XXXVI. THE EFFECT OF EXPOSURE TO LOW TEMPERATURES ON SOME PHYSIOLOGICAL, CHEMICAL AND PHYSICAL PROPERTIES OF AMPHIBIAN MUSCLE.

BY DOROTHY LILIAN FOSTER AND DOROTHY MARY MOYLE.

From the Biochemical Laboratory, Cambridge.

(Received March 6th, 1921.)

FATIGUE in muscle is a phenomenon which has excited the interest of observers ever since it was discovered that an excised amphibian muscle would respond to an electric stimulus by a contraction, but that if the stimuli were continued long enough the response grew weaker, and finally ceased, however strong the stimulus. The muscle was then obviously in a condition similar to that of fatigue in the living animal. The nature of this state of fatigue has been the subject of much research, but the work of Fletcher and Hopkins [1907] was by far the most important. They showed that fatigue is accompanied by the accumulation of lactic acid in the muscle, as is also rigor mortis, however that rigor may have been produced. Since loss of irritability is an invariable accompaniment of rigor, whether in fatigue or in the more complete and irreversible, but similar, state of death, it has been usual to regard this inability to respond to stimuli as due to the accumulation of acid. It seems that, although the equilibrium between a complicated set of factors necessary for irritability is maintained for some time after excision, yet, in the absence of the circulation, the accumulation of products of metabolism, notably lactic acid, finally upsets it. As is described in the following pages, however, it has now been shown that a non-irritable condition can exist without accumulation of lactic acid; it is, therefore, necessary to look upon loss of irritability as a separate phenomenon from the rigor of fatigue and death. Workers with amphibian muscle have occasionally noted that their material was non-irritable, but not rigid, although they have not attributed any significance to this fact, or made any determinations of the acid content of the muscle. Fletcher [1902] showed that muscle kept in oxygen did not become rigid, and Fletcher and Hopkins [1907] noted that their muscle was no longer irritable, though the condition was not one of fatigue.

Obviously, in the non-irritable condition described in the present paper, there is involved another factor, which, under ordinary circumstances of fatigue and death rigor, either does not come into play at all (possibly because

time is necessary for its action) or is masked by the more obvious factor of lactic acid accumulation.

Up to the present we have investigated the effect of prolonged exposure to 0° C. on the irritability, on the power of lactic acid formation under different conditions, and on the osmotic properties of amphibian muscle.

METHODS.

Before adopting a routine method of lactic acid estimation a comparison was made between the method of Fletcher and Hopkins [1907] and that more recently advocated by Wolf [1914]. It was found that Wolf's method had no advantage over the other as regards saving of time, and, in our hands at any rate, it did not give the more exact results claimed for it. Moreover, it is not applicable in all cases; for example, in the determination of the resting minimum.

Hence it was decided to adopt in all details the familiar method of the earlier investigators. At the beginning of this work it was not possible to obtain Merck's charcoal, but it was found that the finest grade of birchwood charcoal, when thoroughly washed, could be used instead. Otherwise, we followed in all respects the methods of extraction and estimation described in the above-mentioned paper, so that further description here is unnecessary.

One other point may be mentioned in connection with methods. Mondscheim [1912] states that lactic acid results are usually too low, because a large proportion, as much as 33 % of the acid, remains in combination with the protein, from which it can only be set free by alkaline hydrolysis. He, however, used extraction with boiling water as his first stage, and it was felt that his results could not apply to extraction with ice-cold alcohol. In order to test this, the residue, after extraction with alcohol in the case of both frog muscle and beef, was hydrolysed with strong alkali as described by Mondscheim, and his procedure followed in detail; but only traces of lactic acid were recovered by this means, and in some cases none at all. It was therefore decided that, as alcohol was used for all extractions, this further treatment was unnecessary.

In studying the effect of exposure to continuous low temperatures on the lactic acid production, it was decided to work at 0° C. because a temperature which is sufficiently low to freeze the muscle fluids will cause rupture of the fibres, and this in itself might be expected to be sufficient to lead to a large production of lactic acid. The method adopted was the following.

