IMPROVED HISTOCHEMICAL METHODS FOR CHLORIDE, PHOSPHATE-CARBONATE AND POTASSIUM APPLIED TO SKELETAL MUSCLE

I. GERSH

Department of Anatomy, Johns Hopkins University

Current histochemical methods for chloride, phosphate, carbonate and potassium can be traced back through Macallum to much earlier work ('08). That these methods suffered from fundamental errors is recognized tacitly by many histologists and is borne out by the paucity of sound scientific contributions derived with their use and by the failure of present day workers to apply them to the elucidation of biological problems. The essential difficulty in the techniques is that they all permit diffusion of water-soluble substances. In addition, the procedures for these histochemical tests failed to take advantage properly of the great sensitivity of reagents for these ions, and the results were not as delicate as they could be. The subtle sources of error are enumerated in great detail by Lison in his handbook and in a more recent paper on the localization of certain ions in gastric glands. The methods introduced in this paper for the identification of chloride, phosphate-carbonate, and potassium are designed to overcome or minimize these difficulties with the aid of recent technical advances.[1] Numerous controls were made at each step to assist in preventing or reducing diffusion without sacrificing unduly the sensitivity of the reactions. The methods

[1] The term 'phosphate-carbonate' is used advisedly to indicate that the method in use demonstrates both of these anions when they are present in large enough amounts, without distinguishing between them; the expression does not refer to the complex salts of phosphate and carbonate such as are present in bone.

enumerated above have been applied systematically to an investigation of their distribution in certain glands and in skeletal muscle. In this paper only observations on the latter are recorded and discussed.

Recently, in connection with the broader problems of membrane permeability and maintenance of osmotic equilibrium in cells, attempts have been made by physiologists and biochemists to estimate quantitatively the inorganic constituents present within the skeletal muscle cell. All of these estimates are based on chemical analyses of the entire muscle, that is, of a complex tissue which includes muscle cells, connective tissue, extracellular tissue fluid and such additional structures as blood vessels and their contents. Two assumptions have been made in all the more recent studies: 1) the composition of the tissue fluid bathing the muscle cell is almost identical with that of the blood plasma; 2) no chloride is present within the muscle fiber—the total amount remaining extracellularly in the tissue fluid. Applying these two assumptions to chemical analyses of whole muscles, calculations have been made of the concentrations of certain inorganic substances within the muscle cell. The most extensive results have been obtained by Fenn working particularly on the sartorius muscle of the frog. He finds that potassium, phosphate and bicarbonate are present within the muscle fiber, in contrast with the assumed complete intracellular absence of chloride. His calculations show that, volume for volume, the intracellular: extracellular ratio is about 30:1 for potassium, a minimum of about 3:1 for inorganic orthophosphate, and about 1:4 for bicarbonate.

Such marked differences between intra- and extracellular phases should be easily demonstrable with adequate histochemical methods. The results recorded in this paper are, within the limits of the method, in remarkable agreement with the conclusions noted above. Chloride cannot be detected within the muscle fiber even under conditions when the chloride content of the whole muscle is greatly increased. The anion is, however, easily visible in the connective tissue surrounding the muscle fibers. Phosphate and carbonate together are

present in appreciable amounts in the muscle cell. Potassium is present in the muscle fiber in amounts vastly greater than that which can be seen in the interstitial connective tissue. These observations were made on the frog sartorius muscle treated experimentally in much the same way as Fenn and his co-workers describe in their series of papers, with the object in view of utilizing their comparable quantitative data in the interpretation of the results.

METHODS

The essential features of the methods are: 1) The retention of substances in or very close to their original inter- and intracellular sites by the use of the Altmann freezing-drying technique. 2) The sectioning of the frozen-dried material (embedded in paraffin) at a definite thickness in order to make possible rough comparative studies, to control more rigidly the conditions of the subsequent procedure, and to permit ready access of the reagents to the cells. 3) The removal of the paraffin by petroleum ether to allow the silver reagents to penetrate the section. 4) The use of silver nitrate solutions designed to precipitate specifically chloride ions, alone or together with phosphate and carbonate ions, and of a solution of sodium cobalti-nitrite to visualize potassium specifically. 5) The reduction of the precipitated silver salts by exposure to an arc light in order to make them visible.

