Solution standardized by titration \bar{c} $\frac{1}{10}$ oxalic acid solution.

The following precautions minimize error in the analysis:-

1. Careful weighing of tissue and bottle.
2. Slow primary heating and not too high temperatures during ashing.
3. Complete digestion as aided by time, heating and stirring.
4. Repeated rinsing ^{of} ~~by~~ ashing dishes and funnels with distilled water.
5. Proper pH for precipitation.
6. Careful filtrations.
7. Calcium free reagents.
8. Freshly standardized permanganate solution.
9. End point of last titration must not be exceeded.

III. HISTOLOGICAL.

In choosing pieces of tissue for histological study, those which would be most representative of the whole tissue were chosen and the accuracy has been further enhanced by removing pieces from several different areas in the same sheet of muscle and averaging the results. In the case of the sartorius for instance, the blood supply comes in at the center of the muscle and is distributed toward the ends. For histologic study pieces from neither ends nor center have been chosen, in the former where the vascular supply is below representative and in the latter where the supply is more than would be representative. Sharp instruments were used in cutting out the pieces in order to avoid, as far as possible, collapsing the vessels.

The process of fixation, blocking, sectioning and staining is here summarized in outline.

I. Fixation and Embedding.

1. 24 hours in Zenkers fluid.

2. 24 hours washing in tap water.
3. 6 hours in 50% alcohol.
4. 24-48 hours in 70% alcohol.
5. 12-24 hours in 80% alcohol.
6. 6 hours in 95% alcohol.
7. 2 hours in absolute alcohol.
8. Cleaning in Xylol.
9. 4 hours at 50° in 2 parts xylol, 1 part paraffin .
10. 2 hours or more at 60° in paraffin.

II. Sectioning.

Sections 5-10 microns were cut with Spencer microtome and fixed to slides in usual manner.

III. Staining.

Mallory's connective tissue stain was used.

Xylol → absolute alcohol → 95% alcohol → 70% alcohol → distilled water → acid fuchsin → .1% aqueous solution for 5 minutes → rinse → aniline blue 1%, orange G .2%, phosphomolybdic acid 1% for 20 minutes → rinse quickly in water 95% alcohol → absolute alcohol → xylol → balsam.

By this method connective tissue stains blue, muscle tissue stains red, and blood corpuscles stain yellow. Sections so prepared were placed in the Edinger projection apparatus and the outlines of the tissue, magnified seventy-five diameters, were projected and drawn on "American Linen Record" paper (18" x 32", 36 lbs. per ream). This paper has been carefully tested by Doctor Jackson and found to vary extremely little in weight at different parts of the sheet. The various areas occupied by different tissues i.e. connective tissue, blood, and muscle tissue, were cut out and weighed and the proportion

of each to the whole was calculated. The appearance of the outlines of samples of smooth and striated muscle showing areas occupied by muscle, connective tissue and blood is shown in ^{Plate II} ~~Figure IV~~. It was not found necessary to use serial sections. Variations from the general proportions even from one end of the tissue to the other were found to be surprisingly slight. Both cross and longitudinal sections were studied, but the former are probably more accurate.

It was found as expected that the same tissues from different animals of the same species vary greatly in the proportions of contaminating tissues present. In the dog, striated muscle seems to vary more in this regard than smooth. Contamination of both of these tissues by blood is relatively very slight, rather more in striated than in smooth. As already stated, it is inadvisable to calculate the areas occupied by lymph spaces in fixed sections on account of shrinkage of tissues in fixing agents. Therefore, the only choice lay in utilizing the rough method of estimation on fresh tissue described previously. As a rough estimate these spaces may be said to occupy 5-10% of the total volume.

In addition to the experiments recorded in the tables, fifteen more experiments were undertaken as follows:-

I. Five experiments on dogs in which cardiac muscle was included among the tissues analysed. The same precautions were observed as in the preparation and analysis of tissues of the first twenty-five animals used. Averages from the results on the three types of muscle shows that cardiac muscle differs only slightly from smooth muscle in its calcium content, though it invariably contains slightly ^{smaller} ~~larger~~ amounts of this element.

The general averages calculated as per cent fresh tissue are as

follows.

| | |
|-----------------|--------|
| Smooth Muscle | .0171% |
| Cardiac Muscle | .0199% |
| Striated Muscle | .0328% |

II. Five experiments on cats undertaken in the same manner. The results, though showing greater calcium values, still observed the same ratio as to calcium content of the three types of muscle i.e., smooth, cardiac, striated.

III. Five experiments on rabbits (no retractor penis) again showing different per cent value for calcium still preserved the same muscle calcium ratio.