The frogs were pithed and the hind limbs skinned as carefully as possible, and then severed above the girdle. The limb pairs were then arranged on long glass slides, about ten or twenty on a slide; each slide fitted into a glass tube about 2½ feet long, and 2½ inches wide. A little Ringer's solution was run in under the slide, and the tube then closed by rubber stoppers, carrying glass leading tubes. The end limb pair was used as a test, and not included in the subsequent estimations. Thin copper wires were twisted round the legs of

this pair, and then passed up one of the leading tubes, to the terminals of an induction coil. The primary circuit consisted of an accumulator (1.4 volts) and key. When the limbs were exposed to atmospheres of oxygen or nitrogen, one stopper had a second leading tube which could be attached, *via* the necessary bubblers, to the cylinder of the gas in question. The tube at the opposite end was attached to a bubbler, so that it could be ascertained whether a steady flow of gas was being maintained. Three or four tubes, according to the number of limb pairs required, were tied to a board, which was then placed in a trough in a sink, and the whole kept filled with crushed ice as long as the experiment lasted. Special arrangements were made to allow for drainage. The whole procedure, from the pithing of the first frog to the covering with ice, generally took ten minutes.

I. THE EFFECT OF EXPOSURE TO 0° C. ON THE IRRITABILITY OF FROG MUSCLE.

The irritability was tested twice a day by make and break induction shocks, the coil being moved out until the maximum distance (minimum shock) was found which would produce a response at make and break respectively. By plotting time against position of the coil, a curve of declining irritability was obtained. Such a record was kept for two experiments, but in all later ones, when the approximate time was known, the limbs were left for a week before being tested at all. The duration of irritability varied with the time of the year and the condition of the frogs, but the minimum period, in air or oxygen, was a week¹. An interesting observation, made by other workers, was confirmed; namely, that the muscles cease to be irritable in a definite order, beginning with those of the thigh, and ending with those of the toes.

The muscles rendered non-irritable in air or oxygen in this way were quite flaccid and clear, showing no trace of the stiffness and opacity familiar in muscles which have been kept in air at higher temperatures, and which appeared at this low temperature in muscles kept in nitrogen.

The curves obtained for air and oxygen atmospheres were very characteristic. There is a rapid falling off of irritability, followed by a period of little change, and again a rapid fall to non-irritability. The meaning of these stages is not yet clear. The curves for nitrogen atmosphere show a rapid fall only. The curves for one experiment are appended.

Fig. 1 shows the curve for winter frogs, which remain irritable longer than spring frogs. Note the marked difference in effect between air and nitrogen. The lactic acid determinations confirm this in a striking way.

II. A. THE EFFECT OF EXPOSURE TO 0° C. ON THE "SURVIVAL" PRODUCTION OF LACTIC ACID IN FROGS.

When it was found that muscles could be rendered non-irritable simply by exposing them to a temperature of 0° C., it was decided to investigate the survival production of lactic acid under these conditions, in order to see

¹ The limb pair used as test always lost irritability sooner than the others, presumably owing to the poisonous action of the copper.

whether the physiological change was accompanied by an alteration in the chemical properties of the muscle.

When the resting minimum lactic acid was estimated in the non-irritable muscles of Experiment I, it was found to be strictly comparable with that of fresh untreated muscle, *i.e.* 0.01–0.03 %. This means that there was no measurable formation of lactic acid in the muscles kept at 0°, either in oxygen or in air. When normal, fresh muscle is maintained in air at room temperature, there is a slow steady production of lactic acid, equal increments being formed in equal lengths of time, as was shown by Fletcher and Hopkins. As a control for Experiment I, ten limb pairs were placed, with all precautions against infection, in sterile Petri dishes, and kept at room temperature for 53 hours. At the end of that time there was not the slightest trace of a putrefactive smell and all limbs were stiff (Expt. VIII). These muscles contained 0.112 % lactic acid as compared with the probable 0.02 % of fresh muscle. As is well

Fig. 1. Curve of declining irritability at 0° C. Break shock record.

known, on the other hand, in an atmosphere of oxygen, there is no measurable accumulation of lactic acid upon survival at room temperature [Fletcher and Hopkins, 1907].

This experiment on non-irritable muscles has been repeated several times, and always with the same result, that prolonged exposure to a temperature of 0° produces non-irritability without increase of lactic acid content, whether the muscle has been exposed to air or pure oxygen. That is to say, at this low temperature, the tension of oxygen in air is as effective as that of pure oxygen at higher temperatures. The following experiments were made, the details being given in the table (p. 341), together with figures quoted from Fletcher and Hopkins [1907] for comparison.

