CHEMICAL STUDIES OF MUSCLE CONTRACTURE.

II. THE DISTRIBUTION OF PHOSPHORUS IN FROG MUSCLE DURING DELAYED RELAXATION.

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Fatigue contracture is produced in an isolated frog gastrocnemius when it is stimulated electrically until it no longer responds to the stimulus. A kymographic record of the contracture shows that the apparent failure in response of the muscle is due to a failure in relaxation, for the height of the contracture is often higher than the initial contraction. Cessation of the stimulus is followed by a relatively slow relaxation.

A condition which simulates fatigue contracture can be produced in frog muscles by the intraperitoneal injection of hypertonic solutions of non-toxic substances. The requirement of the ideal contracture so produced is that the extirpated muscle shortens immediately when the first stimulus is applied and relaxes so slowly that subsequent stimuli applied at an appropriate rate reach it during the shortened state. In order that the criterion for the production of contracture be adequate, the rate of stimulation must be well below the tetanic rate for an excised muscle from an untreated frog. A rate of about thirty shocks per second is required to produce complete tetanus in a previously unstimulated frog gastrocnemius. In the present work a rate of six per second has been used and we believe this rate is sufficiently slow to indicate a marked change in relaxation time when it produces tetanus.

The earliest observations of the effect of water-absorbing substances on the musculature of frogs appear to have been made in 1866 by Hause-
mann and Umeathun (quoted by Santesson). These investigators found that the subcutaneous injection of large doses of glycerol, and of concentrated solutions of sodium chloride or sugar caused muscular rigidity to develop. Santesson (16) has given a review of the literature on the effects of glycerol and of veratrin on frog muscle, and found in his own work that glycerol had a direct action on the muscle which predisposed it to the development of contracture, and agreed with earlier workers that this action was associated with dehydration.

In 1884 Ringer (14) found that intraperitoneal administration of sodium phosphates and of sodium bicarbonate in frogs caused dystonic movements of the limbs and rigidity. When the gastrocnemii were isolated from the animals and stimulated directly by an electric current they showed an increased relaxation time. It appears, however, that he did not connect the phenomenon with dehydration but rather with a specific effect of the bicarbonate and phosphate ions.

The observation of Urano (19) made in 1907 regarding the relation of phosphoric acid and creatine in living muscle is, in light of recent discoveries, prophetic. To quote, "Die oben angedeutete Vermutung dass Kreatin und Phosphorsäure aus demselben im lebenden Muskel vorgebildeten Komplex stammen, ist schon nicht schlechterdings zurückzuweisen." His conclusions were based on the rate of diffusion of the two substances out of living and dead muscle.

Throughout the literature on muscle tonus the idea has been expressed that creatine is in some way connected in physiologic function to muscle tone. Pekelharing (13) found an increased excretion of creatine in the urine of men following prolonged standing in the military position of attention, and it appeared logical to conclude that the creatine was set free by the tonic muscles and then excreted. Mitsuda and Uyeno (11) reported an increase in creatine in frog muscles during nicotine contracture. Scaffidi (17), however, found that fatigue did not alter the creatine content of muscle.

In view of recent work it seems most probable that in resting muscle practically all of the creatine exists in combination with phosphoric acid, and if we recall Urano's findings, the slow diffusibility of the two substances from living muscle indicates that phosphocreatine is bound by protein. The creatine content of rabbit muscle was found to be 367 mg. per cent by Cabella (2) and 528 mg. by Baumann and Hines (1). An approximate average of these values shows the creatine concentration in muscle to be 0.03 M. The maximum phosphocreatine concentration found by Davenport and Sacks (4) in the same sort of muscle was 0.027 M, a value which agrees within the limitations of technical error with the concentration of creatine itself. Hence the relationship between bound and free creatine should have greater significance in the physiology of muscle than total creatine.