The details of the method are as follows: Tissues or pieces of organs are frozen in liquid air and dried in a vacuum at temperatures varying from —60 or —62°C. to —79°C. After complete dehydration they are transferred to Grübler's paraffin (m. p. 50° to 52°C.), infiltrated in vacuo for 10 to 15 minutes at temperatures up to 60°C., and embedded. During the transfer from the vacuum tube to the paraffin, strict precautions are taken to prevent condensation of water on the dried tissues. Just before use, the paraffin is heated in a vacuum at 100°C. or higher for 15 minutes or until bubbling stops. Sections 15 μ thick are cut and these are mounted toward one edge on chemically clean large cover slips. They

are pressed down by the finger and after just melting over a very small flame are pressed down again. It has been found repeatedly that no error is introduced in pressing the section down with the finger. At this point sections to be tested for potassium are removed to the cold room, where they are treated in a manner that will be described later (p. 315). The subsequent treatment of sections to be studied for chloride and phosphate-carbonate proceeds in the following way:

The sections attached to the cover slip are submerged in a watch glass filled with anhydrous petroleum ether (b. p. = 20° to 40° C.), freshly distilled over sodium. The petroleum ether is replaced frequently. The watch glass is covered with another at all times except during the actual manipulations. When the paraffin is dissolved, the cover slip with the section attached is removed rapidly and a flame is applied immediately. The petroleum ether is burnt off and the warm cover slip is allowed to cool. With a little practice, the cover slip can be manipulated in such a way that the section does not slip off or become charred during this process. When the section approaches room temperature it is covered by a drop of one of the silver reagents.

Reagent 1 consists of a 60% solution of silver nitrate diluted with a sufficient quantity of concentrated phosphoric acid to prevent the precipitation of rather large concentrations of phosphates and then saturated with silver chloride; after filtering, 2 to 3 drops of water are added to every 10 cc. of reagent. Reagent 2 consists of a 60% solution of silver nitrate saturated with silver phosphate and silver chloride; after filtering, it is diluted with water as above. The reagents are kept tightly stoppered and in the dark except when in use. They are filtered just before using.

The fluid is decanted from the cover slip after a few seconds. A drop of chemically pure glycerine is placed over the section on the cover slip, which is then reversed over a chemically clean slide. The sections thus prepared in doublets are simultaneously exposed to a carbon arc radiation for the same time interval. The distance between the carbon arc and the slide is such that the section is not warmed during the exposure.

The sections last for a short time and it is best to study them directly after the radiation is completed. They may be examined by transmitted light or with dark field illumination. The reduced silver representing chloride, or phosphate-carbonate, or both, as the case may be, is visible as a yellow to brown, diffuse, homogeneous coloring with or without brown or black particles. With the dark field, the diffuse colorations are in most cases analyzed into finely particulate, reduced silver granules appearing usually orange or rust colored; also by this method the granules visible as such by transmitted illumination are more easily differentiated from the background. The highest power lens used with the dark field condenser was the 4 mm. high-dry apochromat with a numerical aperture of 0.95. The light required for the use of an oil immersion lens is too intense to be used with safety on these preparations because of its great reducing capacity.

The first reagent precipitates specifically the maximal concentration of chloride that can be detected by this method in the presence of phosphate and carbonate ions. Phosphate is kept in solution due to the excess phosphoric acid added to the reagent; carbonate is decomposed by the excess acid. A section treated with this reagent will reveal specifically only chloride. The second reagent precipitates approximately the same chloride concentration that is affected by the preceding acidified reagent, and in addition maximal concentrations of phosphate and some carbonate. After reduction of the silver precipitates, a section treated with this reagent will usually be darker than a similar section treated with the second reagent; the difference represents phosphate and carbonate present in the section, since approximately the same concentration of chloride is present in each.