Experiment I. Muscles kept at 0° till non-irritable.

(a) In air. Lactic acid, 0.017 %.

(b) In oxygen. (1) Lactic acid, 0.021 %; (2) lactic acid, 0.023 %.

Experiment II. The effect of different atmospheres at 0°. Muscles kept at 0° till non-irritable. Survival lactic acid.

(a) In air. Lactic acid, 0.042 %. (The high result in this case was due to experimental error—the final residue not being pure.)

(b) In oxygen. Lactic acid, 0.017 %.

At first it was thought that the muscle might be incapable of forming lactic acid at this low temperature, but this was shown not to be the case as soon as we exposed the muscle to an atmosphere of nitrogen. Then we found the survival production of lactic acid, so characteristic of anaerobic conditions at normal temperatures. The rate of production is delayed, it is true, but this is only to be expected, and the "flattening of the curve" produced by a fall in temperature from 18° to 12° was shown by Fletcher and Hopkins [1907].

Hence this non-production of lactic acid on survival at 0° is not due to the inability of the muscles to form the acid, but probably due to the slowing down at that temperature of the processes which result in the production of lactic acid, so that the tension of oxygen in atmospheric air is sufficient to remove it as fast as it is formed and the conditions are equivalent to those of normal muscle in oxygen at higher temperatures.

(c) (1) Nine limb pairs kept 48 hours in nitrogen at 0°. Lactic acid, 0.130 %.

(2) Ten limb pairs kept six days in nitrogen at 0°. Lactic acid, 0.248 %.

II. B. THE CHEMICAL PROPERTIES OF NON-IRRITABLE MUSCLE.

In the preceding section it is shown that it is possible to avoid the survival production of lactic acid in frog's muscle by exposure to 0° till irritability is lost. It now remained to see if it is possible for lactic acid to be produced under any circumstances in the non-irritable muscle.

We first tried the effect of keeping the muscle at room temperature for 24 hours, and found only a very trifling increase in lactic acid. Whether more would be formed if it were possible to keep the muscle longer at room temperature is not known, as even at the end of 24 hours putrefaction is just beginning.

Experiment I.

(c) Muscles rendered non-irritable by exposure for 12 days to a temperature of 0° in air, and then left overnight at room temperature. Lactic acid, 0.042 %.

Experiment III.

(a) Ten limb pairs rendered non-irritable by keeping at 0° in air for eight days. Then left for 24 hours at room temperature. Lactic acid, 0.036 %.

In one case the muscles were left in ice for four days after all signs of irritability had disappeared, and it was found that there was no increase of lactic acid.

Experiment II.

(d) Muscles kept at 0° in air for four days after loss of irritability. Lactic acid, 0.035 %.

It has been shown above that muscles kept in nitrogen at 0° slowly develop lactic acid. It remained now to see whether muscle rendered non-irritable by keeping at 0°, would produce lactic acid if kept in nitrogen for a further period, at the same temperature. It was found that the non-irritable muscle behaved exactly like a fresh untreated muscle, in that, in the absence of oxygen, there was a production of lactic acid. A characteristic maximum was not reached, but any survival processes which do persist must be very considerably slowed at 0°, and hence a maximum was hardly to be expected in the time allowed.

Experiment V.

Ten limb pairs were rendered non-irritable by exposure, in air, to a temperature of 0° for seven days. The tube was then cleaned, the limbs replaced, and a current of nitrogen passed through the tube continuously for five days. At the end of that time the muscles were slightly opaque, but did not show much rigidity. Lactic acid, 0.148 %.

The non-irritable muscle was next subjected to those conditions which, in fresh muscle, cause maximum production of lactic acid; namely, heat, mechanical injury and the action of certain substances, *e.g.* toluene and chloroform. It was found that, in these circumstances, the muscle behaved in no way differently from normal fresh muscle, a maximum being produced in each case.

Experiment II.

(e) Heat rigor in non-irritable muscle.

The limbs, after removal from the ice, were spread on the bottom of thin-walled, covered beakers sunk in a thermostat at 45°, and maintained at this temperature for 1½ hours. This treatment produced the characteristic condition of stiffness and opacity associated with heat rigor. Lactic acid, 0.229 %. (N.B. These were summer frogs, which would account for the rather low maximum.)

(f) Effect of toluene on non-irritable muscle.