In the present study the reckoning of phosphocreatine may be taken as a guide to the behavior of creatine, as well as phosphoric
acid, and we shall endeavor to show that a low phosphocreatine value is related to the tendency of frog muscle to show contracture.

EXPERIMENTAL.

Individuals of *Rana pipiens* which weighed about 100 gm. were used for all experiments. They were caught during the months of October and November and were used for the experiments within 1 to 3 weeks after capture. Except when otherwise stated, they were kept in an aquarium at room temperature (25°) but were supplied with running water at a temperature of about 10°.

The physiologic data were obtained in the form of kymographic tracings when the muscle was loaded relatively isotonically with 50 gm. by the following method. The inertia of the load was reduced to an insignificant value by suspending a weight of 50 gm. to the writing lever by means of a rubber band 1.5 mm. thick. When the muscle had been clamped to the rigid frame of the apparatus with a bone clamp attached to the femur, the 30 gauge wire leading to the writing lever was secured to the calcaneus tendon by a small hook, the lever was adjusted for height, and the 50 gm. weight then clamped at whatever height it happened to be when hanging freely on the rubber band. Such a system was not strictly isometric since the muscle reacted against a 50 gm. tension on the band.

Direct electrical stimulation from the secondary of an induction coil was transmitted through two silver electrodes at each end of the muscle. The strength of the stimulus was adjusted to be just maximal for the average normal muscle. The primary circuit interrupter was adjusted to give makes and breaks uniformly spaced at the rate of 3 each per second. The temperature of the muscle during stimulation was approximately 25° so that a low temperature (which is known to favor contracture) was not a factor.

All muscles were prepared for chemical analyses by first freezing with a slush of carbon dioxide snow and ethyl chloride, then cross-sectioning by hand with a thin bladed laboratory knife into slices preferably not thicker than 0.2 mm., and dropping the still frozen sections into 5 per cent trichloroacetic acid solution. A complete description of the technique has been reported previously (3, 15). On account of the small size of the muscles, it was
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necessary to limit the number of determinations of phosphorus to the inorganic, phosphocreatine, and total acid-soluble values. Inorganic phosphate was determined by the procedure of Sacks and Davenport (15), the A values by direct readings on the filtrates, and the total phosphorus by wet ashing aliquots of the filtrate with 0.5 cc. of concentrated sulfuric acid, eliminating the carbonaceous material with a few drops of 2 per cent phosphorus-free hydrogen peroxide, and adding (after diluting) molybdate solution and reducing agent to the digested material. The colorimetric method of Fiske and Subbarow (7) was used throughout.

Direct comparisons of data have been made between muscles obtained from the same animal. The conditions of comparison were somewhat variable and hence require a rather detailed description. We endeavored to establish an average value for the inorganic, phosphocreatine, and total phosphorus in resting, untreated frogs of the type to be used. The spinal cord was blocked by injecting about 0.1 cc. of 95 per cent ethyl alcohol into it in the lumbar region. The gastrocnemius of one leg was frozen while the skin was left intact, the limb ligated near the pelvic articulation, and amputated. After 2 hours the second gastrocnemius was frozen and removed in the same manner. This procedure established a control type of experiment for subsequent work in which contracture-producing substances were administered to the frogs after the removal of the first limb. It was found that the amputation of the first limb did not in itself affect the distribution of phosphorus compounds in the second limb during the time interval used. The inorganic phosphate found in the resting untreated muscles varied between 21 and 32 mg. per cent of phosphorus. The A value (phosphocreatine plus inorganic phosphate) varied between 71 mg. and 95 mg. and the totals between 138 mg. and 160 mg. Hence a phosphocreatine phosphorus value of about 60 mg. could be considered usual, and the remaining phosphorus fractions amounted to 65 mg. Muscles isolated from decapitate animals gave a slightly higher inorganic value, a lower A value (therefore less phosphocreatine) and a larger (T−A) remainder. The values for inorganic and phosphocreatine phosphorus are in very good agreement with the data obtained by Eggleton and Eggleton (6).