Sections to be tested for potassium are transferred on the cover slip to a fairly large cold room whose temperature varies from $-1°$ to $+1°$C. during occupancy. The whole procedure is carried out and the sections are observed with instruments and reagents which have remained at these low temperatures continuously for the duration of the experi-

ments. This is an essential element in increasing the sensitivity of the method.

The paraffin is removed from the sections with anhydrous petroleum ether heated on a warm bar. The petroleum ether is burned off as in the test for chloride, and when the cover slip cools to room temperature, the section is covered with one drop of a 12% solution of sodium cobalti-nitrite prepared according to the method of Biilmann (Treadwell and Hall, '16). The reagent is drained off directly and replaced by glycerine. The cover slip with its section is mounted on a slide, section side down.

Observed with the microscope, potassium appears as crystals of sodium potassium cobalti-nitrite. With the oil immersion lens, these crystals are just visible as brilliant yellow short rods with rounded ends against a pale yellow diffuse background. The precaution to maintain reagents, cover slips, sections, slides, instruments and microscope all at close to 0°C. was taken because the crystals are exceedingly insoluble at this low temperature, though they are quite soluble at ordinary room temperatures. A thin commercial mineral oil was used instead of cedar oil because the latter became two viscous for use with the microscope in the cold room. The petroleum ether was warmed on a hot plate in order to shorten the time required to dissolve the paraffin from the sections.

RESULTS

Chloride in frog sartorius muscle as determined with the use of acidified silver nitrate almost saturated with silver chloride (reagent 1). In all the muscles examined histochemically, no chloride could be observed in the muscle cell. All the chloride visualized by this method was present in the extracellular phase, the tissue fluid spaces of the reticular and collagenous connective tissues. Differences in the chloride concentration of muscles subjected to different experimental conditions (as determined quantitatively by Fenn and coworkers) were manifest only as differences in the amount of chloride visible in the interstitial connective tissue.

In sections of muscles carefully dissected and frozen immediately on removal, the connective tissue appeared as a very pale yellow to a frank yellow in contrast with the complete absence of color in the muscle fibers. Viewed through the dark-field condenser, or by direct transillumination, this color was resolved with high power lenses into a variable number of minute, scattered, black or brown granules distributed evenly outside the muscle walls in the connective tissue. The quantitative variations in the choride concentration of normal muscles is matched by the variable number of black granules visible in sections of the same thickness from different muscles. In some, the reduced silver was very scarce, while in others the particles were closely spaced.

When the chloride concentration in this muscle was decreased by its removal and immersion in 4.5% glucose at 22°C. for 3 to 5 hours, generally no chloride was visible on sectioning. Occasionally sections from some muscles treated in this way showed the minimum number of granules which were visible in the normal, untreated muscle.

When the muscle chloride concentration was increased, the only change visible on sectioning was a consistent, increased amount of chloride visible in the intercellular connective tissue; no chloride appeared in the muscle cell except occasionally in a fiber at the periphery which may have been damaged in the manipulations. The chloride concentration was increased in several ways: 1) by immersion of the sartorius muscle for 3 to 5 hours at 22°C. in isotonic Ringer's solution, 2) by a similar immersion in Ringer's solution containing twice the usual concentration of solutes, 3) by the intraperitoneal injection into the live frog of 1 cc. of 10% NaCl 15 minutes before removal of the muscle, 4) by stimulation of the muscle of a urethanized frog for 30 minutes preceding its removal. The last was accomplished by stimulation of the thigh with a 60 cycle A.C. current at 1.5 to 2.5 volts by means of a copper plaque sewed to the skin over the urostyle and a platinum wire passing through the gastrocnemius muscle and wound around the skin. The current was con-

tinuous in some cases and interrupted in others at the rate of 30 to 40 per minute.