The tube, after removal from the ice, was placed on the bench at room temperature, and the ends plugged with cotton wool, soaked in toluene. Lactic acid, 0.261 %.

Experiment IV.

(a) Effect of toluene (repeated).

In this experiment the tube containing the non-irritable limbs was connected at one end to the pump, and at the other to a flask containing toluene;

so that a continuous current of toluene vapour could be drawn through the tube for 24 hours. Lactic acid, 0.185 %.

In this same experiment a series of non-irritable muscles was exposed to toluene at 0° with the following result.

(b) Effect of toluene on non-irritable muscle at 0°.

Lactic acid, 0.024 %.

The effect of injury, both at room temperature and at 0°, was next investigated; in the latter case it was found that the production of the lactic acid maximum was delayed.

Experiment I.

(d) Effect of injury.

The muscle was dissected from the limbs after removal from the ice, put through a mincer and finally rubbed to a paste in a mortar. It was then allowed to stand at room temperature over night to make sure that sufficient time had elapsed for a maximum production of acid [Fletcher, 1911]. Lactic acid, 0.291 %.

Experiment II.

(g) Effect of injury at 0°.

The non-irritable muscle was minced at 0°, and left at 0° over night. Lactic acid, 0.218 %.

An interesting confirmation of the effect of injury was obtained by the action of extreme cold. The freezing point of amphibian muscle is -0.47° , and at this temperature ice crystals separate. At still lower temperatures the whole muscle freezes solid. This means a rupture of the muscle fibres and is equivalent to mechanical injury. Fletcher showed that severe freezing and subsequent thawing was analogous to mechanical injury, in that it resulted in the production of lactic acid [1914]. This was confirmed by us, and further it was found that freezing non-irritable muscle to a temperature of -8° resulted in the formation of lactic acid. In the cases of both fresh and non-irritable muscle, lactic acid was found to be present, both when the muscle was thawed before estimation, and when the temperature during extraction was not allowed to rise above 0°.

Experiment VI.

(a) Effect of cold storage on non-irritable muscle.

A series of frog's limbs were rendered non-irritable by exposure to 0° for two weeks, and then placed in cold storage at -8° for three weeks. Every care was taken to prevent rise of temperature until the end of the extraction. Lactic acid, 0.163 %.

(b) Effect of cold storage and subsequent thawing.

Seven of the frozen limb pairs, obtained as above, were allowed to thaw before being extracted. Lactic acid, 0.174 %.

Summary of experimental results					Controls at higher temperature				
No. of expt.	Nature of experiment	Special treatment	State of muscle	% lactic acid	Expt.	Treatment	% lactic acid	State of muscle	Reference
I (b)	The effect of various atmospheres	Oxygen 18 days at 0°	Non-rigid, non-irritable	0.021	VIII	Oxygen at 13° 23 hrs.	0.028	—	F. and H.
II (b)	"	"	"	0.023					
I (a)	"	Air 12 days at 0°	"	0.017	VIII	Air at 16° 53 hrs.	0.083	—	F. and M.
II (a)	"	—	—	0.042					
II (c) (1)	"	Nitrogen 2 days at 0°	Non-irritable	0.130	X	Hydrogen at 16° 35½ hrs.	0.148	Non-irritable more or less rigid	F. and H.
II (c) (2)	"	5 days at 0°	Non-irritable and rigid	0.248					
II (e)	Chemical properties								
	Heat	7 days at 0° 1½ hrs. at 45°	Non-irritable	0.229	V	Heat at 45°	0.231	—	F. and H.
II (f)	Toluene	7 days at 0° + 24 hrs. exposure to toluene at 22.5° do.	Non-irritable	0.261	XX	Chloroform at 20°	0.329	—	F. and H.
IV (a)			Non-irritable	0.185					
I (d)	Injury	12 days at 0° minced and left over night at room temp.	Non-rigid, non-irritable	0.291	I	Minced and left in hy. drogen at 15°	0.399	—	F. and H.
II (g)		12 days at 0° and minced and left at 0° over night	"	0.218	II	Minced and left in hy. drogen at 10°	0.197	—	F. and H.
V	Nitrogen	7 days in air at 0° followed by exposure to N at 0° 5 days	Non-irritable	0.148					
I (c)	Other properties of non-irritable muscle								
	Thawing	Muscle at 0° in air 12 days, at room temp. over night	Non-irritable and non-rigid	0.042					
VI (a)	Freezing	Muscle at 0° 15 days, -8° 3 weeks	Non-irritable when frozen	0.163	VII (a)	Fresh muscle at -8° 4½ hrs.	0.085	—	F. and M.
II (d)	Prolonged exposure to 0°	Muscle at 0° 7 days till non-irritable and then 4 days more	Non-irritable	0.035	VII (b)	Fresh muscle 65 days at -8°	0.188	—	F. and M.