When an extirpated gastrocnemius was stimulated until it de-
developed fatigue contracture, Fig. 1, 1S, and frozen during stimulation, the distribution of phosphorus in the fractions studied was

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**Fig. 1.** Kymographic tracings of extirpated frog gastrocnemii. The lower line shows stimulation rate and time; the down stroke occurs at the make and the up stroke at the break in the primary. Each stroke equals \( \frac{1}{4} \) second. The chemical data obtained at the time of freezing (end of each chart) are recorded with corresponding numbers in Fig. 2.

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found to be as represented in Fig. 2, 18. By comparison with the unstimulated gastrocnemius (1U) extirpated from the opposite
limb of the same frog one sees that the phosphorus of inorganic phosphate has risen from 35 mg. to 56 mg. The phosphocreatine phosphorus has been reduced from 54 mg. to 13 mg. while there are 20 mg. which have been transferred to the more stable re-
mainder. This relatively large amount of phosphocreatine which was present in that particular muscle was higher than that usually found in fatigue contracture and probably includes some which was resynthesized anaerobically (10, 12) during a delay of a few seconds in completing freezing. A more extreme condition is seen in 2S (Figs. 1 and 2) wherein the phosphocreatine is absent following stimulation superimposed upon an artificially produced contracture. Here the determinations of the inorganic phosphate and the A value checked. Note that the latter is lower than for resting muscle even though there has been dehydration as indicated by the abnormally high total phosphorus.

By the procedure of simultaneously recording physiologic behavior with chemical findings we hoped to obtain information regarding the constancy with which the distribution of phosphorus compounds seen in fatigue contracture occurred in the contractures produced by dehydration.

A repetition of Ringer's (14) experiments gave variable results. The intraperitoneal injection of 5 ce. of a solution of a mixture of Na$_2$HPO$_4$, 0.3 M, and NaH$_2$PO$_4$, 0.05 M, caused twitching and some rigidity, but when the gastrocnemii were removed from frogs so treated and tested by stimulation at the rate of 6 shocks per second only relative degrees of contracture were obtained. Fig. 1, 7S, shows the record usually obtained while 2S shows the more extreme condition. The administration of 2 ce. of 1.2 M NaHCO$_3$ produced variable degrees of contracture and since it was more toxic than phosphate the animals frequently died within an hour. When the survivors were kept in a refrigerator overnight after bicarbonate administration and then warmed to room temperature, the extirpated muscles usually went into contracture on stimulation as shown in Fig. 1, 3S.

The degree of hydration of the muscles was checked by the determination of protein nitrogen in the tissue residues after extraction of acid-soluble phosphorus. The increase in nitrogen paralleled the increase in total acid-soluble phosphorus and varied from 10 to 30 per cent. In one frog the total phosphorus in the muscle removed before the administration of bicarbonate was 150 mg. per cent. 2 hours later the second leg was removed and a total phosphorus of 205 mg. found. This was the most extreme example of dehydration observed.
A review of our preliminary data suggested that the degree of contracture was related to the degree of dehydration, and that coincidentally there was usually a reduction in phosphocreatine in the resting companions of the muscles which developed definite contractures on stimulation.

Glycerol administered in doses of 2 cc. of 50 per cent solution caused marked muscular rigidity, and gastrocnemii removed in about 1 hour from frogs so treated responded to stimulation uniformly by contracture. Characteristic responses are shown in Fig. 1, 5S and 6S. Fig. 2, 5S shows the distribution of phosphorus compounds in one gastrocnemius before administration of glycerol. This muscle was stimulated for fifteen shocks and then frozen during the next fifteen and gave a response like the beginning of tracing 1S, Fig. 1 (normal relaxation). Note that the phosphocreatine is still 39 mg. per cent after stimulation (5S, Fig. 2). 5S', Fig. 2, shows the condition 1 hour after the administration of glycerol and after a comparable stimulation. Note the small amount of phosphocreatine and the degree of dehydration indicated by the apparent increase in total phosphorus. The phosphocreatine was already low in an unstimulated muscle after glycerol as shown by 6U, Fig. 2, and the type of response by its homologue from the same animal is shown by 6S, Fig. 1.