The color range of the connective tissue in sections of muscles treated as described in the preceding paragraph varied from the frank yellow of the uppermost normal range to brown or black. These resolved themselves into minute black granules of variable density; rather closely spaced in sections of some muscles, forming a dense veil in the reticulum surrounding the uncut surface of the muscle fiber in others, with still others showing intermediate stages. In general, the order of increasing density of reduced silver particles visible was 1) muscles of injected frogs, 2) stimulated muscles, 3) muscles immersed in Ringer's solution, and 4) muscles immersed in 2 X Ringer's solution. In all muscles, whether experimentally manipulated or normal, there was more visible chloride in the collagenous connective tissue than in the reticular connective tissue.

Phosphate-carbonate in frog sartorius muscle determined as the difference between the color intensities produced by a) acidified silver nitrate almost saturated with silver chloride (reagent 1) and b) silver nitrate almost saturated with silver chloride and silver phosphate (reagent 2). In all muscles examined histochemically phosphate-carbonate was observed in the muscle cell and in the connective tissue surrounding it. Differences in the intracellular distribution of these ions were too vague and uncertain to be clearly visible in sections of muscles from normal or injected frogs or of muscles immersed in Ringer's or in 2 X Ringer's solution. The extracellular differences were somewhat more marked but also uncertain.

When treated with reagent 2 the cytoplasm of the muscle cells took on a yellowish color which was uniform throughout the cell as compared with the colorless appearance of the cytoplasm after the application of reagent 1. This indicates a uniform cytoplasmic distribution of phosphate-carbonate which is quite at variance with the linear arrangement of these ions described by earlier histochemists and deduced from recent microincineration studies.

The color intensity of all cells in a section was of the same order except for an occasional darker cell on the periphery which may have been injured during its removal.

The intercellular connective tissue always appeared darker with reagent 2 as compared with reagent 1. It appeared that the added increment of color was greater in muscles immersed in Ringer's solution than in normal muscles. The color differential which indicates the presence of phosphate-carbonate was greater in the collagenous connective tissue than in the reticular connective tissue, indicating the presence of a greater amount of these ions in the former.

Potassium in frog sartorius muscle determined with sodium cobalti-nitrite at a low temperature. The normal frog sartorius muscles used for the study of the localization of chloride and phosphate-carbonate were also examined for the distribution of potassium. The crystals representing potassium pack the whole cell cytoplasm uniformly. There is a certain variability in the density of the crystals which one would expect in view of the individual differences Fenn and his co-workers found in such muscles in his quantitative analyses. But the variability is within a certain recognizable range. This uniform distribution was absent only when a known source of error was introduced, as by the addition of slight traces of water to the section by condensation, or by warming the slide or cover slip. In these cases, the crystals were very much larger, and were sharply limited to the dark band. This artifactual distribution of potassium within the muscle cell corresponds with that given by Macallum ('08) and also with that which one would expect from the sharp localization of mineral ash in the dark line after microincineration. A few crystals which were generally larger were scattered in the collagenous and reticular connective tissue spaces. They were more numerous in the former. The distribution of potassium in resting muscles from injected frogs, in stimulated muscles or in muscles immersed in either of the Ringer solutions showed no great differences from that in normal muscles.

This may be due to the fact that the crystals in the sections are so numerous that a slight increment or decrement would be undetectable.

Incidentally, the distribution of potassium in the nucleated red blood corpuscles present in the sections may be noted. Numerous very tiny yellow crystals of sodium potassium cobalti-nitrate packed the cytoplasm surrounding the clear oval nucleus which was entirely devoid of them.

DISCUSSION

A true understanding of the results described in the preceding section is naturally conditional on an appreciation of the nature and limitations of the methods employed. Hence, these subjects will be extensively discussed as a prelude to a discussion of the distribution of the ions in skeletal muscle.