F. and H. = Fletcher and Hopkins [1907].

F. and M. = present authors.

Experiment VII. The effect of cold storage on fresh muscle.

(a) Ten limb pairs were placed in cold store at -8° for $4\frac{1}{2}$ hours. When removed they were frozen hard, and the temperature was not allowed to rise above 0° during the extraction. Lactic acid, 0.085 %.

(b) Ten limb pairs were left in cold store for 65 days, and then extracted with the same precautions as before. Lactic acid, 0.188 %.

SUMMARY OF PARTS I AND II.

It is shown that by continuous exposure to a temperature of 0° it is possible to produce a condition in frog muscles in which they are non-irritable but at the same time non-rigid and to all outward appearance similar to normal fresh muscle.

This loss of irritability is reached without the survival production of lactic acid if the muscle has been kept in air or oxygen. In nitrogen, however, the loss of irritability is much more rapid and is attended with formation of lactic acid. In nitrogen, too, the muscles become rigid and opaque.

Certain chemical properties of the muscle usually associated with irritability and the act of contraction were then investigated to see if the loss of this physiological property could be correlated with any chemical manifestation in the muscle. No such correlation was found. Apart from the absence of "survival" lactic acid the non-irritable muscle formed lactic acid in exactly the same circumstances as fresh irritable muscle. The explanation of this condition in the muscle was then sought in its physical properties, and Part III deals with this side of the investigation.

III. PHYSICAL PROPERTIES OF THE NON-IRRITABLE MUSCLES.

As has been described in Part I of this report, the non-irritable muscles obtained by the prolonged action of a temperature of 0° were found, as regards the chemical property usually believed to be most intimately connected with changes in irritability, *i.e.* lactic acid content, to be practically identical with fresh, irritable muscle. It was therefore necessary to look for changes in the physical properties of the muscle. For several reasons, possible alterations in the permeability of the semi-permeable membranes appeared worthy of first attention.

According to the theory of excitation put forward by Keith Lucas [1910], the special condition for excitation is the accumulation of certain ions in the neighbourhood of a membrane. This accumulation is caused by the fact that the membrane is impermeable to these ions, and it is therefore possible that an increase of permeability of the membrane would abolish excitability. Again, a possible explanation of the fact that in the non-irritable muscle left for 24 hours at room temperature no lactic acid is formed, would be a change in the permeability of some membrane preventing the coming together of enzyme and substrate.

As the conductivity method of measuring permeability was difficult, owing to the stimulating action of the electric current on the normal control muscle, the osmotic method was used. It should be noted that the results obtained by both these methods are capable of two interpretations. For instance, diminished conductivity and osmotic pressure may both be caused *either* by increased permeability of the membrane, *or* by decrease in the number of conducting and osmotically active particles. This latter factor may play an important part. For instance, it was suggested to us by Professor Hopkins that, during the long standing of the muscle at 0°, it is likely, from a physico-chemical point of view, that the colloids may adsorb dissolved electrolytes, thus fixing them and making them incapable of conduction or osmotic activity. The presence of these ions on the membrane might cause a change in its permeability, and might also result, according to Lucas' hypothesis, in a loss of excitability, owing to the non-mobility of the ions.

We also thought that it might be instructive to be able to compare the osmotic properties of these special non-irritable muscles with the osmotic properties found by Fletcher [1903] for muscle which had more or less lost excitability (muscle in death rigor, and fatigued muscle).

Method.

The non-irritable muscles were obtained in the usual way.

Nine gastrocnemii were removed as carefully as possible, and placed in a covered Petri dish on ice. Loops of cotton were tied to the tendons, and the muscles then weighed, in sets of three, in tared, covered watch glasses. Eight gastrocnemii from freshly pithed frogs were similarly treated as controls. The muscles were then suspended in different solutions by hanging the cotton loops on wire hooks, which passed through the corks of the conical flasks containing the solutions.