Since dehydration appeared to be the key to one means of producing contracture in frog muscles we tried hypertonic solutions of both glucose and urea. The latter was given in doses of 2 cc. of saturated solution, and the former the same volume of a 75 per cent solution. The effects of both are comparable to those of the other contracture-producing substances (4 and 8, Figs. 1 and 2).

DISCUSSION.

Previous work by others (6, 8, 10, 12) has shown that muscular fatigue is associated with a decrease in phosphocreatine. Fiske (9) has stated that "The hydrolysis of phosphocreatine seems now to be the principal factor permitting contraction to take place to a limited extent without the appearance of fatigue. . . . "

In our work the distribution of the phosphorus compounds studied was the same in the contracture resulting from dehydration as in fatigue contracture. There was also a close relationship
between a low phosphocreatine content and the development of contracture and at the same time there was a large amount of free inorganic phosphate in the muscles.

We endeavored to interpret the results on the basis of exhaustion of the muscles by the repeated excitation from the central nervous system before they were extirpated, since glycerol and bicarbonate produced twitchings and dystonic movements to a marked degree. This explanation does not suffice for glucose and urea since animals given these substances were quiet, yet their extirpated muscles exhibited contracture on stimulation. That the muscles were not exhausted was shown also by their repeated responses to stimulation provided sufficient time was given for relaxation to occur between stimuli.

Dulière and Bouckaert (5) have reported that contractures accompanied by increased acidity in muscle are accompanied by absence of phosphocreatine, but that the alkaline rigor of hypoglycemic death produced by insulin is similarly accompanied by a disappearance of phosphocreatine.

Schwartz and Oeschmann (18) found that the $A$ value (phosphocreatine plus inorganic phosphate) was lower in the gastrocnemii of frogs during contracture produced by monobromoacetic acid than in relaxed muscles. Their findings agree with ours and indicate that during fatigue and contracture there is some of the phosphocreatine phosphorus which is not accounted for by the inorganic phosphate set free, but is transferred to the more stable compounds contained between the total value (T) and the A value (4, 6).

Since our work has dealt with a type of contracture which can be produced in the frog by the administration of large doses of hypertonic solutions of physiologic substances, they may not be comparable to contractures produced by drugs such as monobromoacetic acid, chloroform, nicotine, and veratrin. The type of contracture produced by dehydration appears to be readily reproducible, is dependent primarily upon an increase in the relaxation time of the muscle, and is capable of sustaining a greater load than that usually used in the study of drug contractures. Our data do not prove that slow relaxation is invariably associated with a change in the distribution of acid-soluble phosphorus from the normal, but indicate a similarity in the mechanism of production of fatigue and dehydration contracture.
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We have been impressed with the similarity of muscular dehydration with its coincident tetany-like symptoms in frogs and the occurrence of tetany in man during disease which causes dehydration. Wechsler (20) lists a number of pathological conditions which are often associated with tetany. Among these are cholera, severe diarrheas in children, pyloric stenosis in infants, and rickets.

SUMMARY.

1. The intraperitoneal injection of hypertonic solutions of sodium phosphates, sodium bicarbonate, glycerol, glucose, or urea produced in frogs an increase in relaxation time of their extirpated gastrocnemii of approximately 5 times the normal.

2. The distribution of inorganic phosphate and phosphocreatine in the muscles from the treated frogs resembled that in muscles from untreated animals during fatigue contracture.

3. Dehydration of the muscle appeared to be the exciting cause of the artificially produced contractures.

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