Discussion of methods. Numerous controls extending over a long period of time have convinced the writer that with proper precautions there is no significant detectable diffusion of chloride, phosphate, carbonate or potassium, and that the observed distribution of these substances in cells and tissue fluids actually does represent essentially their real localization. Reasons are also given in this discussion of the methods for believing that the methods are also quite sensitive and specific. There is, in the final analysis, no complete proof that the distribution of ions described in this paper actually represents their real localization in the living cells. This fundamental difficulty occurs not only in the particular problem under investigation, but is quite general in the biological sciences. There are two principal means of circumventing this problem. The first is to show that when certain precautions are not taken, certain errors are introduced. In other words, the procedure indicated is to introduce certain known errors in technique (as, for example, the addition of water to anhydrous petroleum ether used in this procedure) and then observe the difference in the distribution of chloride or phosphate-carbonate. That difference is then the result of the diffusion of these ions caused by the introduction of water in the reagent

used. In the same way, each step in the procedure must be controlled. Finally when every step is controlled in this way, the assumption is that the precautions taken in the procedure are adequate, and that the distribution of the substances studied represents approximately their real distribution. It follows, then, that subsequent refinements of technique that have escaped the writer up to now will improve the method further. In this sense, the procedure outlined in this paper will serve as at least a beginning toward the elaboration and development of a useful, accurate technique for the detection of these various ions. A second way of determining the adequacy of the histochemical tests is to compare the results obtained by means of them with those yielded by other techniques—in this particular case, with the theoretical conclusions from chemical analyses of skeletal muscle. As the following paragraphs will emphasize, such a comparison shows that there is a striking agreement in the composition of the intra- and extracellular phases of skeletal muscle with respect to chloride, phosphate-carbonate and potassium as determined histochemically by the methods described in this paper and by chemical analyses of whole muscle.

The objects attained by the procedures described in the section on Methods are: 1) limitation of the diffusion of chloride, phosphate, carbonate, and potassium to the point where it becomes insignificant, 2) a greater sensitivity of the reactions so that minimal concentrations of these ions can be detected. The steps in the procedure that limit the diffusion of the ions that are being studied are: a) almost instantaneous freezing in liquid air and removal of water present in the tissues at low temperatures, b) direct infiltration of the dried tissue in paraffin freed from water and low boiling point oils by heating in a vacuum above the boiling point of water, c) absence of an affixative (Mayer's albumen) containing water, which if present at a later point, causes diffusion of salts, d) use of an anhydrous reagent (petroleum ether) to remove the paraffin, e) burning off the petroleum ether instead of slow evaporation, and the use of a low-boiling fraction. During the burning process, the temperature of the

cover slip (and the section) is raised above that of the surroundings thus preventing a condensation of atmospheric water on the section, such as takes place during slow evaporation from the cover slip due to the cooling effect.

The steps taken in the procedure to assure great sensitivity of the reactions for chloride, and phosphate-carbonate all follow from solubility product relations. They are: a) the very high concentration of silver ion in the reagent, b) the use of a minimal volume of water in the reactions, c) the addition of silver chloride and silver phosphate to a point just short of saturation (as a precaution against evaporation and precipitation of excess silver chloride and phosphate on the section from the reagent preceding the application of glycerine) in the reagents used to detect chloride and phosphate-carbonate, respectively, d) the substitution of light to reduce the precipitated silver chloride, phosphate and carbonate instead of the customary reducing solutions.

The sensitivity of the reaction for potassium is enhanced by: a) use of a concentrated reagent, b) use of a minimal amount of water, c) conduction of the test and observation of the sections in a cold room with reagents and instruments all at the same uniformly low temperature.

In spite of the precautions taken to increase the sensitivity of the methods, there is necessarily an irreducible amount of these ions in sections which escape detection. Hence conclusions drawn from histochemical studies with these methods apply definitely only to ion concentrations above a certain limited but not definitely known range; below this the methods are entirely inoperable.