The following solutions were used:

Flask 1. 0.2 % NaCl	3 non-irritable muscles.
2. 0.75 % NaCl	„ „
3. 7.5 % cane sugar	„ „
4. 0.2 % NaCl	Control. 3 muscles.
5. 7.5 % cane sugar	„ 3 „
6. 0.75 % NaCl	„ 2 „

Each flask contained 600 cc. of solution.

The sodium chloride solutions are of the same strengths as those used by Fletcher [1903], the 0.2 % being hypotonic for normal muscle, and the 0.75 % isotonic at 17°. The 7.5 % cane sugar is, according to Meigs [1919], isotonic.

The sets of muscles were removed from the solutions about once every 1½ hours and weighed, the time being noted. The cotton loops were thoroughly blotted with filter paper, and the muscles dried by drawing across filter paper two or three times. This procedure was made as uniform as possible.

The weighings were repeated at regular intervals for about 12 hours and then a final weighing was obtained at the end of 24 hours.

This procedure was followed in Experiments X and XI with certain additions. The effect of distilled water, as well as that of the isotonic and hypotonic solutions already described, was investigated. It should be pointed out that the muscles used in Experiments IX and X were from summer frogs and those of Experiment XI were from winter frogs. This would account for individual variations [see Fletcher, 1903].

The curves from three experiments are appended (Figs. 2, 3 and 4).

Fig. 2. Expt. IX. The osmotic properties of normal and non-irritable muscle.

Fig. 3. Expt. X. The osmotic properties of normal and non-irritable muscle.

Fig. 4. Expt. XI. The osmotic properties of normal and non-irritable muscle.

RESULTS.

One property illustrative of the condition of the non-irritable muscle is clearly shown in all these curves. The osmotic condition is such that a solution which is isotonic to a normal muscle is hypertonic to the non-irritable muscle and correspondingly a solution which is hypotonic to a normal muscle is less hypotonic to the non-irritable muscle. (N.B. The isotonic cane sugar in Experiment X would seem to be an exception, but it is well known that cane sugar has special effects on muscle [see Bayliss, 1918].)

If these curves are compared with those given by Fletcher [1903] for fatigued muscle, it will be seen at once that, though differing from the normal, they are in no way representative of those for fatigued muscle. As already suggested this osmotic behaviour of the non-irritable muscle can be accounted for in two ways:

- (a) The membrane may have become more permeable.
- (b) There may be a diminution in the number of osmotically active particles—as for instance by adsorption on to the surface of colloids.

Further work must attempt to discriminate between these alternatives. By the kindness of Dr Adrian we have been able to ascertain that the electrical properties show a variation from the normal. It was found that a non-irritable sartorius gave no current of action on stimulation, but there was a demarcation current which was, however, considerably less than the normal.

Thus we have found well-marked physical, though no chemical, differences in the non-irritable amphibian muscle.

More recently we have found that, by treating the non-irritable muscle with various solutions, it is possible to recover a certain degree of irritability.

An amphibian "Ringer" solution, made according to Bayliss' formula, was most efficient but was almost equalled by an isotonic solution of pure sodium chloride. The interval before recovery was very short in these solutions but was unaffected either by exposure to oxygen or to nitrogen atmospheres. This lends support to the view that the loss of irritability is of "ionic" origin. Up to the present it has only been possible to get a slight recovery; the twitch is small and the stimulus required very large. Work on these lines is still proceeding and will form the subject of a future communication.

We wish to express our great indebtedness to Professor Hopkins who proposed this work to us and who has made many valuable suggestions throughout.

This communication formed part of a report to the Food Investigation Board of the Department of Scientific and Industrial Research.

REFERENCES.

- Bayliss (1918). Principles of General Physiology, p. 125.
Fletcher (1902). *J. Physiol.* **28**, 476.
— (1903). *J. Physiol.* **30**, 414.
— (1911). *J. Physiol.* **43**, 287.
— (1914). *J. Physiol.* **47**, 361.
Fletcher and Hopkins (1907). *J. Physiol.* **35**, 247.
Lucas (1910). *J. Physiol.* **40**, 225.
Meigs, quoted by V. Fürth (1919). *Ergeb. Physiol.* **17**.
Mondschein (1912). *Biochem. Zeitsch.* **42**, 105.
Wolf (1914). *J. Physiol.* **48**, 341.