I am not prepared to lay too much stress on the value of figures obtained on rabbits and cats as compared with those of the dog. The amounts of tissue available were much smaller. It has been my experience to note that when very small weights of tissue were used the percentage of calcium to the total was somewhat higher. The pyloric region of the stomach of the dog contains an extensive thickened sheet of musculature. In the rabbit this thickening is not nearly so extensive and is even less in the cat. The muscular layer of the fundus of the stomach is very thin. But since nearly equal weights of smooth, cardiac and striated muscle were always used from cats and rabbits, the comparison would not be changed and it is to be concluded that this same muscle calcium ratio is common to all three species of animals used.

RESULTS.

A summary of the results of chemical quantitative analysis on the various tissues through a series of twenty dogs is given in

Table I. Results from analyses of tissues from the first five dogs are not included because of the variability during the period of establishing a desirable operative technique and chemical procedure. A summary of the results, corrected by the histological methods is given in Table II. The importance of this source of error is quite evident from comparison of the tables, and the uniform reduction of individual variations toward a constant is very gratifying. Curves to show the general averages in uncorrected and corrected analyses and the amount of reduction in error by the method are shown in ^{Plate III} ~~Figure III~~.

CONCLUSIONS.

DISCUSSION.

Were it only possible by methods known to us at the present time, to determine the intimate nature of the exchange of chemical products between living cells of the human body and the circulating medium, many secrets concerning the chemical processes of life might be divulged. If we were only able to learn more definitely the nature of permeability of cell membranes in normal and diseased conditions and changes in this permeability brought about by the presence of foreign substances, e.g. excess of inorganic salts, drugs, hormones, etc. our knowledge concerning physiological processes in the normal cell, and the physiological action of drugs would be greatly enhanced. Almost all contributions to this knowledge so far have been limited to studying the changes in physiological response of tissues to changes in the nature of the circulating medium. Practically nothing has been learned regarding the intracellular chemical changes which must be responsible for this physiological action of the tissue as a whole. We cannot hope with the chemical

and morphological means available to us at this time to shed much light on this most fundamental of all physiological problems.

Quantitative chemical analysis of the contents of pure tissues under variations in environment due to changes in the circulating medium, is naturally the ideal solution. But it seems at this time almost impossible to secure a pure tissue, and chemical and morphological methods are, as yet, far too crude to make this means of solution practical. We will hope, however, that we are slowly advancing toward the solution by striving for an ideal chemical and morphological method and the slightest contribution toward attaining that end, in the way of more accurate quantitative chemical methods and by elimination of a few of the many morphological sources of error, may be worthy of consideration.

CONCLUSIONS.

1. By critical review of various methods used in physiological quantitative analysis, a method has been adopted which seems most satisfactory for the determination of calcium in both blood and solid tissues.
2. A rapid method for the removal of tissues from the body makes it possible to use the term "fresh" tissue in a stricter sense.
3. A new morphological method has been utilized in eliminating some of the important sources of error to quantitative chemical analysis of tissue, due to contamination of the pure tissue by the presence of other types of tissue.
4. The ratio of Blood calcium Tissue calcium is probably a constant in normal dogs.
5. There appears to be a definite relation between the calcium content of the various types of muscle tissue in dog, cat and rabbit, namely, Cardiac < Smooth < Striated.

PLATE I Table 1.

SUMMARY OF CALCIUM
CONTENT OF BLOOD AND TISSUES
Calculated as the number of grams of element in 100 grams