The reagents used appear to be specific for the identification of the ions which they are designed to demonstrate. The great danger in the use of silver salts for the demonstration of chloride and phosphate-carbonate is the non-specific confusing reduction of silver by the reducing substances common to all tissues and by the slow action of light on silver-protein compounds. Sections tested as usual for chloride and phosphate-carbonate, but kept in the dark for 30 minutes without

exposure to light show no trace of reduced silver at the end of that time. After exposure to a bright light for a short time or to daylight for a longer time, sections such as these, or others which have been washed with 1 to 5 cc. of distilled water preceding or following the application of the silver reagents, show a slow progressive reduction of silver by the tissue proteins. This is manifested by a uniform brown tint becoming more pronounced in time. This phenomenon is completely absent in sections observed directly after reduction by light as described, or for a short time thereafter. When the proper precautions are taken there is a wide safety factor between the time required to reduce the chloride, phosphate, carbonate and hydroxide precipitates of silver and the time at which the tissue proteins begin to be reduced. The reduced silver observed in sections should represent specifically chloride alone or together with phosphate and carbonate, depending on the reagent used. A study of the chemical analyses of various structures makes it probable also that none of the substances which interfere with the specificity of the potassium reaction (ammonium, the alkaline earth metals, lithium, or sodium) are present in sufficiently large amounts in skeletal muscle to interfere with the specificity of the reagent for potassium. All the evidence leads to the conclusion that the reagents used as prescribed are specific for the ions they are designed to demonstrate in the structures to which they have been applied.

It will be noted that it is advisable to dehydrate tissues at — 60°C. or — 62°C. if they are to be used for histochemical studies for these ions. This temperature was recommended several years ago by Scott ('33) and more recently by Hoerr ('36). In the course of this investigation there was developed good evidence to show that under certain conditions in skeletal muscle there is some diffusion of chloride and phosphate carbonate during the process of drying if the tissue is dehydrated at — 30°C. This becomes markedly pronounced if the tissues are first kept at — 30°C. for a month and then dried in a vacuum at the same temperature. The behavior of

these substances, however, is in marked contrast with that of ferrocyanide in the rabbit kidney and of calcium phosphate in liver, spleen, bone marrow and lymph node. An exhaustive series of controls demonstrate that ferrocyanide in the mammalian kidney has an identical distribution in sections of material dried at —60°C. and at —30°C., within the limits of the method employed to demonstrate this substance. In the same way, no evidence could be uncovered of the diffusion of calcium phosphate ingested by phagocytes. The stability of ferrocyanide during dehydration may be related to its high eutectic point or to some other factor. The indiffusibility of calcium phosphate during dehydration may be referable to its high eutectic point; it is certainly related to its marked insolubility in water solutions. The behavior of calcium phosphate in the rat and dog will be described in two forthcoming papers.

The methods described in this section suffer from several possible sources of error. Although they have been utilized successfully in several specific problems, the procedures cannot be applied universally and indiscriminately to a variety of organs and processes. During the mounting of sections on the cover slip, when the paraffin is being melted and pressed down again, and again after the paraffin is dissolved by the petroleum ether, there is a shifting of the paraffin and of the section; together with it there may be a passive movement of inorganic and organic, free or attached particles. This may result in a purely mechanical shifting of the inorganic constituents that destroys their strict localization.

It must be emphasized at this point that whereas the tests for chloride and phosphate-carbonate are specific under certain conditions, an evaluation of the total quantity of the latter depends on the method of difference. That is, the total quantity of phosphate-carbonate is indicated by the difference in color intensity and number of granules between a section demonstrating phosphate, carbonate and chloride, and another showing only the same amount of chloride. When phosphate-carbonate is small in amount, or when the chloride content is high, an error in interpretation may be introduced.

The amounts of chloride, phosphate, carbonate and potassium present in each section are so minute, and their diffusibility so great, that the admission of small traces of water is capable of disturbing the strict localization so essential for histochemical work. Attempts have been made throughout to avoid such pitfalls and to accept for study only those sections which have been carried through the procedure under the most favorable conditions.