| DOG | BLOOD | BLOOD PLASMA | PLASMA | STOMACH | RETRACTOR | SARTOR. | TENDON | |
|-------|-------|--------------|--------|---------|-----------|---------|--------|-------|
| VI* | .0082 | | .0187 | | .0210 | .0260 | .0342 | .0194 |
| VII | .0064 | | .0139 | | .0180 | .0208 | .0327 | .0184 |
| VIII | .0059 | | .0139 | | .0148 | .0239 | .0294 | .0170 |
| IX | .0060 | | .0138 | | .0142 | .0250 | .0309 | .0168 |
| X* | .0080 | | .0178 | | .0210 | .0277 | .0346 | .0196 |
| XI | .0063 | | .0141 | | .0190 | .0256 | .0328 | .0184 |
| XII | .0060 | | .0139 | | .0144 | .0244 | .0335 | .0180 |
| XIII | .0063 | | .0138 | | .0147 | .0254 | .0319 | .0173 |
| XIV | .0064 | | .0140 | | .0205 | .0266 | .0314 | .0200 |
| XV | .0061 | | .0137 | | .0175 | .0265 | .0291 | .0186 |
| XVI* | .0082 | | .0188 | | .0240 | .0290 | .0354 | .0196 |
| XVII | .0064 | .0066 | .0140 | .0146 | .0180 | .0281 | .0366 | .0184 |
| XVIII | .0063 | .0065 | .0137 | .0139 | .0165 | .0250 | .0342 | .0176 |
| XIX | .0062 | .0060 | .0142 | .0139 | .0154 | .0187 | .0284 | .0174 |
| XX | .0074 | .0073 | .0147 | .0146 | .0206 | .0263 | .0301 | .0190 |
| XXI | .0062 | .0065 | .0138 | .0136 | .0133 | .0194 | .0334 | .0151 |
| XXII | .0058 | .0059 | .0136 | .0134 | .0174 | .0201 | .0363 | .0169 |
| XXIII | .0064 | .0060 | .0140 | .0143 | .0154 | .0265 | .0301 | .0169 |
| XXIV | .0065 | .0062 | .0140 | .0137 | .0191 | .0230 | .0313 | .0172 |
| XXV | .0066 | .0066 | .0138 | .0139 | .0147 | .0184 | .0281 | .0170 |
| Aver. | .0060 | | .0146 | | .0175 | .0243 | .0332 | .0179 |

12-21-6M
*Dogs fasted only one day. Not included in general averages.

PLATE I Table 2.

SUMMARY OF CALCIUM CONTENT
OF SMOOTH AND STRIATED MUSCLE.

1. Without correction for blood, lymph and connective tissue.

2. After making quantitative histological corrections.

| DOG | 1. STOMACH | 2. STOMACH | 1. RETRACT. | 2. RETRACT. | 1. SARTOR. | 2. SARTOR. |
|-------|---------------|---------------|----------------|----------------|---------------|---------------|
| XII | .0143 | .0132 | .0244 | .0155 | .0335 | .0253 |
| XV | .0175 | .0140 | .0265 | .0181 | .0291 | .0247 |
| XVIII | .0165 | .0132 | .0250 | .0170 | .0342 | .0270 |
| XIX | .0154 | .0133 | .0187 | .0144 | .0284 | .0225 |
| XX | .0206 | .0155 | .0263 | .0190 | .0301 | .0232 |
| XXI | .0133 | .0126 | .0194 | .0156 | .0334 | .0253 |
| XXII | .0174 | .0135 | .0201 | .0153 | .0363 | .0265 |
| XXIII | .0154 | .0142 | .0265 | .0197 | .0301 | .0226 |
| XXIV | .0191 | .0145 | .0230 | .0180 | .0313 | .0242 |
| XXV | .0147 | .0136 | .0184 | .0141 | .0281 | .0210 |
| Aver. | .0164 | .0138 | .0228 | .0167 | .0305 | .0242 |

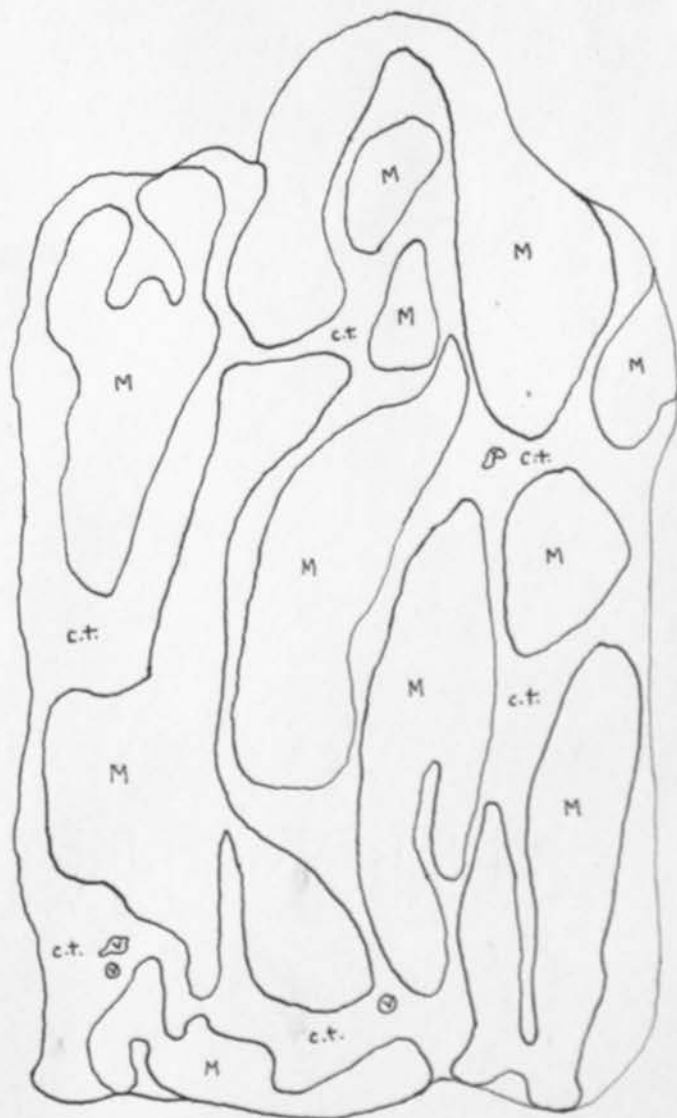
Curves are presented in Plate III, showing the great variations in individual analyses, and the reduction of individual variations after histological corrections.

Though the percentage of contaminating tissue in individual muscle tissues is extremely variable, the following general averages are obtained:

Smooth muscle from stomach-----84% pure muscle

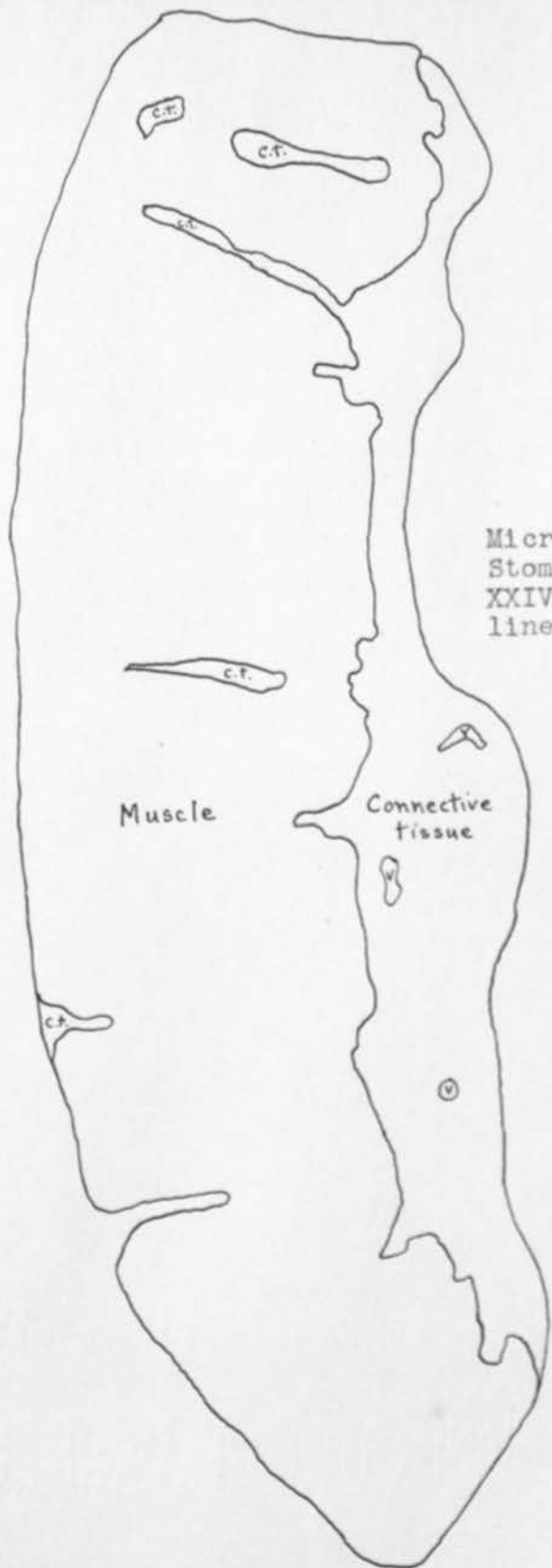
Smooth and striated from retractor---72% pure tissue

Striated muscle from sartorius-----81% pure tissue



Microscopic Section of the
Sartorius Muscle Dog XXII.
Projectoscopic Outline and
Magnification 50 Diameters.

PLATE II Figure 2.



Microscopic Section of the
Stomach Musculature of Dog
XXIV. Projectoscopic Out-
line Magnification 50 Diam.

Muscle

Connective
tissue

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Milligrams
Calcium 35

30

25

20

15

10

5

0

XII

XV

XVIII

XIX

XX

XXI

XXII

XXIII

XXIV

XXV

Dog No.

Sartorius
uncorrected.

Sartorius
corrected

Retractor
uncorrected

Stomach
uncorrected

Retractor
corrected

Stomach
corrected