Finally, another possible source of error in histochemical investigations of inorganic substances has been emphasized by Hoerr, who pointed out that proteins contain a certain residium of water which cannot be removed in a vacuum even at temperatures higher than those customarily used. The extreme measures which must be taken to deprive the proteins of this residium would suggest that the water may be 'bound' by capillarity, or that it may be an essential part of the organic and inorganic molecular structure, or that it may be included in some other way so as to be not available as water of solution and subsequently be responsible for diffusion of solutes. If this is so, then the water remaining in tissues after they have been dehydrated in the vacuum is of no immediate concern in histochemistry. The following experiment proves that this is in fact the case. Glass tubes with an inner diameter of 5 mm. were closed at one end with DeKhotinsky cement. Through the open end, serum was added to a height of about 1 cm. They were frozen by immersion in liquid air. The tubes were removed to the air for a moment and covered with an equally high layer of serum in which crystalline sodium ferrocyanide or sodium thiocyanate had been dissolved. The tubes were again immersed in liquid air, and after freezing they were transferred to a vacuum chamber and dried at —30°C. After drying, several tubes were kept in a vacuum desiccator at —30°C. and at room temperature, in a lightly stoppered container at —30°C. and at room temperature. Immediately after drying, and at intervals thereafter up to 3 months from the time of dehydration, one of each set of tubes was removed, and brought to room temperature.

The de Khotinsky seal was carefully removed from the bottom of the column of dried serum. The solid serum was then chipped away, without disturbing the column above, up to a point about 2 mm. from the ferrocyanide or thiocyanate level. No trace of these salts could be detected under these conditions in any fraction of the lower level of ferrocyanide-free serum. At the end of 3 months, the tubes still contained about 0.6% of water as determined by the conventional method of heating a weighed sample.

Because it is intimately related to the nature of the methods employed it is desirable at this point to insert a general statement regarding the interpretation of the findings. The methods for the demonstration of chloride and potassium as used in the present investigation visualize the total amount of these substances present in a microscopic section of definite thickness. In two specimens of equal thickness an increased amount of chloride in one may indicate one of two things: 1) An increase in the amount of fluid containing chloride with no change in its concentration. For example, when considering the reticular connective tissue, if the amount of reticular connective tissue (+ tissue fluid) is doubled and the concentration of chloride in the tissue fluid remains the same, then more chloride will be visible in the sections of the dried material. 2) An increase in the concentration of chloride in the fluid with no change in the volume of the tissue fluid. Again considering the reticular connective tissue, if the volume of reticular tissue (+ tissue fluid) remains the same, but the concentration of chloride in the tissue fluid is doubled, then more chloride will be observed with the microscope in sections of the dried material. Variations in the observed amount of chloride in the preparations, then, may indicate differences in the amount of chloride-containing fluid, or in the concentration of chloride in the fluid, depending on the amount of fluid present. The amount of free water present in cells and intercellular material cannot be determined by the method in use. However, in the absence of definite knowledge on the subject, there is a great body of knowledge derived from other sources which can be applied to certain aspects of the problem.

Attempts will be made in this paper to interpret variations in the total amount of chloride as observed in sections with differences in the amount of chloride-containing fluid and with fluctuations in the concentration of chloride in the fluid.

Discussion of results. The results described in the preceding section serve as an actual demonstration of what had been assumed or deduced regarding the intracellular distribution of these four ions in striated muscle fibers. Potassium is present in very large amounts in the cytoplasm, phosphate-carbonate is present in moderate amounts, and chloride is completely absent. Assuming this complete absence of chloride from the cytoplasm, biochemists and physiologists made quantitative estimates of the inorganic intracellular composition of muscle fibers (Fenn). Accordingly, it is believed that the frog sartorius muscle cells contain potassium, sodium, magnesium, calcium, bicarbonate and phosphate. The substantiation by direct histochemical methods of this basic assumption lends greater force to the subsequent derived composition of muscle cells as given above.

Potassium and phosphate carbonate are represented as being uniformly distributed in the muscle fiber, with no apparent relation to its longitudinal or transverse striations. This is again in agreement with the conclusion to which Fenn was led: that "the distribution of potassium in the normal untreated cell is (not) essentially different from the distribution of water." Such findings are in conflict with the sharply localized distribution of ash in the dark bands after micro-incineration and with the earlier obviously faulty histochemical methods.

The muscle chloride, then, was found to be confined entirely, under all the conditions of this investigation, to the reticular and collagenous connective tissue framework, dissolved in the tissue fluid which infiltrates and permeates the interstitial tissue. This distribution was assumed by Fenn and his co-workers, but its demonstration gives greater credence to the calculations of Fenn and co-workers for the frog sartorius muscle and of Hastings and Eichelberger ('37) for mammalian muscle of the proportion of a skeletal muscle which

is not occupied by muscle fibers but rather is given over to the intercellular, tissue-fluid-containing spaces. The tissue fluid spaces occupy a calculated volume of about 14% in the normal frog sartorius muscle, and about 18% in the rectus abdominus muscle of the dog.

In addition to chloride, the tissue fluid contains visibly demonstrable phosphate-carbonate and potassium. All four ions were present in greater amounts in the collagenous tissue than in the reticular connective tissue. This is probably referable to the fact that in sections of striated muscle there are no large areas of reticular fibers, comparable to those occupied by collagenous fibers. The greater amount of these ions in the latter as observed in sections, then, may reflect only differences in the amount of tissue fluid present in different volumes of connective tissue.

The observed increased amount of chloride in the tissue fluid spaces of muscles stimulated indirectly is again a confirmation of the conclusions drawn from quantitative analyses of whole muscle. It is to be correlated with the increase in the tissue fluid and the lymph flow which is known to take place in contracting muscles.

SUMMARY AND CONCLUSIONS

1. Methods are described for the histochemical study of chloride, phosphate-carbonate, and potassium in certain structures.

2. These methods were utilized in a study of the distribution of these ions in the sartorius muscle of the frog. Phosphate-carbonate and potassium, but no chloride was detected in the muscle fiber. All four ions were present in the extracellular tissue fluid spaces.

3. These results are on very close agreement with certain conclusions drawn recently from chemical analyses of whole muscle.

LITERATURE CITED

FENN, W. O. 1936 Electrolytes in muscle. Physiol. Rev., vol. 16, pp. 450–487.

FENN, W. O., AND D. M. COBB 1934 The potassium equilibrium in muscle. J. Gen. Physiol., vol. 17, pp. 629–656.

——— 1936 Electrolyte changes in muscle during activity. Am. J. Physiol., vol. 115, pp. 345–356.

FENN, W. O., D. M. COBB AND B. S. MARSH 1934 Sodium and chloride in frog muscle. Am. J. Physiol., vol. 110, pp. 261–272.

GERSH, I. 1938 The fate of colloidal calcium phosphate in the dog. Am. J. Physiol. (In press.)

——— 1938 Histochemical studies on the fate of colloidal calcium phosphate in the rat. Anat. Rec., vol. 70, pp. 331–349.

HASTINGS, A. B., AND L. EICHELBERGER 1937 The exchange of salt and water between muscle and blood. I. The effect of an increase in the total body water produced by the intravenous injection of isotonic salt solutions. J. Biol. Chem., vol. 117, pp. 73–93.

HOERR, N. L. 1936 Cytological studies by the Altmann-Gersh freezing-drying method. I. Recent advances in the technique. Anat. Rec., vol. 65, pp. 293–317.

LISON, L. 1936 Histochemie Animale. Méthodes et Problèmes. Paris: Gauthiers-Villars.

——— 1936 Recherches histochimiques sur la sécrétion chlorhydrique de l'estomac. Zeit. f. Zellf. u. mikr. Anat., Bd. 25, S. 143–159.

MACALLUM, A. B. 1908 Die Methoden und Ergebnisse der Mikrochemie in der biologischen Forschung. Ergeb. d. Physiol., Bd. 7, S. 552–652.

SCOTT, H. G. 1933 A critical study and review of the method of micro-incineration. Protoplasma, vol. 20, pp. 133–151.

TREADWELL, F. P. 1916 (Translated by W. T. Hall.) Analytical Chemistry. Vol. 1, 4th English edition, p. 81. New York. John Wiley & Sons, Inc.